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Review

Secretions of plant-parasitic nematodes: a molecular update

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Abstract

The interaction between sedentary endoparasitic nematodes and plants is fascinating, because these animals have developed an ingenious way to manipulate the plant's gene regulation and metabolism to their own advantage. They are able to form highly specialized feeding structures in the plant root to satisfy their nutritional demands for development and reproduction. This ability makes them extremely successful parasites with severe consequences for agriculture. Triggered by these economical losses, detailed studies of the parasitic interaction have been performed, which resulted in an extensive descriptive knowledge. However, the underlying biochemical and molecular events of this intimate relationship have still not been elucidated. It is generally accepted that secretions produced by the nematode are responsible for the dramatic alteration of specific cells in the host plant. In the past few years, the identification of genes coding for secreted proteins was a breakthrough in plant nematode research. However, the available information is still too limited to allow the formulation of a comprehensive model, mainly because the sequences of many of these genes are novel with no similar sequence found in the existing databases. A new challenge in the coming years will be the functional analysis of these putative parasitism genes.

Keywords: Pharyngeal gland; Syncytium; Giant cell; β-1,4-endoglucanase; Chorismate mutase; Secretome

1. Introduction

Parasites have long been neglected in the leading history of biology. In the nineteenth century they were portrayed as being degenerate creatures (Lankester, 1880) and the little evolution they experienced was seen as the result of being dragged along by their host. Parasitology suffered a long time from this stigmatisation. However, during the second half of last century, more detailed studies have thoroughly changed this view. It is getting clear that parasites are complex and extremely well adapted creatures (Bianco and Maizels, 1989; Thomas et al., 2002). According to recent hypotheses, parasites should be seen as one of the main driving forces of evolution (Hamilton and Zuk, 1982). In addition, new evidence proves their ecological value in controlling population densities (Torchin et al., 2003). A considerable amount of important parasites belong to the diverse phylum nematoda. It is estimated that billions of people, especially in developing countries, are infected with nematodes (de Silva et al., 2003; Colley et al., 2001). Moreover, by infecting livestock and crops, parasitic nematodes cause huge economical losses (Bird and Koltai, 2000).

This review focuses on plant-parasitic nematodes, in particular on so-called sedentary endoparasites. These animals are fascinating because they spend almost their entire life cycle embedded within the roots of higher plants,

Abbreviations: J, juvenile.

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sucking nutrients from a feeding site. This type of existence gives them numerous benefits, which may help to increase the number of offspring (Burrows, 1992; Tytgat et al., 2000). For example, the surrounding plant tissue provides protection from predation and insulates the nematode to some extent from unpredictable environmental conditions. Furthermore, by changing behaviour from migratory to sedentary, vital energy is saved.

Due to their economic importance, cyst forming species such as *Heterodera* spp. and *Globodera* spp., and root-knot nematodes such as *Meloidogyne* spp. are best characterised.

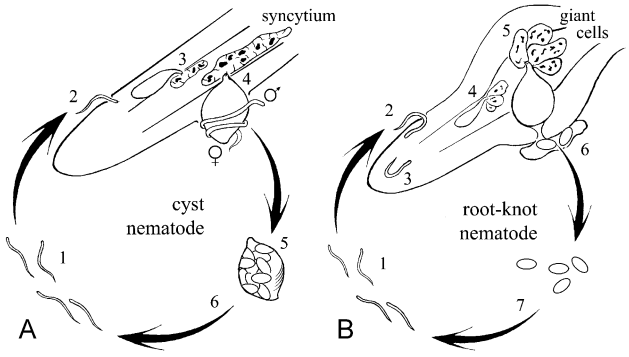

Fig. 1. (A) Life cycle of a cyst nematode (1–2 months). (1) Second stage juveniles are attracted to the root. (2) After penetration, they migrate intracellularly towards the vascular cylinder of the plant root in a quite aggressive way, using their hollow protrusible stylet to perforate cell walls of successive cells. (3) The destructive behaviour suddenly changes into a subtle exploration pattern upon arrival near the vascular cylinder of the plant. At that point a single cell is selected and transformed into a large syncytium by incorporation of neighbouring cells through cell wall breakdown. During nematode development, the syncytium continuously increases in volume by integrating more adjacent cells. The juveniles undergo three additional moults before reaching the adult stage. Females lose their worm shaped morphology and their body enlarges spectacularly. The vermiform males regain their mobility, leave the root and migrate in the soil, attracted to the females by pheromones. (4) After copulation a new generation of nematodes starts to develop within the female body. (5) A first moult of J1 to J2 occurs inside the eggs, which are retained in the protective cyst formed by tanning of the cuticle at death of the adult female. (6) Under favourable conditions, the J2s hatch and migrate towards a new host root. (B) Life cycle of a root-knot nematode (1–2 months). (1) Second stage juveniles are attracted to the root. (2) They enter the plant closely behind the root tip and migrate intercellularly, separating cells at the middle lamella. (3) To circumvent the barrier formed by the endodermis, they migrate towards the root tip and turn around when arriving in the apical meristematic region. Subsequently they go back up in the vascular cylinder towards the zone of differentiation where they induce a permanent feeding site. (4) Five to seven procambial cells around the nematode's head are stimulated to go through repeated rounds of mitosis uncoupled from cytokinesis, leading to multinucleated giant cells. (5) Differentiation of giant cells is often accompanied by proliferation and hypertrophy of nearby pericycle and cortical cells resulting in a pronounced galling of the surrounding root tissue. After feeding for about 2 weeks, the juveniles quickly moult three times. Although males can be formed, reproduction of most root-knot nematodes occurs exclusively parthenogeneticly. (6) Eggs are deposited on the root surface in a protective gelatinous matrix secreted by the female. The females usually stay embedded in the root gall and their cuticle does not tan or harden. A first moult of J1 to J2 occurs inside the eggs. (7) Under favourable conditions, the J2s hatch and migrate towards a new host root.

Their complex life cycle consists of distinct free living and parasitic stages (Fig. 1; see legend for details). The first task of the infective second stage juveniles (J2s) is to enter a host. Inside the root they form a complex multinucleated feeding structure, which will be the only source of nutrients for the parasite. Cyst nematodes form syncytia by cell wall dissolution and subsequent fusion of neighbouring cells. During nematode development, the syncytium continuously increases in volume by integrating more adjacent cells. A fully developed syncytium may have incorporated several hundred cells. The feeding site of root-knot nematodes consists of five to seven multinucleated giant cells formed by repeated cycles of mitosis without cytokinesis (Jones, 1981).

Although syncytia and giant cells differ from each other in ontogeny and cellular structure, they both function as a nutrient sink, which serves to satisfy the nutritional demands of the developing animal. The common function of both cell types is reflected in an analogous ultrastructure. Both feeding structures are hypertrophied and multinucleated cells with a dense granular cytoplasm, an extensive amount of small vacuoles, proliferated nuclei and an increased quantity of cellular organelles like mitochondria, plastids, free ribosomes, golgi apparatus and endoplasmatic reticulum (Wyss et al., 1992). These modifications point towards the high metabolic activity of these cells (Jones and Northcote, 1972), a fact which is also observed at the molecular level. Indeed, many plant genes have been identified that are differently expressed in the feeding sites compared to normal cells. However, only a few of them have been studied in detail (for a comprehensive review, see Gheysen and Fenoll, 2002).

2. Signals from the nematode

Plants cannot have evolved for the purpose of feeding nematodes. The induced feeding site is of no use for the plant host but is an essential requisite for the nematode to complete its life cycle. As such, it should be seen as an extended phenotypic effect of nematode genes. In addition, the ability of root-knot nematodes to induce giant cells with the same basic design in several thousands of host species suggests that this nematode interferes with key switches at the level of plant cell differentiation. Similarly, cyst nematodes induce syncytia that have a uniform structure. By doing so the parasites are believed to exploit existing developmental pathways to modify specific plant cells to their benefit. For example, both cyst and root-knot nematodes induce a re-activation of the plant cell cycle (Niebel et al., 1996; Goverse et al., 2000) and affect the structure of the cytoskeleton (de Almeida Engler et al., 2004). Extensive efforts have been performed to characterize the molecular triggers of the nematode involved in this manipulation. It is generally accepted that secretions (many of which are proteins) produced by the nematode are responsible for

the dramatic plant cell alterations (Hussey, 1989a). Genes coding for such secretions are considered as parasitism genes in a way that they were evolved to promote parasitism in a host (Davis et al., 2000). Their molecular identification has made significant progress in the past few years and these accumulated data will be presented in this review.

Linford (1937) suspected for the first time that root-knot nematodes use their stylet to inject specific compounds into the initial feeding cell. He postulated that those secreted molecules play a decisive role in the redifferentiation of the plant cell. Further observations are consistent with this suggestion. For example, the ectoparasitic nematodes *Xiphinema* and *Longidorus* spp. are able to induce a giant cell-like feeding structure (Wyss et al., 1988; Griffiths and Robertson, 1984) while they do not enter the root tissue. The only contact between these nematodes and their feeding cells is made by their long stylet.

Stylet secretions of endoparasitic nematodes are produced in three large unicellular pharyngeal glands, which are optimally adapted for high secretory activity (Fig. 2). One is located dorsally in the nematode body and the other two are subventrally positioned (Endo, 1984). Secretion granules produced by the dorsal gland are transported through a cytoplasmic extension and are released in the pharynx close to the base of the stylet, whereas the two subventral glands empty their granules immediately posterior to the muscular pump chamber. This morphology led to the idea that subventral gland secretions can only pass posteriorly towards the intestine and as a consequence only function in intracorporal digestion (Hussey, 1989a). However, experimental evidence is now available for a role of the subventral glands during migration of the second stage juveniles (Smant et al., 1998). Using immunolocalisation, Wang et al. (1999) were able to demonstrate that the nematode secretes cell wall degrading enzymes, originating from these two subventral glands, to soften the plant tissue. Cytological observations confirmed that the subventral glands are highly metabolically active during migration in the host tissue. Their secretory activity decreases with the preparation of the feeding site at which time the dorsal gland activity increases (Bird, 1983; Tytgat et al., 2002). Indeed, during feeding site induction, release of dorsal gland secretions into the cytoplasm of the root cells was observed (Endo, 1987). Therefore, the triggers for feeding site induction are believed to originate mainly in the dorsal gland.

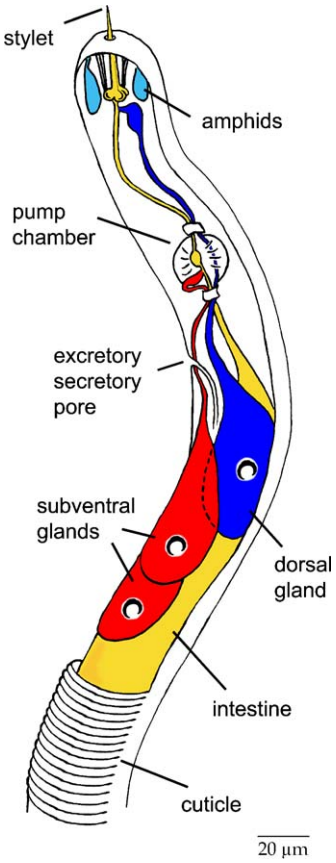

Fig. 2. Schematical representation of the anterior region of a J2 sedentary endoparasitic nematode. The most important secretory organs are indicated.

Secretions originating from the pharyngeal glands have another important function. Once injected into the feeding site, they form a so-called 'feeding tube' (Endo, 1987). The precise function of this tube is not clear, but since those tubes are attached to the stylet orifice during the period of feeding they may function as a molecular sieve for the selective uptake of nutrients, thereby preventing cellular organelles from obstructing the stylet orifice (Böckenhoff and Grundler, 1994). Moreover, a long feeding tube would provide the feeding nematode access to a greater area of the cytoplasm (Hussey and Grundler, 1998). The morphology of those tubes has been described in detail (Hussey and Mims, 1991; Sobczak et al., 1999). However, it is striking that despite the high level of feeding tube proteins produced, the encoding genes have still not been identified. The fact that these components might be similar to other structural cell proteins could be a potential explanation why they are overlooked. De Meutter (2002) isolated a very insoluble compound from extracted secretions of *H. schachtii* of which at least part was protein, deduced from amino acid analysis. However, the resulting amino acid composition did not show any striking similarity to proteins deposited in the database. The closest relative found was preCol-NG (AF043944), a component of the byssal threads of the mussel *Mytilus edulis*. Those threads are the means by which the mussel adheres to its substratum. They are produced in a gland at the base of the mussel foot and harden immediately by a process that looks like polymer injection moulding (Bell and Gosline, 1996).

3. Analyzing and purifying secreted proteins

Over the years, a variety of approaches towards biochemical characterization of putative parasitism factors from

the pharyngeal glands were used (Davis et al., 2000). First analyses were performed on solutions in which the nematode species were incubated. Different enzyme activities could be detected such as cellulases, pectinases and proteinases (Dropkin, 1963; Myers, 1965; Bird and Loveys, 1980; Deubert and Rohde, 1971; Giebel, 1974). Although the presence of some of these enzymes was recently confirmed by different molecular techniques, the biochemical assays were inconclusive and frequently resulted in contradictory data due to technical limitations. Therefore, the evidence for such enzymes of nematode origin remained elusive. The major obstacle for obtaining reproducible results is the small size of most plant-parasitic nematodes (less than 0.5 mm long and only 20 μm wide) and their obligate parasitism, which makes it difficult to collect sufficient material for analysis. In addition, sterile conditions were not always maintained. As a result, bacterial contamination and proteins leaking from dead nematodes could cause reactions falsely attributed to the secreted enzymes. Histochemical and cytochemical methods were also used to localise specific components in the nematode tissue (Bird, 1968; Cardin and Dalmasso, 1985; Sundermann and Hussey, 1988; Aumann and Wyss, 1989). Here too, only limited results were obtained since the cuticle of intact nematodes is impermeable to many reagents. In addition, the small size of the nematode body and its soft inner tissue make sectioning difficult.

Immunoaffinity purification was introduced in an attempt to overcome the limited availability of material for analysis. Various strategies for the preparation of immunogens were evaluated for enrichment of secreted proteins (Davis et al., 1992). Using a fractionated lysate of *G. rostochiensis*, Smant et al. (1998) were the first to succeed in purifying and characterizing a secreted protein (β-1,4-endoglucanase) from the subventral glands. However, except for this example, the success rate of this approach has been limited (Atkinson et al., 1988; Hussey, 1989b; Hussey et al., 1990; Davis et al., 1994; Goverse et al., 1994; De Boer et al., 1996a, 1996b; Yan and Davis, 1997). Despite this disappointing outcome, studies performed with antibodies confirmed previous observations that the expression pattern of secreted components is developmentally regulated. Subventral gland cell antigens are more abundant in preparasitic J2s, whereas antigens from the dorsal gland are more abundant in later parasitic stages (Atkinson and Harris, 1989; Hussey et al., 1990; Davis et al., 1994). It is obvious that those changes correlate with key events in the establishment of the parasitic interaction (Tytgat et al., 2002).

A more straightforward method, although technically demanding, is the direct purification of secretions and the analysis of their components. In a first attempt, subcellular granules were isolated from *M. incognita* J2s using isopycnic centrifugation on Percoll. SDS-PAGE analysis showed 15–20 major protein bands, and acid phosphatase activity was detected (Reddigari et al., 1985), confirming previous histochemical observations. Later, Veech et al. (1987) manually collected stylet secretions produced around the oral aperture of adult females of *M. incognita* and *M. arenaria* using glass micro-needles. In this batch of secretions, nine major proteins were spotted. Due to the low quantities available, further characterization was not possible. Moreover, the exudate preparations suffered from severe microbial contamination. For this reason the technique was further improved by the introduction of a sterilisation procedure. In addition, it was discovered that chemical reagents stimulate the nematodes to secrete more. In particular, 5-methoxy-*N*,*N*-dimethyltryptamine (DMT) (Goverse et al., 1994), an agonist of serotonine, increased pharyngeal pumping in cyst nematodes. Resorcinol has the same effect on root-knot nematodes (McClure and von Mende, 1987).

Robertson et al. (1999) stimulated *G. rostochiensis* with DMT and detected at least 10 proteins after concentration of the secretions by ultrafiltration and separation on SDS-PAGE. Western Blot was used to demonstrate the presence of superoxide dismutase and proteases were identified using in-gel activity assays. However, the genes encoding those proteins could not be cloned due to a lack of sufficient protein material for further characterization. Goverse et al. (1999) was able to partially fractionate a small compound (smaller than 3 kDa) from secretions of *G. rostochiensis* juveniles that co-stimulated the proliferation of tobacco leaf protoplasts in the presence of synthetic auxin and cytokinin analogues. This mitogenic compound, postulated to be a peptide, might be co-responsible for feeding cell development upon nematode infection.

Advancement in this area depended on the development of improved microtechniques. In the last years, a number of analytical tools with high sensitivity have become available. By combining 2D-gelelectrophoresis with microsequencing, two endoglucanases and a novel protein were characterized in secretions of *H. schachtii* (De Meutter et al., 2001). A similar technique was used for *M. incognita* and resulted in the identification of a calreticulin (MI-CRT) and a 14-3-3 protein (Jaubert et al., 2002a). Both proteins have multiple functions including the regulation of signalling and metabolic pathways and the control of cell cycle progress (Abad et al., 2003). The fact that calreticulin is also found in secretions of human parasitic nematodes (Kasper et al., 2001) and its specific expression restricted to the subventral glands made it an interesting protein to characterize in more detail. Recent evidence showed its role as a chaperone for maturation of secreted proteins (Debrabant et al., 2002). Further functional analyses will help to reveal its function in the parasitic process.

4. Looking at genes and transcripts encoding secreted proteins

Another approach to select candidate parasitism genes is to focus on the transcriptome of the nematode. A simple, rapid and cost-effective method for gene discovery is the

construction of cDNA-libraries and random single-pass sequencing of the clones (McCarter et al., 2003). These expressed sequence tags (ESTs) can then be used for homology searches (Popeijus et al., 2000a; Dautova et al., 2001). For at least nine plant-parasitic nematode species EST-sequencing projects are going on at present (www.nematod.net/Phyla/index.php) and allowed the cloning of a considerable amount of interesting genes such as pectate lyases (Popeijus et al., 2000b), β-1,4-endoglucanases, β-1,4-xylanase (Dautova et al., 2001) and polygalacturonase (Jaubert et al., 2002b). Despite the relative success, this strategy also has its limitations. Abundant mRNAs are over-represented while rare transcripts will be hard to isolate (McCarter et al., 2000). Moreover, the minimal number of nematodes required to achieve a representative cDNA-library is at least one hundred thousand. This threshold is the reason why cDNA libraries are predominantly constructed from the preparasitic stages, which are technically easier to sample, and not from the more interesting parasitic stages (Smant et al., 2002). As a result the EST sequences characterised today are biased towards genes expressed during the penetration and migration of the nematode in the plant root. The many cell-wall degrading enzymes characterised using this approach confirm this presumption. Furthermore, since nematode genes involved with parasitism are likely to be pioneer sequences, they are difficult to distinguish among the many other genes without homologues in the public databases.

Limited amounts of cDNA from minute quantities of biological material can be amplified by means of the polymerase chain reaction (PCR). Different research groups exploited this combination of cDNA technology with PCR to investigate RNA populations from the parasitic stages. Additional techniques were used to enrich the gene pool for interesting genes. Most of the methods are based on the fact that parasitism genes are differentially expressed in time and space during the life cycle of the nematode. For example, Ding et al. (1998) used a differential display approach to compare the gene expression between preparasitic and parasitic J2s of *M. incognita*. Tags that were specifically or highly expressed in the early parasitic J2 were cloned. Two out of 54 cDNA sequences were further analysed. One cDNA codes for a secreted cellulose-binding protein (MI-CBP-1) produced by the subventral glands. The actual function of this protein is still not known. However, it was shown that a recombinant bacterial cellulose-binding domain influences plant cell elongation in a dose–response manner (Shpigel et al., 1998). The other cDNA clone (*mi-msp-1*) is similar to a venom allergen antigen AG5-like cDNA (Ding et al., 2000). Animal parasitic nematodes secrete such AG5-like proteins during the invasion of the host (Zhan et al., 2003). They may play a role in the transition of the nematode from the free living to the parasitic stage.

Later, differential display was replaced by a more stringent technique, cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) (Bachem et al., 1996). By comparing 5 different life stages of *G. rostochiensis*, Qin et al. (2000) generated transcript profiles of about 16 000 tags. Approximately 400 of them showed an interesting expression pattern and were further analysed by means of whole mount in situ hybridisation. 30 pharyngeal gland-specific clones coding for putative secretory proteins were isolated in this way (Smant et al., 2002). Several of these clones showed significant homology with RanBPM, a 55 kDa protein (BPM55) initially thought to be involved in microtubule nucleation (Nakamura et al., 1998). However, it was later found that these original data were based on a truncated protein (Nishitani et al., 2001). The proper function of RanBPM remains to be determined but yeast two-hybrid screening elucidated different interacting proteins, reflecting its complex biological interrelationships (Wang et al., 2002a,b; Rao et al., 2002; Ideguchi et al., 2002; Umeda et al., 2003; Emberley et al., 2002). Without doubt, a more profound knowledge in this molecular network will help to reveal the function of similar proteins produced by the nematode.

By differential hybridisation of cDNA libraries for mRNA expressed in posterior and anterior regions of *M. javanica* parasitic J2s, Lambert et al. (1999) isolated a gene (*Mj-cm-1*), highly expressed in the pharyngeal glands with similarity to a bacterial gene coding for chorismate mutase. This enzyme has been identified in plants, fungi and bacteria (Romero et al., 1995) but is normally absent in animals. Homologues were also found in the cyst nematodes *H. glycines* (Bekal et al., 2003) and *G. pallida* (Jones et al., 2003). Chorismate mutase functions in the shikimate pathway and converts chorismate to prephenate, which is a precursor of aromatic amino acids. MjCM-1 is synthesised in the pharyngeal glands and probably (but not experimentally demonstrated yet) injected into the feeding site where it may alter the synthesis of chorismate derived compounds among which are precursors of cell wall synthesis, plant hormone biosynthesis and plant defence compounds. In this way, chorismate mutase may affect the normal development of the root tissue. Remarkably, in *H. glycines* polymorphisms in the chorismate mutase genes are correlated with different abilities of the nematode to infect resistant soybean cultivars (Bekal et al., 2003). Another secreted protein identified with differential hybridisation is a pectate lyase MJ-PEL-1 (Doyle and Lambert, 2002), which likely functions in softening the root tissue to facilitate nematode migration.

Gao et al. (2001a) analysed the transcriptome on an even smaller scale and constructed a cDNA-library from RNA obtained by microaspiration of pharyngeal gland cells from parasitic stages of *H. glycines*. This library was subjected to different molecular techniques to select parasitism genes, postulating that the most interesting candidate genes are highly expressed in the pharyngeal glands during early onset of parasitism and have a signal peptide for secretion. Suppression substractive hybridisation with a gland cell specific library as tester and cDNA from the intestinal region as driver was used to enrich the cDNA pool for gland cell specific genes (Gao et al., 2001a). Ten clones with a signal

peptide for secretion were isolated. Four of them were expressed specifically in the dorsal gland and expression of another one could be localized in the subventral glands. Alternatively, the signal peptide selection method of Klein et al. (1996) was used to enrich the gland cell specific library for those genes coding for secreted proteins (Wang et al., 2001). Doing so, another four cDNAs were cloned which encoded putative extracellular proteins and which hybridised to transcripts within the dorsal pharyngeal gland cell of the parasitic stages of *H. glycines*. Finally, De Boer et al. (2002a) further extrapolated the use of a gland specific library by spotting 1358 uncharacterised cDNA clones on a microarray. Developmental expression profiles of the tags were determined by hybridising the array with probes from both parasitic and non-parasitic life stages. Despite this high-throughput approach, no novel gland specific genes were identified. The gland extract libraries proved to be of low complexity and consisted largely of ribosomal RNA genes. It is likely that the lack of success was due to the technical limitations of the approach, namely the difficulty of gland aspiration, combined with library construction from extremely small amounts of starting material (Karrer et al., 1995). Nevertheless, based on EST analysis of gland specific libraries combined with data mining and in situ hybridisation, a profile of the *H. glycines* secretome was made by Gao et al. (2003). They presented 51 new gland-expressed candidate parasitism genes, of which 38 constitute completely novel sequences. A similar gland-cell specific library was created from the root-knot nematode *M. incognita*. Of 2452 clones sequenced, 37 unique clones with signal peptide were specifically expressed in the dorsal (24) or subventral (13) gland (Huang et al., 2003). Different (putative) parasitism genes identified in cyst and root-knot nematodes are summarized in Table 1.

5. Secretory organs

Although most of the work is focussed on secretions originating from the pharyngeal glands, other organs should not be overlooked. Secretions can originate from different sources such as the cuticle, the amphids, the excretory/secretory (E/S) system and the rectal glands. Like the pharyngeal glands, it is likely that the function of those organs changes upon the adoption of the parasitic stage of the life cycle (Jones et al., 1994).

The amphids are the primary chemosensory organs in the head of the nematode. Antibodies directed against amphidial secretions hamper host finding (Perry, 2001), indicating that those organs may be involved in the early steps of host–parasite recognition (Fioretti et al., 2002). A gene coding for a putative avirulence protein (MAP-1) was isolated after AFLP fingerprinting of near-isogenic lines of *M. incognita* (Semblat et al., 2001). The putative protein has no homologues in the database, but polyclonal antibodies against a synthetic peptide of MAP-1 clearly labelled the amphidial secretions.

The excretory/secretory system secretes proteins and glycoproteins that may be involved in the formation of the glycocalyx, a layer covering the cuticle (Bird et al., 1988). However, none of these secreted components have yet been characterised and the actual function of the E/S system is still a matter of speculation. It is presumed that E/S secretions play a similar function as the better-characterised cuticular secretions such as the protection of the parasitic nematodes against the host defence system (Spiegel and McClure, 1995). The potato cyst nematode *G. rostochiensis* was shown to secrete peroxiredoxin from the epidermis (Robertson et al., 2000). This enzyme removes hydrogen peroxide, produced at the plant–nematode interface, thereby shielding the nematode from the oxidative stress response of the plant (Waetzig et al., 1999). A similar protective function is dedicated to FAR-1, a fatty acid and retinol binding protein (Prior et al., 2001). Trapping of fatty acids can inhibit their lipoxygenase-mediated modification. This may have far-stretching consequences for the plant defence response since peroxidation of linolenic acid by lipoxygenase is one of the first steps leading to the formation of jasmonic acid, a systemic plant defence signal transducer (Conconi et al., 1996). As such, the cuticle is involved in a sophisticated interaction between the nematode and its host by disguising the invading animal for the plant tissue. Forrest et al. (1989) suggested that the cuticle might also play a role in the formation of the feeding plug that functions as a seal at the place where the stylet is inserted in the feeding site. However, earlier observations point to the amphids as the origin of the feeding plug (Endo, 1978).

The most active glands of adult root-knot females are the rectal glands, secreting large amounts of glycoproteins that form the voluminous gelatinous matrix in which the nematode deposits its eggs. This matrix provides protection to the eggs against desiccation (Wallace, 1968). Although Orion and Kritzman (1991) showed that the matrix possesses antimicrobial activity, Papert and Kok (2000) found a specific bacterial population inside the matrix. It is possible that those particular bacteria have a probiotic function, protecting the eggs against pathogenic microorganisms. Because the nematode itself stays inside the gall, the matrix is excreted in the environment through a canal in the plant tissue ending at the root surface. To form such a canal, the matrix itself possesses cellulytic activity (Orion et al., 1987) that may aid in the extrusion of the eggs out of the gall. The production of these cell wall degrading enzymes is presumably also performed by the rectal glands (Rosso et al., 1999), giving these glands an important role in the nematode–host interaction.

6. Non-protein signals

Until now, secretion of cell wall degrading enzymes was only discussed in the light of migration; either of the nematode itself or of the eggs it produced. However, it is

possible that these enzymes play another important role. It is known that a certain sub-class of fragments released from cell wall polysaccharides can function in plants as molecular signals involved in the regulation of growth, development and defence responses (Darvill et al., 1992; Dumville and Fry, 2000). These so-called oligosaccharins may be created in an artificial way by partial hydrolysis of various polysaccharides, however certain oligosaccharins were detected at biologically relevant concentrations in the spent media of plant cultures (Smith et al., 1999). Although the complex signalling processes of oligosaccharins in plants have hardly been studied, there seems to exist an extensive cross talk between these molecules and conventional phytohormones.

In contrast to the lack of research data on oligosaccharins in feeding site development, the involvement of phytohormones has been postulated for a long time (Kochba and Samish, 1972; Dropkin, 1972; Viglierchio, 1971). Cellular changes occurring upon nematode infection, such as hypertrophy, hyperplasia, lateral root induction, karyokinesis without cytokinesis and the breakdown of cell walls, can be partially mimicked by application of exogenous auxin (Giebel, 1974; Fan and Machlachlan, 1967). These observations supported the idea of a functional role for auxin in nematode feeding sites (Hutangura et al., 1999). Indeed, measurements of auxin levels in galls induced by root-knot nematodes revealed higher auxin concentrations (Balasubramanian and Rangaswami, 1962; Bird, 1962) or a different composition of indole compounds compared to surrounding tissues (Yu and Viglierchio, 1964; Viglierchio and Yu, 1968). The locally increased levels can be explained by retention of phytohormones in the nematode feeding site or by a stimulus from the nematode that releases the hormone from its inactive conjugated form. Also, the sensitivity of the plant cell itself can change. Another possibility is that the nematodes themselves produce phytohormones. It is known for a long time that indole compounds are formed as end products of tryptophan metabolism in animals. Auxins were detected in extracts of *H. schachtii* (Viglierchio and Yu, 1968; Johnson and Viglierchio, 1969) and in egg masses and extracts of different *Meloidogyne* species (Yu and Viglierchio, 1964; Setty and Wheeler, 1968). Other papers report on the presence of cytokinins in *Meloidogyne*. Dimalla and van Staden (1977) have found cytokinins in lysates of *M. incognita* females, in hatched juveniles and in egg masses and Bird and Loveys (1980) identified them in exudates of *M. javanica* J2s. Using new sensitive techniques such as capillary electrospray-liquid chromatography coupled to tandem mass spectrometry the presence of auxin, its homologues and its precursors and different types of cytokinins were shown in secretions and lysates of *M. incognita* and *H. schachtii* preparasitic juveniles (De Meutter, 2002; De Meutter et al., 2003).

The fact that the nitrogen fixing symbiotic bacteria *Rhizobium* induces similar structures on plants as root-knot nematodes intrigued scientists for decades. Both organisms cause galling of root tissue and are able to influence phytohormone levels. There are, however, considerable differences between nodules of rhizobia and galls of nematodes both on histological and molecular level. Nodules are structured tissues with their own meristem and different developmental zones, whereas galls are tumour like structures resulting from cell proliferation surrounding the giant cells. Comparing gene expression showed that only 2 out of 192 *Medicago truncatula* ESTs were upregulated after nematode infection, whereas 38 were upregulated in nodules (Favery et al., 2002). However, it is remarkable that EST sequencing revealed chitine synthase and nodL homologues in *M. incognita* (Bird and Koltai, 2000; Scholl et al., 2003). *Rhizobium* uses those enzymes to construct Nod-factors which are host-specific mitogenic components, consisting of a β-1,4-linked *N*-acetyl-D-glucosamine backbone bearing various substituents including a fatty acid residue. These oligosaccharin-related molecules play a pivotal role during nodule initiation and developing. Whether similar components are used by nematodes remains to be explored. Also, research towards other secondary metabolites is still lacking. That such components can be important in specific parasitic interactions was shown by Doss et al. (2000). They identified bruchins in extracts of pea weevils (*Bruchis pisorum* L.), an economical important insect pest of pea. These bruchins are lipid like structures that are able to stimulate cell division resulting in neoplastic growth of undifferentiated plant cells.

7. Phylogeny of parasitism genes

The β-1,4-endoglucanase genes form the best-characterised family of parasitism genes from plant-parasitic nematodes. Alignments revealed the remarkable observation that the closest relatives are found among the bacterial endoglucanases (De Meutter et al., 1998; Smant et al., 1998; Rosso et al., 1999). Nevertheless, the structure and organisation of the cellulase genes was studied in detail and confirmed that they exhibit a typical eukaryotic gene structure with introns, polyadenylation signals and characteristic eukaryotic promoters with normal regulatory elements (Yan et al., 1998, 2001). The endoglucanases are not the only parasitism genes that share significant homology with bacterial genes. Pectate lyase, MI-CBP-1 and chorismate mutase are other examples. Moreover, using a high-throughput genomic screen based on the comparison of EST data from *Meloidogyne* with prokaryotic and eukaryotic sequence databases, six new candidates were presented (Scholl et al., 2003). Four of them have highest similarity to genes of the nitrogen-fixing soil bacteria rhizobia.

These data point to an ancient acquisition of microbial genes by means of horizontal gene transfer as being the evolutionary origin of several nematode parasitism genes (Yan et al., 1998). Although this is an interesting hypothesis, it is extremely difficult to prove and as a result remains

speculative (Kurland et al., 2003). The striking similarity with *Rhizobium* genes indicates that ancestors of these bacteria may be the donors of those genes. Both organisms occupy similar ecological niches, in close contact with plant roots. Looking for prokaryotic organisms occupying the same physical niche, other interesting candidates can be found, such as ectoparasitic bacteria, living on the cuticle of nematodes or obscure prokaryotic organisms living intracellularly in different nematode tissues (Bird and Bird, 2001). Endosymbionts have been described in many filarial nematodes and some plant-parasitic nematodes such as *G. rostochiensis*, *H. glycines* and *Xiphinema brevicollum* (Shepherd et al., 1973; Taylor and Hoerauf, 1999; Endo, 1979; Walsh et al., 1983; Coomans et al., 2000). However, detailed investigation on these endosymbionts has been hampered by the inability to culture these organisms outside their host. Advances in molecular techniques have provided new tools for their characterisation (Vandekerckhove et al., 2000). In addition, a lot can be learned from studies of similar organisms in arthropods. Many of those obligate endosymbionts are found in hosts that live on restricted diets such as aphids feeding solely on plant phloem sap. This suggests that those organisms play a nutritive role for the host, providing a beneficial function in helping to overcome the problems of a one sided diet (Douglas, 1998). It is possible that chorismate mutase is a relict from such an endosymbiotic organism.

In the case of filarial nematodes there is strong evidence for a consistency between the phylogeny of *Wolbachia* endosymbionts and that of the filarial nematode hosts (Bandi et al., 1998; Casiraghi et al., 2001). This phylogenetic pattern suggests that symbiont and host share a long co-evolutionary history, leading to reciprocal co-adaptation and dependence. Indeed, tetracycline treatments targeted towards *Wolbachia* strains are detrimental to their filarial hosts (Bosshardt et al., 1993; Hoerauf et al., 1999). Recently, a *Wolbachia* genome fragment of 11kb containing 12 open reading frames, was identified on the X chromosome of its host insect (Kondo et al., 2002), which proves that horizontal gene transfer from this kind of bacteria is indeed possible.

Such horizontal transfer of genetic information provides an important means for the evolution of novel genotypes by combining genomes with different biochemical capabilities. In general, one could say that this delivers a powerful tool in order to generate rapid diversification.

8. Functional analysis

Sequence data is most useful when one can transform the obtained information into biological relevant knowledge (Grant and Viney, 2001). Such knowledge can be obtained based on comparisons of the sequences with well-described genes in annotated databases. For example, the successful characterisation of the many cell wall degrading enzymes secreted by the nematode is mainly due to the detailed studied counterparts in plant-parasitic and saprophytic bacteria and fungi. However, many candidate parasitism genes are novel genes without homologues in the databases (Gao et al., 2001b, 2003; Wang et al., 2001; Huang et al., 2003), or in some cases some homologues are found of which the precise function remains unknown. It was previously assumed that the enormous research efforts devoted to the free-living microbivorious nematode *Caenorhabditis elegans* would help to characterize those genes (Riddle and Georgi, 1990; Bird et al., 1999). Indeed, it is clear that many genes in plant-parasitic nematodes are likely to have a functional counterpart in *C. elegans*. However, the specific parasitism genes isolated thus far are mostly not present in this bacterial feeding nematode (Gao et al., 2003). As a result, functional genomics on the model nematode is limited in respect to understanding parasitism mechanisms.

Fortunately, a lack of information in the database does not mean that the functional analysis is just stumbling in the dark. Computer prediction tools are important in the elucidation of gene function. Looking for candidate parasitism proteins secreted by the nematode, we can profit from characteristics of those proteins, for example the presence of an N-terminal signal peptide that guides them through the cellular secretory pathway. Different prediction tools are available such as SignalP (Nielsen et al., 1997) and PSORT (Nakai and Kanehisa, 1992) to look for such signals. Further information can be obtained by looking for specific protein domain architectures. Nevertheless, experimentally obtained data are essential to confirm those predictions. Reliable evidence includes expression analysis of the gene and immunolocalisation of the corresponding protein.

Since the most interesting proteins of the parasitic interaction are probably released by the nematode in the plant tissue, analysis should not be focused on the nematode only. Localisation of the secreted protein in plants at a cellular or subcellular level might help us to better understand its specific task in the parasitic process (Wang et al., 1999). The green fluorescent protein (GFP), from the bioluminescent jellyfish *Aequorea victoria*, has intriguing properties that make it a versatile marker to monitor biological processes (Billinton and Knight, 2001). In our department, we fused different putative parasitism proteins of *H. schachtii* to GFP to examine its subcellular localisation in BY-2 plant cells. This provides information that can be integrated in a functional description (Tytgat et al., 2004). For example, a strong labelling of the nucleus may indicate that the protein interacts with the DNA of the host and functions as a transcription factor. The fact that 15 out of 51 putative parasitism genes presented by Gao et al. (2003) have a putative nuclear localisation signal, may indicate that the nematode is able to control its host in such a way. Tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow-2) are easily monitored using microscopic techniques and therefore have become a powerful research

Table 1
Summary of (putative) parasitism genes of cyst and root-knot nematodes

Accession	Name	Gene product	exp[a]	Sp[b]	M[c]	Reference
Protection against plant defence response						
Y09293	*Gp-far-1*	fatty acid and retinol binding protein	E	Gp	9	Prior et al., 2001
AJ493677	*Gr-gpx-1*	glutathione peroxidase	E	Gr	3	Jones et al., 2004
AJ243736	*Gr-tpx-1*	peroxiredoxin	E	Gr	9	Robertson et al., 2000
Cell wall modifying proteins						
AF004523	*Gr-eng1*	β-1,4-endoglucanase	SVG	Gr	1	Smant et al., 1998
AF182392	*Gt-eng1*	β-1,4-endoglucanase	SVG	Gt	2	Goellner et al., 2000
AJ299386	*Hs-eng1*	β-1,4-endoglucanase	SVG	Hs	2	De Meutter et al., 1998
AF006052	*Hg-eng1*	β-1,4-endoglucanase	SVG	Hg	1	Smant et al., 1998
AF100549	*Mi-eng1*	β-1,4-endoglucanase	SVG	Mi	2	Rosso et al., 1999
AF004716	*Gr-eng2*	β-1,4-endoglucanase	SVG	Gr	1	Smant et al., 1998
AF182393	*Gt-eng2*	β-1,4-endoglucanase	SVG	Gt	2	Goellner et al., 2000
AF006053	*Hg-eng2*	β-1,4-endoglucanase	SVG	Hg	1	Smant et al., 1998
AJ299387	*Hs-eng2*	β-1,4-endoglucanase	SVG	Hs	2	De Meutter et al., 1998
AF044210	*Hg-eng3*	β-1,4-endoglucanase	SVG	Hg	6	Yan et al., 2001
AY043224	*Hg-eng4*	β-1,4-endoglucanase	SVG	Hg	4	Gao et al., 2002a
AF455757	*Mj-pel-1*	pectate lyase	PG	Mj	8	Doyle and Lambert, 2002
AF127915	*Gr-pel-1*	pectate lyase	SVG	Gr	3	Popeijus et al., 2000b
AY026357	*Hg-pel-1*	pectate lyase	SVG	Hg	2	De Boer et al., 2002b
AF520566	*Hg-pel-2*	pectate lyase	SVG	Hg	4	Gao et al., 2003
AY098646	*Mi-pg-1*	polygalacturonase	SVG	Mi	3	Jaubert et al., 2002b
AF224342	*Mi-xyl-1*	β-1,4-endoxylanase	SVG	Mi	3	Dautova et al., 2001
AJ311902	*Gr-expb2*	expansin	SVG	Gr	5	Qin et al., 2004
AF049139	*Mi-cbp-1*	cellulose-binding protein	SVG	Mi	11	Ding et al., 1998
AF469058	*3B05*	cellulose-binding protein	SVG	Hg	4	Gao et al., 2003
Putative parasitism genes						
AY160225	*Hg-cm-1*	chorismate mutase	PG	Hg	2	Bekal et al., 2003
AF095949	*Mj-cm-1*	chorismate mutase	PG	Mj	8	Lambert et al., 1999
AJ457834	*Gp-cm-1*	chorismate mutase	SVG	Gp	3	Jones et al., 2003
AF013289	*Mi-msp-1*	venom allergen like protein	SVG	Mi	11	Ding et al., 2000
AF374388	*Hg-vap1*	venom allergen like protein	SVG	Hg	12	Gao et al., 2001a
AY033601	*Hg-vap2*	venom allergen like protein	SVG	Hg	4	Gao et al., 2001a
AF402771	*Mi-crt*	calreticulin	SVG	Mi	7	Jaubert et al., 2002a
AJ251758	*Gr-A41*	RanBPM homologue	DG	Gr	5	Qin, 2001
AJ251757	*Gr-A18*	RanBPM homologue	DG	Gr	5	Qin, 2001
AJ271910	*Gr-sxp-1*	SXP/RAL-2	E	Gr	3	Jones et al., 2000
AJ270995	*Gr-ams-1*	SXP/RAL-2	A	Gr	3	Jones et al., 2000
AY286305	*Hs-ubi1*	ubiquitin extension protein	DG	Hs	5	Tytgat et al., 2004
AY288520	*Hs-ubi2*	ubiquitin extension protein	DG	Hs	5	Tytgat et al., 2004
AF473831	*G3H07*	ubiquitin extension protein	DG	Hg	4	Gao et al., 2003
AF469060	*4G06*	ubiquitin extension protein	DG	Hg	4	Gao et al., 2003
AF468679	*Hg-cht-1*	chitinase	SVG	Hg	4	Gao et al., 2002b
AF469059	*4F01*	annexin	DG	Hg	4	Gao et al., 2003
AJ300178	*Gp-nex*	annexin	A	Gp	9	Fioretti et al., 2001
AF500024	*G8H07*	SKP1-like protein	DG	Hg	4	Gao et al., 2003
AF502391	*G10A06*	RING-H2 zinc finger protein	DG	Hg	4	Gao et al., 2003
AY134440	*msp21*	acid phosphatase	SVG	Mi	4	Huang et al., 2003
AF344862	*Hgg-15*	salivary proline-rich glycoprotein	DG	Hg	12	Gao et al., 2001b
AF273735	*hsp8*	ERp99	DG	Hg	10	Wang et al., 2001
AY142117	*msp34*	sodium/calcium/potassium exchanger	SVG	Mi	4	Huang et al., 2003

Notes to Table 1:
Only genes expressed in the secretory organs (including the epidermis) and coding for proteins experimentally determined to be secreted or containing a signal peptide for secretion are listed. Pioneer genes are not included.
Gao et al. (2003) presented 53 candidates parasitism genes of *H. glycines*; 39 code for proteins with unknown function of which 38 were pioneers.
Huang et al. (2003) presented 37 candidates parasitism genes of *M. incognita*; 33 code for proteins with unknown function of which 27 were pioneers.

[a] Exp = expression patterns analysed with in situ hybridisation or immunolocalisation: E, epidermis; SVG, subventral glands; D, dorsal gland; A, amphids; PG, pharyngeal glands.

[b] Sp = nematode species: Gr, *Globodera rostochiensis*; Gp, *Globodera pallida*; Gt, *Globodera tabacum*; Hg, *Heterodera glycines*; Hs, *Heterodera schachtii*; Mi, *Meloidogyne incognita*; Mj, *Meloidogyne javanica*.

[c] M = Method used to isolate the gene: 1—immunopurification; 2—homology-based cloning; 3—EST analysis; 4—EST analysis of gland library; 5—cDNA-AFLP; 6—functional screening; 7—protein purification; 8—differential hybridisation; 9—cDNA library screening; 10—yeast signal-P selection; 11—differential display; 12—suppression subtraction hybridisation.

tool for protein localization studies (Nagata et al., 1992; Geelen and Inzé, 2002). Moreover, the possibility to synchronise division of those cells makes it an accurate model system to analyse the basic mechanisms underlying plant cell biology and to track specific proteins during the plant cell cycle.

Additional information can be obtained by analysing the influence of a nematode protein on plant development by overexpressing the protein in plant roots. Expression of the chorismate mutase of *M. javanica* in hairy root cultures of soybean resulted in a distinct reduction of lateral root formation (Doyle and Lambert, 2003). In addition, the lateral roots that developed had an abnormal morphology and histological observation showed an inhibition of vascular tissue development. The altered phenotype could be rescued by the addition of auxin. This observation resulted in the hypothesis that chorismate mutase lowers auxin concentrations in the plant root by competition for chorismate. Doing so, the nematode would be able to inhibit vascularisation of the initial giant cells.

Besides overexpression of a specific gene in the plant, another strategy is to inactivate the gene in the nematode. An elegant and sensitive way is provided by RNA interference (RNAi) and is based on the ability of double stranded RNA (dsRNA) to direct sequence specific degradation of homologous RNA molecules (Fire et al., 1998; Boutla et al., 2002). This technology is well-studied in plant and animal model organisms and has been used extensively to characterise gene function. A protocol for RNAi on plant-parasitic nematodes was published by Urwin et al. (2002) and is based on octopamine mediated uptake of dsRNA. This technique may result in valuable data about the specific function of putative parasitism genes in the infection process. Instead of soaking the nematodes in dsRNA, one could also express dsRNA of nematode origin in plants. Although not demonstrated yet, it is possible that dsRNA can be delivered to the feeding nematode to interfere with normal gene function in the nematode, on the condition that the RNA molecule or the silencing complex is not excluded by the molecular sieve of the feeding tube. These new approaches open novel perspectives to study the plant–nematode interaction and may support research towards the development of nematode resistant plants.

9. Conclusions

Thanks to the extensive use of different molecular techniques and the effort of many research groups, characterisation of proteins secreted by plant-parasitic nematodes is making progress. Recently it was proven that both cyst and root-knot nematode use cell wall degrading enzymes to soften plant tissue during migration. Beside those enzymes, nematodes secrete proteins that protect them against the plant defence responses. However, despite the many cloned putative parasitism genes, the trigger for feeding site induction is still not elucidated. Possibly, it is produced by one of the novel genes identified but not characterised yet. The real challenge for the future will be unravelling the role of these genes in the parasitic process.

Considering the amount of different proteins secreted and the complexity of the parasitic interaction, it is likely that a combination of proteins is necessary to transform the plant cell. As such, we should not focus on specific genes, but search for possible interrelations between the candidate parasitism genes. For example, the fact that several putative parasitism genes of cyst nematodes code for proteins involved in ubiquitylation provides indications that this process may be important in nematode parasitism.

Even if we are still far from a general and comprehensive picture describing the molecular details of the plant–nematode interaction, our knowledge is evolving rapidly and will eventually result in elucidating the secreted code of the nematode.

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