Genetics of Nematode Parasitism 10

It is well known that various genetic factors influence the host plant and either turn it into a resistant to the nematode pest or enable the nematode to overcome the resistance of the host plant (Sidhu and Webster 1981). Most notably, all phytonematodes are equipped with a stylet to pierce cell walls and allow solute exchange between plant and parasite. Furthermore, plant-parasitic nematodes have well-developed secretory gland cells associated with their esophagus that produce secretions released through the stylet into host tissues. Interestingly, the development of enlarged secretory cells associated with the esophagus also exists in nematode parasites of animals but is notably absent from microbivorous nematodes like *C. elegans.* In the case of the root-knot nematodes and cyst nematodes, as is the case with the other tylenchid phytonematodes, there are three esophageal glands, one dorsal and two subventral glands.

The success of nematodes as phytoparasites is measured by their capacity to develop and reproduce on host plants and rarely on ability to cause disease. Parasitism in nematodes, in most studies, is being measured in terms of rates of development and reproduction but not in terms of virulence (Triantaphyllou 1986). Physiological races and biotypes along with nematode–host interactions are the major concepts to consider. Nematodes possess genes for parasitism but not genes for virulence. Parasitism genes simply are the ones which enable nematodes to overcome the effects of genes for resistance. Substitution of parasitism for virulence in nematology will not change the widely used concepts of "virulence versus aggressiveness." However, aggressiveness may be a suitable term to use with regard to nematodes.

"Parasitism" may be defined in several ways, and unfortunately, a lack of consensus about the meanings of host–parasite (pathogen) terminology still exists in plant pathology. The most common definition of a parasite is "an organism living in or on another living organism, obtaining from it part or all of its organic nutriment and commonly exhibiting some degree of adaptive structural modification." This broad definition encompasses a wide range of potential nematode parasitism genes that have evolved specifically or perhaps were "procured" and modified from other successful parasitic organisms, to promote parasitism in a host. Nematodes should be considered first as parasites, and if disease results in the host, the parasites become pathogens. The products of nematode parasitism genes may be manifested as morphological structures that provide access to parasitism of a particular host, or they may play critical physiological roles in the interaction of the nematode with its host.

10.1 The Role of Various Secretions in Parasitism

It is well known that in phytonematodes, stylet penetrates the wall of a plant cell, injects gland secretions into the cell, and withdraws nutrients from the cytoplasm. Migratory-feeding nematodes

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

The evolutionary adaptations of nematodes for plant parasitism led to the development of the protrusible stylet as well as marked morphological and physiological modifications of the esophagus (Bird 1971). Secretory gland cells in the nematode esophagus are the principal sources of secretions involved in plant parasitism, and these gland cells enlarged considerably as nematodes evolved from microbial-feeding nematodes to become parasites of higher plants. Likewise the function of the secretions produced by the esophageal gland cells also evolved to enable nematodes to feed on plant cells and modify them into complex feeding cells. Recent discoveries also suggest that some genes encoding esophageal gland secretions of plant-parasitic nematodes may have been acquired via horizontal gene transfer from prokaryotic microbes. This treatise focuses primarily on discoveries made in identifying parasitism genes in cyst and root-knot nematodes because these nematodes induce the most dramatic and evolutionarily advanced changes observed in host cell phenotype. Cyst and root-knot nematodes have evolved to alter gene expression in specific root cells to modify them into very specialized and metabolically active feeding cells, called syncytia or giant cells, respectively (Hussey et al. 2002a, b). Cell fusion following cell wall degradation gives rise to the syncytia, whereas abnormal cell growth following repeated mitosis uncoupled from cytokinesis produces the giant cells. Major roles of nematode secretions include egg hatching, penetration and migration in plant tissue, induction and maintenance of feeding site, feeding tube formation, and digestion of host cell contents.

10.1.1 Amphidial Secretions

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. They also capture and transport chemotactic stimuli to the sensillar membrane. Secreted proteins include annexin (Gp-nex-1), calcium-dependent phospholipid-binding protein, putative collagen, and gene in J2 amphids and hypodermis of adult female. The amphidial glands are the largest and most complex of the anterior sensory organs of nematodes. A gene coding for a putative avirulence protein (MAP-1) was isolated after AFLP fingerprinting of near-isogenic lines of *M. incognita*. The putative protein has no homologues in the database, but polyclonal antibodies against a synthetic peptide of MAP-1 clearly labeled the amphidial secretions. The dendritic nerve extensions of the amphidial neurons are surrounded by secretions of the amphidial gland cells (Aumann 1993). The secretions may protect the nerve dendrites against microbial attack. The amphidial secretions of the plant-parasitic nematode *Heterodera schachtii* are composed of glycoproteins with terminal galactose units (Aumann 1989). Several lectins with different carbohydrate specificities bind to the amphidial and "excretory" system secretions of this (Aumann and Wyss 1989) and other nematode species. The carbohydrates may be bound to the protein backbone either N-glycosidically via

N-acetylglucosamine and asparagines or O-glycosidically via N-acetylga-lactosamine and serine or threonine.

Dendritic processes project into the amphidial cavity and are bathed in secretions produced by the amphidial sheath cell (Duncan 1995). These secretions are highly glycosylated. Initial studies indicating the presence of carbohydrate residues in amphidial secretions came from the observation that the adhesion of some nematophagous fungi appeared to occur exclusively to chemosensory structures. It was shown that this was mediated by a lectin–carbohydrate interaction (Nordbring-Hertz and Mattiasson 1979; Jansson and Nordbring-Hertz 1983), with the carbohydrate being located in the amphidial secretions. Concurrent work using lectin binding carried out on the closely related *Heterodera schachtii* indicated that its amphidial secretions are composed exclusively of O-glycans (Aumann 1994). O-glycan linkages are known to be the major constituents of mucus, although they are also found in some cell membrane-associated molecules. Amphidial secretions were very resistant to proteolytic attack, often a consequence of O-glycosylation due to the relative resistance of the glycosylated regions to protease degradation. It is thought that this resistance is due to the attached carbohydrate residues blocking access to the peptide core as the removal of the carbohydrate allows subsequent protease digestion. Another effect of O-glycosylation may be to extend the functional domain of a molecule out from the cell surface, thus allowing interactions with extracellular molecules.

Secretions collected from *G. pallida* using the two different methods were analyzed using SDS-PAGE electrophoresis (Duncan 1995). Secretions were also used for antiserum production, giving two antisera, Luffness antiserum and ES antiserum. These were subsequently used for immunoblotting and indirect immunofluorescence studies. Indirect immunofluorescence studies indicated that the two antisera recognized different nematode components. This was further confirmed by immunoblotting studies which revealed that Luffness antiserum recognized a number of nematode proteins and was capable of differentiating both between and with species of *G. pallida* and *G. rostochiensis*. In contrast, ES antiserum recognized only two proteins which appeared to be conserved between the two species. Observations also indicated that presence of a nematode lectin component present in amphidial secretions with apparent specificity for N-acetylgalactosamine. Experiments were also performed to examine different methods of inducing secretions. Previous research had shown that the serotonin agonist 5-methoxy dimethyltryptamine (DMT) is an effective inducer of nematode esophageal secretions. Comparison of DMT-induced secretions with ES secretions using SDS-PAGE electrophoresis revealed that the protein profiles were similar, although some proteins were more abundant following induction with DMT. Treatment of *G. pallida* with DMT followed by indirect immunofluorescence with Luffness antiserum revealed an increased and altered distribution of antibody binding on the nematode surface.

It has been postulated that carbohydrate residues may have important functions in transduction of a chemosensory signal. Low concentrations of nematicides can impair responses to chemoattractants with no effect on motility. Exposure to the carbamoyloxime nematicide, aldicarb, resulted in the hypertrophy of the internal dendrite terminals within the amphidial sheath cell, a reduction in surface volume of the dendritic processes, and the appearance of electron-lucent granules in the cytoplasm of the amphidial sheath cell. Interestingly, these neuroanatomical effects were restricted to the amphids and not observed in the sheath cells or dendrites of the labial or cephalic sensilla. It was therefore suggested that aldicarb may have an effect via disruption of cholinesterase activity that has been reported. Amphidial secretions may be involved in initiation and/or maintenance of the host–parasite relationship. Amphidial secretions have a function in pathogenesis or the establishment of infection. It has been postulated that the feeding plug which is secreted by cyst nematodes once the feeding site is established may originate from the amphids. However, later studies suggest that feeding plug material may in fact be secreted through the cuticle.

10.1.2 Esophageal Glands Secretions

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

Esophageal gland cell secretions injected through the stylet by sedentary parasites transform root cells in susceptible plants into metabolically active feeding cells. These gland secretions modify, directly or indirectly, gene expression to induce profound morphological, physiological, and molecular changes in the recipient cells to enable them to function as a continuous source of nutrients for the nematode parasitic stages. The removal of the nematode at any point during the parasitic interaction results in degeneration of the feeding cells, suggesting the need for a constant and specific stimulus from the nematode to maintain the modifications in the parasitized cell. The gland secretions may be deposited outside the plasma membrane or injected directly into the cytoplasm of the recipient cell through the stylet orifice. In either case, specific molecules in the secretions could bind to plant cell receptors to elicit a signal transduction cascade to modulate gene expression in the cell. Alternatively, the secretions could enter the nucleus to directly modify gene expression in the recipient plant cell.

The development of monoclonal antibodies that bind to secretory antigens within the esophageal gland cells has been critical in the study of secreted proteins from cyst and root-knot nematodes (Hussey and Grundler 1998). The monoclonal antibodies have been used to monitor the developmental expression of different esophageal antigens at various stages of nematode development (Smant et al. 1998). During feeding, sedentary endoparasitic nematode species, viz., *Globodera, Meloidogyne, Heterodera*, and *Rotylenchulus*, also inject dorsal gland secretions that form unique tubelike structures called feeding tubes within the cytoplasm of the feeding cell. Feeding tubes function in the selective and efficient removal of nutrients from the cytoplasm of the large modified cells by the feeding nematode. Microinjection studies with fluorescent probes of different molecular weights showed that the walls of feeding tubes serve as a molecular sieve during nutrient uptake by the parasite. Dorsal gland (DG) secretions induce feeding site, produce feeding tube and modify the cytoplasm- syncytia, whereas subventral gland (SVG) contains cell wall-degrading enzymes like cellulase and proteolytic enzymes, chorismate mutase (aromatic amino acid synthesis) and SVG contains proteins (induces feeding cell).

10.1.3 Cuticular Secretions

The cuticle protects nematodes from plant defense response. The cuticle is a multifunctional exoskeleton. It is a highly impervious barrier between the animal and its environment. It is essential for the maintenance of body morphology and integrity and has a critical role in locomotion via attachments to body-wall muscles. It includes a peroxiredoxin that catalyzes the breakdown of hydrogen peroxidase; retinol protein; fatty acid-binding protein, which bind to linolenic and linoleic acid; precursors of plant defense compounds; and jasmonic acid signaling.

Parasitism gene products in nematodes include cellulase or endo-ß-1,4-glucanase, pectate lyase,

polygalacturonase, xylanase, expansins, chorismate mutase, chitinase, annexin, calreticulin, and small bioactive peptides. Feeding behavioral sequence in phytonematodes includes exploration, insertion of the stylet into host cells, injection of secretion, ingestion of host cytoplasm, and retraction of the stylet from host cell. Nematode parasitism is a complex and dynamic interaction with major activities like hatching stimuli, attraction to the host, penetration, recognition of tissue, feeding site formation, modification of host tissue, and an active response from the host. Parasite specificity in nematodes depends on the body adaptations, diverse habitats, and diverse niches.

The change in nematode morphology is accompanied by biochemical and ultrastructural changes in the surface cuticle (SC). The cuticle is a complex structure that is involved in the motility, maintenance of morphology, and interactions with the external environment. Molecules expressed at the SC of these parasitic nematodes represent the primary host–parasite interface and together with secreted–excreted products are probably the first signals perceived by the host. Nematode surfaces have a coat which contains different carbohydrates probably in the form of glycoproteins. Among the nematode's secretory products, stylet secretions are believed to play a role in the penetration and migration through root tissue, modification and maintenance of root cells as feeding sites, formation of feeding tubes, and digestion of host cell contents to facilitate nutrient acquisition by the nematode (Hussey 1989). These secretions are produced by two subventral and one dorsal esophageal gland cells and are secreted through the stylet into the plant tissue during parasitism.

Molecules expressed at the surface cuticle (SC) of plant-parasitic nematodes represent the primary plant–nematode interface and together with secreted–excreted (S-E) products are probably the first signals perceived by the host (Lima et al. 2005). These molecules, which are released into plant tissue, probably play important roles in the host–parasite interactions. They characterized these antigens that help in the identification of nematode targets useful for novel control strategies, which interfere with the nematode infection of plants. Three monoclonal (MAbs) and three polyclonal (PAbs) antibodies produced to S-E products of *Meloidogyne* spp. and *Heterodera avenae* were used to examine their reactivity toward *M. incognita* and/or *M. arenaria* second-stage juveniles and adult females. The three PAbs showed cross-reactivity with *M. incognita* and *M. arenaria.* Antibody Roth-PC 373 strongly recognized molecules present in the SC, amphids, and intestine, antibody Roth-PC 389 recognized the nematode amphids and metacorpus, while antibody Roth-PC 419 bound to molecules present in the subventral glands. Reactivity of the MAbs was only tested against *M. arenaria*. Monoclonal antibody Roth-MAb T116C1.1 showed intense reactivity with molecules present in the amphidial and phasmidial glands. Monoclonal antibodies Roth-MAb T46.2 and T42D.2 labeled the nematode amphids and molecules present in the nematode esophagus (metacorpus), respectively.

10.2 Niches Occupied by Phytonematodes

1. *Aerial*: Several nematodes feed on the aerial plant parts like stem, foliage, and flowers, for example, *Aphelenchus, Bursaphelenchus,* and *Anguina*.
2. *Subterranean*: Those nematodes which feed on the underground plant parts including roots, tubers, corms, suckers, etc., for example, M*eloidogyne, Heterodera, Xiphinema,* and *Longidorus*.

10.3 Convergent Specializations of Feeding Modes

Specializations in plant-parasitic lifestyles include migratory ectoparasitism and burrowing endoparasitism as well as various types of independently evolved, sedentary ecto- and endoparasitism and a full range of intermediates. These lifestyles relate to divergent parasite-specific host reactions, and in each case, the major pathology is the result of secretion products of pharyngeal glands injected into the host cell (Hussey 1989). Sedentary parasites

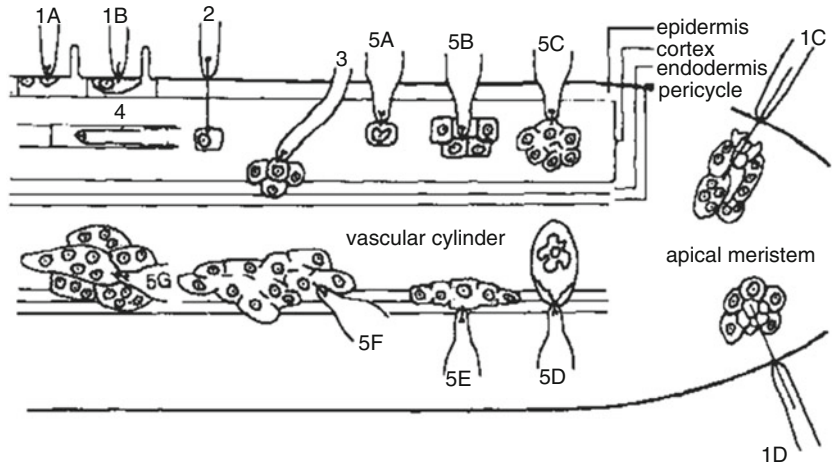


Fig. 10.1 Feeding site phytonematodes: 1A, *Tylenchorhynchus*; 1B, *Trichodorus*; 1C, *Xiphinema*; 1D, *Longidorus*; 2, *Criconemella*; 3, *Helicotylenchus*; 4, *Pratylenchus*; 5A, *Trophotylenchulus*; 5B, *Tylenchulus*; 5C, *Verutus*; 5D, *Cryphodera*; 5E, *Rotylenchulus*; 5F, *Heterodera*; 5G, *Meloidogyne*

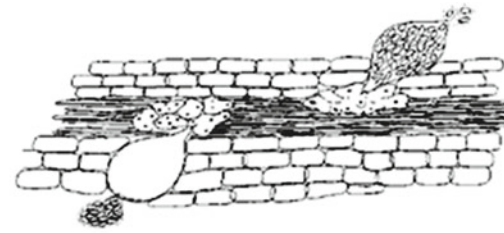
are associated with modifying and regulating host cell function to yield feeding sites acting as a metabolic sink and sustaining the parasite through its life. These feeding sites include various types of nurse cells (Mundo-Ocampo and Baldwin 1992), often specific to the nematode species. Nurse cells include single uninucleate giant cells as in *Sarisodera* sp., some *Meloidodera* (Heteroderinae) and *Rotylenchulus* sp. (Hoplolaimidae), multinucleate giant cells as in *Meloidogyne* or multinucleate syncytia as in many other Heteroderidae, and some other *Rotylenchulus.*

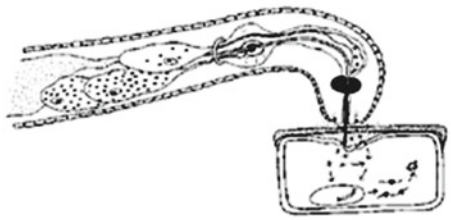
Host responses specific to particular nematode taxa have proven useful as characters in phylogenetic analysis of Heteroderinae, but a broader understanding of the evolution of the mode and direction of plant parasitism has been largely speculative. For example, a common perspective is that feeding sites among sedentary root-knot and cyst nematodes reflect the most elaborate and putative derived adaptations known among plant parasites (Davis et al. 2000a, b), a hypothesis testable in the context of phylogenetic trees. While acknowledging the limitations of preliminary data (taxon sampling, alignment issues, information content), some clades of plant parasites emerge with enough support to allow us to address several taxonomic and evolutionary hypotheses with a modicum of confidence. Emerging understanding of the patterns of these clades also provides a framework for mapping modes of parasitism, including the evolution of sedentary endoparasitism. Host responses specific to particular nematode taxa have proven useful as characters.

Nematodes mechanically injure plants (rubbing and probing) and bring about several physiological changes in the host, create openings for the entry of other microorganisms (interaction with other pathogens), transmit other disease-producing agents, and increase the plant's susceptibility to environmental stress.

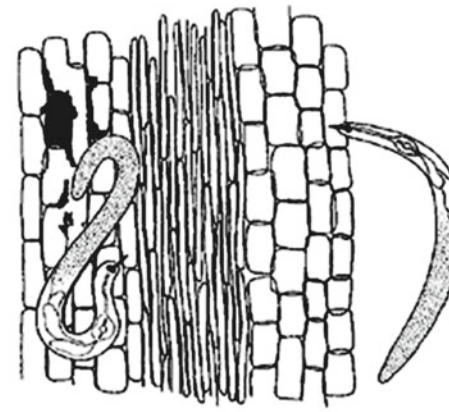
Phytonematodes are well-known obligate parasites. Some species have evolved rather simple feeding strategies, while other nematode species are highly adapted for more sophisticated parasitic relationships with host plants. A majority of research has focused upon plant response to nematode parasitism, primarily the complex modifications that some plant-parasitic nematodes induce in host plant cells and plant resistance to nematode challenge. Recent research is now providing insights into the molecular and genetic basis of the "nematode side" of plant–nematode interactions. Nematode parasitism genes may be active in any or all parts of the parasitic cycle of plant nematodes (Fig. 10.1), including "preparasitic" life stages (before invasion of the plant) and "parasitic" life stages (after invasion of the plant).

- Active feeding by nematode via the stylet
- Feeding cells serve as a nutrient sink for nematode
- Nematode stimulus maintains feeding site
- Feeding tubes aid ingestion of nutrients
- Successful nematode growth and reproduction

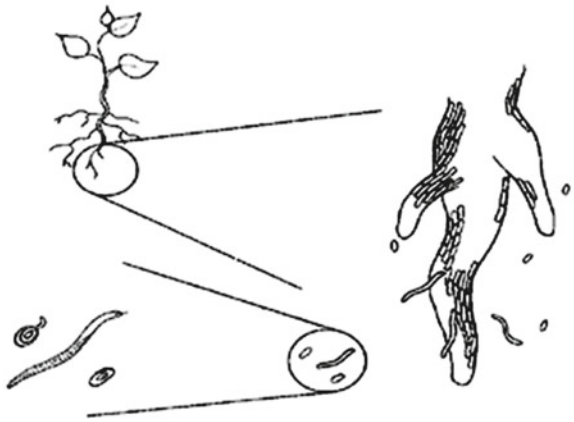




- Nematode signals trigger feeding site formation
- Esophageal gland secretions released through stylet
- Interaction of plant and nematode signals
- Gene expression is modified in parasitized cells
- Avirulent nematodes elicit defense in resistant genotypes



- Ectoparasites feed externally by inserting stylet
- Endoparasites enter roots to feed
- Mechanical and/or enzyme-aided migration within roots
- Nematodes select specific cells for feeding
- Resistant response to avirulent nematodes



- Egg hatch is influenced by root exudates
- Motile nematodes active in soil environment
- Nematodes respond to root signals
- Soil microbial activity affects nematodes
- Nematodes recongnize specific root tissues

Fig. 10.2 Progressive stages of parasitism by phytonematodes (from *bottom*)

Plant-parasitic nematodes have evolved diverse parasitic strategies and feeding relationships with their host plants to obtain nutrients that are necessary for development and reproduction. The vast majority of plant-parasitic nematode species are soil dwelling and feed from plant roots (Fig. 10.2). In highly specialized pathogens like root-knot nematodes, second-stage juveniles (J2) invade the roots of plants at the growing tip. They migrate between the cells and establish a permanent feeding site close to the developing vascular cylinder. There they molt three times, without

feeding between molts, to become adults. At the feeding site, they induce the formation of multinucleate "giant cells," of which they feed. Eggs are pushed to the surface of the root. The first molt takes place within the eggshell, and the second-stage juveniles hatch and disperse in the soil to search for a host.

These biotrophic parasites, depending upon species, feed from the cytoplasm of unmodified living plant cells or have evolved to modify plant cells into elaborate discrete feeding cells. Plant-parasitic nematodes use a hollow, protrusible feeding structure, called a stylet, to penetrate the wall of a plant cell, inject gland secretions into the cell, and withdraw nutrients from the cytoplasm. Migratory-feeding nematodes remove cytoplasm from the parasitized cell, frequently causing cell death, and then move to another cell to repeat the feeding process. Other nematodes become sedentary and feed from a single cell or a group of cells for prolonged periods of time. For this sustained feeding, the sedentary parasites dramatically modify root cells of susceptible hosts into elaborate feeding cells, including modulating complex changes in cell morphology, function, and gene expression. These feeding cells become the sole source of nutrients for sedentary endoparasites such as *Meloidogyne* (root-knot nematode) or *Heterodera* and *Globodera* (cyst nematode) species. Similarly, in sedentary ectoparasites such as the ring nematode, *Criconemella xenoplax*, a single feeding cell is utilized as a nutrient source for several days before the nematode moves on to establish another feeding site.

10.4 Cellular Changes

The root-knot nematodes, *Meloidogyne* spp., and the cyst nematodes, *Heterodera* and *Globodera* spp., are sedentary parasites of roots of many crop plant species that collectively incite billions of dollars in annual crop losses around the world. While both nematode groups use very similar parasitic strategies to complete their life cycles, they employ different mechanisms to carry out their strategies. In each group, the motile juvenile molts to the second stage (J2) and hatches from the egg in soil. The infective J2 follows environmental and host cues in soil to locate tissues near the plant root tip that it will penetrate. Infective juveniles of root-knot nematodes and cyst nematodes differ somewhat in their means of migration and apparent preference for feeding location near the vascular tissue of host plant roots, which shall not be revisited here (Davis et al. 2004). More substantial differences become obvious once feeding commences. If initiation of feeding is successful, the sedentary parasitic phase ensues, leading to nematode growth and three subsequent molts to the reproductive adult stage. Both root-knot nematodes and cyst nematodes transform initial feeding cells into elaborate feeding sites that share a dense cytoplasm, altered cell walls, duplication of their genetic material, and increased metabolic activity. However, root-knot nematode and cyst nematode feeding sites differ in ontogeny and appearance.

The root-knot nematode induces substantial enlargement and changes in a small group of initial feeding cells around the nematode head and turns each of them into a discreet "giant cell" from which the nematode feeds in sequence (Fig. 10.3a). In each giant cell, the nucleus undergoes repeated divisions resulting in a multinucleate state. A cyst nematode, on the other hand, induces changes in a single initial feeding cell, which then are reciprocated in neighboring cells, including cells that are not necessarily in direct contact with the nematode. These changes culminate in the fusion of many modified cells, sometimes involving over 200 cells, to form one large multinucleate cytoplasm called a syncytium (Fig. 10.3b). Nuclei of syncytial cells undergo endoreduplication of their DNA content but do not divide.

The elaborate changes in morphology of both syncytia and giant cells are accompanied by dramatic alteration in gene expression in the affected plant cells (De Meutter et al. 2003). Interestingly, root-knot nematodes and cyst nematodes in general also differ in the fact that most root-knot nematode species have broad host ranges, whereas cyst nematodes have much

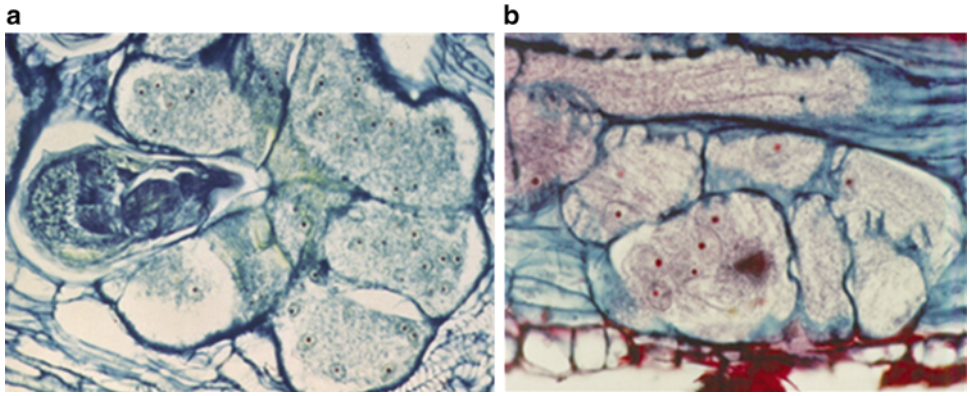


Fig. 10.3 Cross sections of feeding cells induced by sedentary endoparasitic nematodes in plant roots. (**a**) Multinucleate giant cells. (**b**) Multinucleate syncytium

smaller groups of host plants. A current hypothesis is that both nematodes use different strategies to induce their respective feeding sites and that giant cell induction by the root-knot nematode targets a plant mechanism that is widely conserved among plant species, thereby allowing parasitism of many host plants. On the contrary, for the formation of syncytia, cyst nematodes may target molecular plant mechanisms that are divergent among different plants, and therefore, individual cyst nematode taxa can only infect relatively small groups of plants (Baum et al. 2007).

Interrelationship of the genetic factors makes a host plant resistant to a parasite. Interrelationship involves a susceptible host plant on which a parasite can develop and reproduce freely. Genetics of nematode parasitism occur due to physiological variation and nematode–host interaction.

10.5 Physiological Variation

It mainly refers to the intraspecific variation, which means variation among populations of the same species. Knowledge about the species status of a given organism, particularly whether it comprises a biological species, is important before its physiological variation is recognized. The usage of some terms for identifying intraspecific variation includes the following.

10.5.1 Pathotype

An intra-subspecific classification of a pathogen distinguished from others of the species by its pathogenicity on a specific host(s). It is more preferred for potato cyst nematode, *Globodera rostochiensis* and *G. pallida*. This term is more appropriate to equivalent populations of amphimictic nematodes (Sidhu and Webster 1981). Pathotypes are differentiated based on the different breeding lines of the same plant species (Stone 1985). However, Sturhan (1985) reported that since resistance genes are often transferred through introgressive hybridization from one plant species to another, application of this definition may be ambiguous. This term should be used to individuals within a population that exhibit the same phenotype, i.e., possess the same host range. This way, a particular field population may represent one race only, which may comprise one or more pathotypes.

10.5.2 Biotype

"A group of genetically identical individuals sharing a common biological feature" is the definition of a biotype. It can be used to recognize intraspecific variation in parasitic capabilities of nematodes. In a complex genetic system, a single individual contains more than one biotype. It is

more preferred for stem and bulb nematode, *Ditylenchus dipsaci.* This term may be used to refer to parthenogenetic nematode populations with different host preferences and to identify intraspecific variation in parasitic capabilities of nematodes.

10.5.3 Race

It refers to a subdivision of a pathogen, distinguished from other members of the species by specialization for pathogenicity to different cultivars of a host. The varieties of a host species used to identify physiological races of a pathogen are known as differential hosts or host testers. Differential hosts are chosen on the basis of differences in their resistances to the pathogen, but the genes for resistance present in them are usually not known. Ideally, each of the differential hosts should posses a single resistance gene different from those present in others; such a set of differentials is known as ideal differentials. This term, implying host race, is used mostly for the soybean cyst nematode, *Heterodera glycines*, and for root-knot nematodes, *Meloidogyne* spp. (Table 10.1) (Dong et al. 1997*).* Host races are differentiated based on the genes for resistance from various plant species. This term should be applied to phyletically related populations, for instance, those that share several common characters apart from possessing the same host range (Sturhan 1985).

Physiological variation is very commonly seen in soybean cyst nematode, potato cyst nematode, cereal cyst nematode, stem and bulb nematode, and root-knot nematode.

Table 10.1 Races of *H. glycines* on standard soybean differentials

Differential host					
Race	Pickett	Peking	PI90763	PI88788	Lee
Group I					
3	R	R	R	R	S
6	S	R	R	R	S
9	S	S	R	R	S
14	S	S	S	R	S
Group II					
1	R	R	R	S	S
2	S	S	R	S	S
4	S	S	S	S	S
5	S	R	R	S	S

Group I and group II are separated based on parasitic ability on PI88788
R resistant or female index <10 %, *S* susceptible or female index >10 %

10.5.4 Nematode–Host Interaction

It deals primarily with inheritance of plant resistance through introgressive hybridization and a very few with inheritance of nematode parasitism. Nematodes possess balanced type of parasitism presumably through coevolution with their hosts. Gene-for-gene relationship occurs in host–pathogen interaction (Ellingboe 1984). It occurs when plant-parasitic genes behave as dominant and complimentary genes for parasitism behave as recessive, and vice versa. Major and minor genes for resistance are matched with major and minor genes for parasitism, respectively, though this may not always be true (Triantaphyllou 1986). It is normally more complex when a given gene for resistance interacts with more than one gene for parasitism (phenotypes show immune/resistance/susceptible response). The genetics of parasitism in most instances may be extrapolated from the knowledge about the inheritance of resistance.

One of the efficient ways to investigate the inheritance of resistance or parasitism is to test the progeny of appropriate crosses against genetically homogenous nematode or plant populations. The host or the parasite later simplifies the genetic system of the interaction and allows genetic analyses which may give confirmed results.

10.6 Genetic Models of Plant Parasitism by Nematodes

Analysis of mutants has been an extremely powerful approach to unravel complex biological mechanisms in organisms such as *C. elegans*, *Arabidopsis thaliana*, and *Drosophila melanogaster*. Unfortunately, the artificial generation of

phytonematode mutants altered in their parasitic behavior is still technically challenging and, in most cases, presumably lethal. Thus, plant nematologists have to confine their studies to the genetic variation offered by nature. A well-known group of naturally occurring variants among plant-parasitic nematodes is those revealed by their (in)ability to reproduce on host plants that carry major resistance genes. Most of the reported variants in nematode virulence can be explained by gene-for-gene relationships with their hosts, similar to what is observed with many microbial plant pathogens (Davis 2000). For one nematode/plant combination, a gene-for-gene relationship has been confirmed by genetic analyses of both interacting partners. Virulence tests of 15 F2 lines, obtained by selfing of the F1 of a cross between a virulent and avirulent line, showed that virulence in *G. rostochiensis* toward the *H1* gene in potato is controlled by a single recessive gene. Although Mendelian proof for both interacting partners remains scarce, evidence is accumulating that such gene-for-gene mechanisms are common among plant/nematode interactions.

The most evolutionary advanced adaptations for plant parasitism by nematodes are the products of parasitism genes expressed in their esophageal gland cells and secreted through their stylet into the host tissue to control the complex process of parasitism (Hussey et al. 2002a, b). Molecular analyses of nematode parasitism genes are revealing the complexity of the tools that enable the nematode to attack plants, and the results paint a more elaborate picture of host cellular events under specific control by the parasite than previously hypothesized. Interestingly, the majority of the parasitism genes discovered encodes proteins unique to plant-parasitic nematodes. Identifying the nematode parasitome, i.e., the complete profile of parasitism gene products secreted through the nematode stylet during the parasitic cycle, is the key to understanding the molecular basis of nematode parasitism of plants. Such knowledge will identify vulnerable points in the parasitic process that can be interfered with to achieve nematode control to limit nematode-induced yield losses in crops.

Phytonematodes deploy a broad spectrum of feeding strategies, ranging from simple grazing to the establishment of complex cellular structures, including galls in host tissues. Various models of feeding site formation have been proposed, and a role for phytohormones has long been speculated by Bird and Koltai (2000) although whether they perform a primary or secondary function was unclear. On the basis of recent molecular evidence, they presented several scenarios involving phytohormones in the induction of giant cells by root-knot nematode and presented the models for horizontal gene transfer. Also discussed is the origin of parasitism by nematodes, including the acquisition of genes to synthesize or modulate phytohormones.

At present more than 25 major resistance genes (R genes) against nematodes have been mapped. With the exception of the first nematode R gene identified, *Hs1pro-1*, the other cloned nematode R genes share various structural features with other plant disease resistance genes that operate in gene-for-gene relationships. Several nematode R genes are members of a family characterized by a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs). Recent cloning of the potato cyst nematode resistance gene *Gpa2* also revealed NBS and LRR domains. Interestingly, the *Gpa2* gene has a remarkably high homology with the virus resistance gene *Rx*. Various studies have shown that *Rx*-mediated resistance against potato virus X is a gene-for-gene mechanism in which the R gene encodes a putative receptor that recognizes the viral coat protein as an avirulence gene product. A major challenge in plant nematology is to identify the avirulence gene products of parasitic nematodes. To reach this goal, various research groups have conducted selections of virulent and avirulent nematode lines. Such lines have been established for *H. schachtii*, *M. incognita*, *H. glycines*, and *G. rostochiensis*. Root-knot nematodes have been subjected to rigorous selection experiments to generate parasitic variants in these asexual nematode species. Selection experiments with *M. incognita* against the *Mi* resistance gene of tomato showed a slow but progressive increase

in the proportion of virulent nematodes after each generation, suggesting a polygenic inheritance.

10.7 Parasitism Genes

Although it is currently not possible to predict the number of members of the parasitome of plant-parasitic nematodes, only a small fraction of the estimated 15,000–20,000 genes (based on the ~ 19,000 genes of *Caenorhabditis elegans*) of a plant-parasitic nematode should be expected to encode proteins that have a direct role in parasitism. The first members of a parasitome to be cloned from plant-parasitic nematodes were ß-1,4-endoglucanases (cellulases) developmentally expressed in the two subventral gland cells of *Heterodera glycines* and *Globodera rostochiensis* (Smant et al. 1998). A smaller cellulase cDNA in *G. rostochiensis* (Gr-eng-2) lacks the CBD, and one (Hg-eng-2) from *H. glycines* is missing both the peptide linker and CBD. The presence of a CBD presumably enhances cellulase activity toward crystalline cellulose. mRNA in situ hybridization and immunolocalization with anti-ENG polyclonal sera confirmed that eng-1 and eng-2 were expressed exclusively within the subventral esophageal gland cells of both nematode species.

Differential screening of gene expression has been the most widely used method to clone parasitism genes expressed within the esophageal gland cells of plan parasitic nematodes. Esophageal gland regions from second-stage juveniles of *M. javanica* were excised, and cDNA was prepared from this tissue by reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA pool was differentially screened against cDNA from the nematode tail region to isolate genes that are upregulated or expressed specifically in the esophageal gland region. A full-length cDNA clone that had homology to a bacterial chorismate mutase was obtained with this screening strategy (Lambert et al. 1999). Expression of Mj-cm-1 is localized within the subventral esophageal gland cells of parasitic *M. javanica* by mRNA in situ hybridization and with antisera generated to the product of Mj-cm-1. Chorismate mutase initiates the conversion of chorismate, the end product of the shikimate pathway, to the aromatic amino acids, phenylalanine and tyrosine. The secretion of Mj-cm-1 into the cytosol of a plant cell could potentially alter the spectrum of chorismate-dependent compounds, which, among other functions, are involved in cell wall formation, hormone biosynthesis, and synthesis of defense compounds in plants. Alternatively, these compounds (tyrosine) could be used by the nematode in cuticle formation. RNA fingerprinting has been used to analyze differential gene expression between preparasitic and parasitic stages of *M. incognita*. A cDNA encoding for a secretory cellulose-binding protein (Mi-cbp-1) was isolated using this method (Ding et al. 1998). Mi-cbp-1 is specifically expressed in the subventral gland cells of *M. incognita*, and in vitro analysis confirmed the secretion of Mi-cbp-1 through the nematode stylet. The N-terminal region of the predicted peptide has no similarity to known proteins, but the C-terminus has strong homology to a CBD. Two of the candidate parasitism genes identified share homology with Ran-binding proteins and are hypothesized to be involved in feeding cell induction (Qin et al. 2002).

Proteinaceous stylet secretions from nematodes that are synthesized in the esophageal gland cells are considered as primary signaling molecules at the plant–nematode interface because the morphology, contents, and activity of the gland cells change in relation to nematode migration within plant tissues, feeding cell formation, and nematode feeding activity (Hussey 1989). The genes encoding these secretions have been termed parasitism genes. The first phytonematode parasitism genes identified encoded cellulases (endoglucanases) synthesized in the esophageal gland cells of cyst nematodes that were expressed and secreted only during nematode migration within roots. These were the first endogenous endoglucanase genes cloned from an animal and phylogenetic analysis, which indicated strong similarity to cellulase genes of soil bacteria, suggesting the potential for ancient horizontal gene transfer as a mechanism of gene acquisition in nematodes (Davis et al. 2000a, b).

A number of nematode parasitism genes encoding other cell wall-modifying proteins, including

the first non-plant expansin (Qin 2004), have since been identified that are expressed in the esophageal gland cells during nematode migration in plant tissues. Beyond cell wall modifications, phytoparasitic nematodes appear to be armed with a suite of stylet secretions to modulate many of the features observed in nematode feeding cells. Genes encoding secreted chorismate mutase (CM) that are most similar to bacterial CM have been isolated from root-knot and soybean cyst nematodes. Chorismate mutase is a pivotal enzyme in the shikimic acid pathway that modulates synthesis of "Phe" and "Tyr," having pleiotropic effects on cellular metabolism and auxin synthesis and as precursors of plant defense compounds. Expression of nematode CM in tissues affected the vascular tissue differentiation and was indirectly related to local indole-3-acetic acid concentrations and cellular partitioning of chorismate (Doyle and Lambert 2002).

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

The signal peptide-selection, microarray, and SSH analyses of gland cell cDNA libraries provided a sampling of parasitism genes expressed within *H. glycines*, but the apparent complexity of the libraries suggested that a more comprehensive approach was necessary to obtain a complete profile of the nematode parasitome (Hussey et al. 2002a, b). The *H. glycines* gland cell library generated by LD-PCR was macroarrayed on nylon membranes for indexing, and ESTs of 3,711 cDNA clones were analyzed. The presence of the signal peptide identified these gland cell proteins as candidates for being secreted through the nematode's stylet and potentially having a biological function in *H. glycines* parasitism of soybean.

In EST analyses of parasitism genes in root-knot nematodes, 37 unique clones from a gland cell-specific cDNA library were expressed within the subventral (13 clones) or dorsal (24 clones) esophageal gland cells of *M. incognita* (Huang et al. 2002). In BLASTP analyses, 73 % of the predicted proteins were novel proteins, and those with similarities to known proteins included a pectate lyase, acid phosphatase, and hypothetical proteins from other organisms. Molecular analysis of genes expressed in the esophageal gland cells is proving to be the most direct and efficient approach for identifying nematode parasitism genes. These direct molecular studies are providing for the first time new and surprising information on the complexity and dynamics of the parasitome of a multicellular parasite (Hussey et al. 2002a, b). Obtaining a comprehensive profile of the parasitome is critical for dissecting the molecular signaling events and regulatory mechanisms involved in nematode parasitism of crops by these economically important pathogens.

Another group of candidate secreted nematode parasitism gene products that may also augment host cellular metabolism includes members of the proteasome (Skp-1, RING-H2, and ubiquitin extension protein) with significant similarity to plant genes involved in selective host cell protein degradation. Several proteins have been identified in nematode secretions, and in some cases, their roles in parasitism have been determined (Table 10.2).

Root-knot and cyst nematode genes with known putative functions in parasitism, mostly based on similarities to characterized proteins in other organisms, are known. In addition to the parasitism proteins with similarity to characterized proteins, there are an even larger number of parasitism genes from root-knot and cyst nematodes for which no similarities to characterized proteins in other organisms exist.

Table 10.2 Some gene products secreted from the esophageal glands of phytonematodes

Gene product	Species in which identified	Organisms with close homologues	Possible function
β-1,4-Endoglucanase (cellulase)	*Globodera rostochiensis*	Bacteria	Cell wall degradation
	G. tabacum		
	Heterodera glycines		
	Heterodera schachtii		
	Meloidogyne incognita		
Pectate lyase	*Meloidogyne javanica*	Bacteria and fungi	Cell wall degradation
	G. rostochiensis		
	H. glycines		
Polygalacturonase	*M. incognita*	Bacteria	Cell wall degradation
Chorismate mutase	*H. glycines*	Bacteria	Alter auxin balance
	M. javanica		Feeding cell formation
	G. rostochiensis		
Thioredoxin peroxidase	*G. rostochiensis*	Animal-parasitic nematodes	Breakdown of H_2O_2, protect against host defenses
Venom allergen-like protein	*M. incognita*	Animal-parasitic nematodes,	Early parasitism?
	H. glycines	*C. elegans*	
Calreticulin	*M. incognita*	Animal-parasitic nematodes	Early parasitism?

10.8 Genetic Analysis of Nematode Parasitism

A parasite must reproduce to successfully complete its life cycle. In this sense, the ability of a *H. glycines* individual to parasitize a soybean plant is measured by reproduction. In general, resistant hosts do not support female nematode development to reproductive maturity. Thus, parasitism of a particular host genotype is a qualitative trait that the individual nematode either possesses or does not. Nematode populations may be additionally described quantitatively by their level of reproduction on a given host plant. Field populations of *H. glycines* are mixtures of many genotypes, some of which may confer the ability to overcome host resistance genes. Selection pressure from growing resistant cultivars can alter the frequency of alleles in the population for reproducing on a resistant host. Because of the importance of soybean cyst nematode as a pathogen, and also the identification and utilization of host resistance by soybean breeders, a considerable body of literature exists on the genetic basis of parasitism in *H. glycines* (Dong et al. 1997). It is generally believed that both major and minor genes (including dominant, partially dominant, and recessive alleles) are all involved to some degree in conferring resistance to *H. glycines* (Triantaphyllou 1987), although it is not clear which genes are essential and which are specific to certain nematode genotypes, if any. Interpretation is complicated by the use of *H. glycines* field populations to evaluate resistant soybean; field populations are highly heterogeneous, both among and within isolates. Results from population measurements usually are biased by this genetic variability, and the frequency of certain genes for parasitism (nematode genes necessary to overcome host resistance) may affect phenotypic designation of either parasitism or the levels of reproduction. Therefore, it is believed that the previous results are not accurate indications of the genetic basis of soybean parasitism in *H. glycines* (Opperman and Bird 1998).

Several parasitism genes are essential genes; however, the converse is not the case. Johnsen and Baillie (1997) estimated that 15–30 % of *C. elegans* genes are essential and this is the largest single class in *C. elegans*. Although mutations at

many other loci can give drastic phenotypes, the functions encoded by these genes appear to be dispensable for reproduction per se, so they are not classified as essential (Opperman and Bird 1998). This assignment is, however, to a large degree, an artifact of the way *C. elegans* is maintained in the laboratory. For example, the second largest class of genes in *C. elegans* is that in which mutation gives an uncoordinated (Unc) phenotype. Because coordinated movement is dispensable for a free-living nematode lying on a Petri plate in a sea of bacteria, the Unc loci are considered to be nonessential. In contrast, the equivalent genes (and many others) are almost certainly essential for obligate parasites such as *H. glycines*. For these nematodes to reproduce, they must locate a host, invade, and select and establish a feeding site, events that certainly require coordinated movement and behavior. Thus, correct interpretation of genetic ablation experiments requires an assay that accurately scores disruption of the specific parasitic interaction being tested. Being able to phenocopy a previously characterized genetic phenotype by reverse genetics would be a powerful confirmation of equivalent function and underscores the power of classical genetics to study parasitism.

Significant progress on the genetics of parasitism in nematodes has been made in plant-parasitic species, particularly *Globodera rostochiensis* and *H. glycines* (Opperman and Bird 1998). This is partly because these nematodes are sexually dimorphic, obligate amphimictic species, making them genetically tractable, but also because plants are experimentally more amenable as hosts than are many animals, especially in the numbers required for classic genetics. Importantly, it has proven possible to score for parasitism traits that enable particular nematode genotypes to evade host defense responses. A gene-for-gene relationship appears to be in operation in the case of the golden potato cyst nematode–potato interaction. Potatoes carrying the dominant H1 gene are resistant to certain pathotypes of *G. rostochiensis*. Pure parasitic and nonparasitic lines of *G. rostochiensis* have been selected, and crosses using these lines have revealed that parasitism is inherited at a single locus in a recessive manner (Janssen et al. 1991). However, results from reciprocal crosses suggested that there is no evidence for sex-linked inheritance of parasitism.

Dong et al. (1997) developed pure lines of *H. glycines* that carry single genes for parasitic ability on soybeans and were used to demonstrate that *H. glycines* contains unlinked dominant and recessive genes for parasitism of various host genotypes; parasitism genes in *H. glycines* were analyzed by crossing two highly inbred lines (>29 generations). A nonparasitic *H. glycines* line, which fails to reproduce on the resistant soybean lines PI88788 and PI90763, was used as the female and recurrent parent and was crossed to a parasitic line that does reproduce on these resistant hosts. The segregation ratio of the progeny lines developed by single female inoculation revealed that parasitism to these soybean lines is controlled by independent, single genes in the nematode. In accord with genetic nomenclature rules for parasitic nematodes, these loci were named ror for reproduction on a resistant host (Dong et al. 1997). In the inbred lines, ror-1(kr1) confers the ability to reproduce on PI88788 and is dominant. The recessive gene, ror-2(kr2), controls reproduction on PI90763. A second recessive gene, ror-3(kr5), controls the ability to parasitize the soybean line Peking. Although not verified, it is an intriguing possibility that some genes controlling parasitism may be acting additively. Examination of F1 data from controlled crosses revealed that the presence of two ror genes results in twice as many females being formed on PI88788 as when only one of these genes is present. This may explain varying levels of aggressiveness between different nematode populations on the same host genotype. It is particularly significant to note that these loci are entirely independent and do not appear to interact; that is to say, no novel host ranges are detected when combinations of ror genes are present in a particular nematode line. In addition to alleles for parasitism of resistant soybeans, there are SCN lines that have been selected to reproduce on tomato (Opperman and Bird 1998). The genes controlling this host acquisition remain to be characterized, either at the genetic or at the molecular level.

10.8.1 Cell Wall-Digesting Enzyme

The major structural component of the plant cell wall is cellulose, the most abundant biopolymer in the world (Davis et al. 2011). Cellulose is composed of successive glucose residues which are inverted 180°, forming a flat ribbon with cellobiose as the repeating unit. These (1,4)-β-linked glucan chains are able to form extensive hydrogen bounds to adjacent glucan chains. Approximately 36 of these crystalline chains are arranged in parallel in 3-nm-thick microfibrils forming insoluble cable-like structures. Cellulose microfibrils are among the longest molecules known in nature, since they are believed to consist of 8,000 (primary cell wall) to 15,000 (secondary cell wall) glucose molecules. Glycoside hydrolases are enzymes that catalyze the hydrolysis of the glycosidic bonds in sugar polymers. These glycosyl hydrolases are classified into different families according to their sequence similarity (Henrissat and Bairoch 1996). Cellulases or endo-1,4-β-glucanases, for example, are capable of degrading cellulose by hydrolyzing the (1,4)-β bonds. Several endoglucanases (or cellulases, EC 3.2.1.4) belonging to different glycosyl hydrolase families have been found in nematodes, facilitating the penetration and migration of the nematode through the plant cell wall.

The sclerotized, protrusible stylet of phytoparasitic nematodes provides a tool to mechanically breach the host plant cell wall. Such stylet activity can be readily observed for nematodes grown in monoxenic plant root culture and has been documented for both ectoparasitic and endoparasitic nematodes in video microscopy (Davis et al. 2011). An early body of evidence suggested that nematodes also secrete hydrolytic cell wall-degrading enzymes to assist in this process. Protein extracts and exudates from a number of phytoparasitic and fungal-feeding nematode species contained cellulolytic, amylolytic, chitinolytic, and pectolytic enzyme activity, suggesting the potential for endogenous production and secretion of cell wall-degrading enzymes from nematodes.

Most of the identified endoglucanases in nematodes belong to glycosyl hydrolase family 5 (GHF5). GHF5 endoglucanases were found in several nematodes belonging to the superfamily of the Tylenchoidea (order Rhabditida, suborder Tylenchina, infraorder Tylenchomorpha) (De Ley and Blaxter 2002). The majority belongs to the well-studied sedentary nematode genera *Heterodera*, *Globodera*, and *Meloidogyne*. Besides these sedentary nematodes, GHF5 endoglucanases have also been identified in the migratory nematodes *Radopholus similis*, *Ditylenchus africanus*, and *Pratylenchus* species. The GHF5 endoglucanases consist of several domains. They all have a signal peptide, which is required to secrete the protein, and a catalytic domain with the actual enzyme activity. Some endoglucanases have an additional carbohydrate-binding module (CBM) at the C-terminal end of the protein, which is thought to aid the enzyme in binding to its substrate.

The root-knot nematodes and cyst nematodes use a mixture of enzymes to soften root-cell walls, which should aid in penetration through the root epidermis as well as migration within root tissues (Yan et al. 1998). To date, there have been cellulase and pectinase genes described for root-knot nematode and cyst nematode species. The discovery of cellulase genes in the soybean and potato cyst nematodes represented the first major breakthrough in parasitism gene discovery. Hewezi et al. (2008) reported that phytocyst nematodes secrete a complex of cell wall-digesting enzymes, which helps in root penetration and migration. *Heterodera glycines* also produces a secretory cellulose-binding protein (Hg CBP). To determine the function of CBP, an orthologous cDNA clone (Hs CBP) was isolated from the sugar beet cyst nematode *H. schachtii*, which is able to infect *Arabidopsis thaliana*. CBP is expressed only in the early phases of feeding cell formation and not during the migratory phase. Transgenic *Arabidopsis* expressing Hs CBP developed longer roots and exhibited enhanced susceptibility to *H. schachtii*. A yeast two-hybrid screen identified *Arabidopsis* pectin methylesterase protein 3 (PME3) as strongly and specifically interacting with Hs CBP. Transgenic plants overexpressing PME3 also produced longer roots and exhibited increased susceptibility to *H. schachtii*,

while a pme3 knockout mutant showed opposite phenotypes. Moreover, CBP overexpression increases PME3 activity in plants. Localization studies supported the mode of action of PME3 as a cell wall-modifying enzyme. Expression of CBP in the pme3 knockout mutant revealed that PME3 is required but not the sole mechanism for CBP overexpression phenotype. They concluded that CBP directly interacts with PME3, thereby activating and potentially targeting this enzyme to aid cyst nematode parasitism.

The identification of endogenous genes encoding multiple types of cell wall-degrading enzymes in phytoparasitic nematodes has confirmed early physiological evidence for their expression and potential roles in plant parasitism. Since the initial identification of endoglucanase genes in cyst nematodes, both a candidate gene approach and extensive EST analyses have been the primary means of gene identification. The genome sequences of both *M. incognita* and *M. hapla* have not only confirmed the presence of multiple cell wall-modifying genes that were found in expressed sequence analyses, but they have revealed how unexpectedly large some of these gene families are, most notably the genes encoding pectolytic enzymes and expansin-like proteins (Opperman et al. 2008). The existence of gene families that encode cell wall-modifying enzymes in nematodes presents the potential for functional redundancy, although the biological significance of this potential remains unclear. The expression of nematode cell wall-modifying enzymes is almost exclusively localized within the esophageal gland secretory cells and developmentally consistent with the putative functional role of these secretions in migratory life stages of phytoparasitic nematodes.

10.8.2 Expansins

Expansins are extracellular, cell wall-loosening proteins involved in growth and cell wall disassembly. They mediate pH-dependent extension of the plant cell wall and growth of the cell. In many plants, they were found to be involved in a variety of growth processes including cell expansion, cell differentiation, and cell wall disassembly and breakdown (e.g., softening of fruits). Expansins belong to relatively conserved protein subfamilies, the alpha-, beta-, and gamma-expansins. Currently, 12 expansins in *Lycopersicum* are known. Alpha-expansins are involved in the auxin- and ethylene-mediated expansion and ripening of tomato fruits. These processes are similar to the syncytium (NFS – nematode feeding site) formation by cyst nematodes (*Heterodera schachtii*, Hs; *Globodera rostochiensis*, Gr), especially in the expansion and ripening phase.

Expansins are known to play an important role in cell wall formation and modification. Therefore it can be anticipated that they are involved in plant–pathogen interactions that go along with major structural changes in cell wall architecture, such as the formation of hypertrophic and hyperplastic tissues (Wieczorek et al. 2006). Expansins were first identified more than a decade ago as the key cell wall factors responsible for "acid growth." Characteristically, expansions induce cell wall extension at an acidic pH optimum in vitro and enhance stress relaxation of isolated cell walls over a broad time range. They comprise two major gene families: a-expansins (EXPA) and b-expansins (EXPB). EXPA proteins bind tightly to cellulose and hemicellulose, but they have no hydrolytic activity against these major polysaccharides of the cell wall. Expansins disrupt non-covalent bonding between cellulose microfibrils and matrix glucans, thereby allowing turgor-driven slippage of microfibrils relative to one another. Comparable studies of EXPB binding and hydrolytic activity have not yet been published, but their wall-loosening action is similar to that of EXPA.

Nematodes secrete proteins with sequence similarity to expansins (Qin 2004). Nematode secretions containing these and other cell wall-loosening proteins may assist the rapid penetration of the nematode into the root tissues. However, the highly orchestrated patterns of altered cell growth and syncytium formation would seem to require more subtle spatial and temporal control of cell wall loosening and growth processes that could not be achieved through nematode secretion alone. In addition to the ability to break down

covalent bonds found in plant cell walls through cellulases and pectinases, there is evidence that the potato cyst nematode also secretes a protein having the ability to break non-covalent bonds (Qin 2004). This activity is accomplished by an expansin-like protein discovered in the potato cyst nematode. Expansins soften cell walls by breaking non-covalent bonds between cell wall fibrils, thereby allowing a sliding of fibrils past each other. Wieczorek et al. (2006) analyzed whether members of the expansin gene family are specifically and developmentally regulated during syncytium formation in the roots of *Arabidopsis thaliana*. PCR was used to screen a cDNA library of 5–7-day-old syncytia for expansin transcripts with primers differentiating between 26 alpha- and three beta-expansin cDNAs. AtEXPA1, AtEXPA3, AtEXPA4, AtEXPA6, AtEXPA8, AtEXPA10, AtEXPA15, AtEXPA16, AtEXPA20, and AtEXPB3 could be amplified from the library. In a semiquantitative RT-PCR and a Genechip analysis, AtEXPA3, AtEXPA6, AtEXPA8, AtEXPA10, and AtEXPA16 were found to be upregulated specifically in syncytia, but not to be transcribed in surrounding root tissue. Histological analyses were performed with the aid of promoter:GUS lines and in situ RT-PCR. Results from both approaches supported the specific expression pattern. Among the specifically expressed genes, AtEXPA3 and AtEXPA16 turned out to be of special interest as they are shoot specific in uninfected plants. It was concluded that syncytium formation involves the specific regulation of expansin genes, indicating that the encoded expansins take part in cell growth and cell wall disassembly during syncytium formation.

Griesser and Grundler (2013) investigated gene expression patterns and localization of expansins in a comparative analysis. Expansins are cell wall-loosening proteins involved in growth and cell wall disassembly. The expression of expansins in syncytia of *G. rostochiensis* in tomato and in syncytia and galls induced in *Arabidopsis thaliana* has already been described. They provided additional information on the expression of 10 tomato expansin isoforms, namely, *LeEXPA1*, *LeEXPA2*, *LeEXPA3*, *LeEXPA4*, *LeEXPA5*, *LeEXPA8*, *LeEXPA9*, *LeEXPA10*, *LeEXPA*11, and *LeEXPA18* in 5- and 10-day-old galls of *M. incognita* with sqRT-PCR. They also determined the quantitative expression of seven differentially regulated tomato expansins in syncytia and galls at different developmental stages. They observed a very high induction of *LeEXPA2*, *LeEXPA5*, and *LeEXPA11* with maxima in 10-day-old syncytia and 5-day-old galls. Other members of the gene family were slightly induced in syncytia, whereas in galls only *LeEXPA2*, *LeEXPA5*, and *LeEXPA11* were found to be upregulated. Previous results on the expression of *LeEXPA5* in galls were confirmed, and new detailed information on expansin expression in nematode feeding site was provided. *LeEXPA4* and *LeEXPA5* were localized in syncytia recently, and these results were confirmed with in situ RT-PCR. *LeEXPA1*, *LeEXPA2*, *LeEXPA9*, *LeEXPA11*, and *LeEXPA18* were also detected in 5- and 10-day-old syncytia and neighboring cells. Especially the expression pattern of *LeEXPA2* and *LeEXPA5* was of interest, because of their low expression in uninfected roots but their high induction in nematode feeding sites. These results confirmed that expansins are differentially regulated during the formation of both syncytia and galls and indicate that these genes are involved in cell wall-modifying processes during plant–nematode interactions.

During syncytium development, extensive cell wall modifications take place. Cell wall dissolution occurs during cell wall opening formation, cell walls expand during hypertrophy of syncytial elements, and local cell wall synthesis leads to the thickening of syncytial cell wall and the formation of cell wall ingrowths. Numerous studies revealed that nematodes change expression of plant genes encoding cell wall-modifying proteins including expansins. Expansins poses unique abilities to induce cell wall extension in acidic pH. Fudali et al. (2008) demonstrated that two α-expansin genes *LeEXPA4* and *LeEXPA5* were upregulated in tomato roots infected with *Globodera rostochiensis*. They also presented the most recent results concerning the involvement of plant cell wall-modifying genes in syncytium development and discussed possible practical

applications of this knowledge for developing plants with resistance against nematodes.

10.8.3 Metabolic Enzymes

These enzymes catalyze the conversion of the shikimate pathway product chorismate to prephenate. This process represents a key regulatory mechanism determining the ratio of the aromatic amino acids phenylalanine and tyrosine on one hand and tryptophan on the other. Consequently, this regulatory activity influences the production of the metabolites that have these amino acids as precursors, among which auxin and salicylic acid are of particular interest in plant–parasite interactions. The plant shikimate pathway is found in the plastids from where chorismate also is translocated to the plant cytoplasm. According to the current understanding of chorismate mutase function, nematode-secreted chorismate mutases will deplete the cytoplasmic chorismate pool leading to an increased translocation of chorismate from the plastids, effectively decreasing synthesis of plastid-produced chorismate-dependent metabolites like auxin or salicylic acid. A lack of salicylic acid production in response to nematode chorismate mutase injection could result in a downregulation of plant defenses.

10.8.4 Ubiquitination/Proteasome Functions

The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. The proteasome is a protein-destroying apparatus involved in many essential cellular functions, such as the regulation of cell cycle, cell differentiation, signal transduction pathways, antigen processing for appropriate immune responses, stress signaling, inflammatory responses, and apoptosis (Hirano et al. 2005). It is capable of degrading a variety of cellular proteins in a rapid and timely fashion, and most substrate proteins are modified by ubiquitin before their degradation by the proteasome. The proteasome is a large protein complex consisting of a proteolytic core called the 20S particle and ancillary factors that regulate its activity in various ways.

The most common form is the 26S proteasome containing one 20S core particle and two 19S regulatory particles that enable the proteasome to degrade ubiquitinated proteins by an ATP-dependent mechanism. Another form is the immunoproteasome containing two 11S regulatory particles, PA28 alpha and PA28 beta, which are induced by interferon gamma under the conditions of intensified immune response. Other regulatory particles include PA28 gamma and PA200. Although PA28 gamma also belongs to a family of activators of the 20S proteasome, it is localized within the nucleus and forms a homoheptamer. PA28 gamma has been implicated in the regulation of cell cycle progression and apoptosis. PA200 has been identified as a large nuclear protein that stimulates proteasomal hydrolysis of peptides. The proteasome is in the final common step of protein degradation and is part of a pathway called the ubiquitin–proteasome pathway. Ubiquitin effectively tags proteins and marks them for presentation to the proteasome, where the protein is digested, and ubiquitin is actually recycled in the cell. Ubiquitin is the marking agent to covalently link the protein and present it to the proteasome structure.

Targeted and timed protein degradation is a final and powerful means to regulate gene expression. Cyst nematodes apparently use this mechanism to alter gene expression in parasitized plant cells since these nematodes appear to secrete proteins involved in polyubiquitination, i.e., the process that specifically decorates proteins with ubiquitin protein molecules, thereby targeting these proteins for degradation.

10.8.5 Venom Allergen Proteins

The venom allergen-like proteins form a family of effectors that seems to be conserved among all parasitic nematodes of plants and animals studied to date. The venom allergen-like protein

of *Globodera rostochiensis* Gr-VAP1 interacts with the apoplastic cysteine papain-like proteases Rcr3pim of *Solanum pimpinellifolium* (Lozano Torres et al. 2013). They reported that Gr-VAP1 and Rcr3pim are both required to activate defense-related programmed cell death and resistance to nematodes mediated by the extracellular plant immune receptor Cf-2 in tomato. Thus, Gr-VAP1 is able to trigger defense responses in a host plant of *G. rostochiensis*, but the virulence function of Gr-VAP1 or of any other venom allergen-like protein of a phytonematode is not known. A specific knockdown of Gr-VAP1 expression in *G. rostochiensis* showed that the effector is indeed important for virulence of infective juveniles in host plants. Similarly, the ectopic expression of venom allergen-like proteins in transgenic plants alters their response to nematodes and other plant pathogens. RNAseq analysis of these transgenic plants has shed light on the molecular mechanisms underlying the virulence function of venom allergen-like protein of plant-parasitic nematodes in plants.

Animal and phytonematodes have the capability to remain within the host for a long time. To do so, they have evolved immunoevasive and immunosuppressive strategies. Secretory proteins produced in the esophageal glands of parasitic nematodes likely include suppressors of plant innate immunity (Lozano et al. 2009). A venom allergen protein from *Globodera rostochiensis* (Gr-vap1) was identified, by cDNA-AFLP, as being strongly upregulated in invasive second-stage juveniles. In situ hybridization microscopy showed specific expression of Gr-vap1 in the subventral esophageal glands. Gr-vap1 codes for a secretory protein, including a single SCP/CAP domain. Temporal expression analysis of Gr-vap1 in different developmental stages revealed upregulation in the motile J2s and adult males. Knocking down Gr-vap1 expression, by RNA interference, significantly reduced the infectivity of nematodes on host plants. Protein interaction studies using Gr-vap1 and a tomato root cDNA library, in a yeast two-hybrid screening, resulted in the identification of various interacting host proteins associated with plant immunity. A pull-down assay confirmed the physical interaction of Gr-vap1 with Rcr3, an extracellular cathepsin-like cysteine protease from tomato. Others showed that Rrc3 is required for disease resistance to fungi and oomycetes in plants. However, heterologous expression of nematode VAPs in *Arabidopsis thaliana* caused enhanced susceptibility toward diverse plant pathogens. It was hypothesized that VAPs are important modulators of innate immunity and as such interfere with different host defense response pathways.

These parasitism protein candidates are similar to known proteins whose functions, however, are still unknown or too diverse. This intriguing group of parasitism proteins contains representatives from root-knot nematodes and cyst nematodes that are collectively called "venom allergen proteins" (vaps). Gene sequences for these venom proteins were first described from hymenopteran insects, and vaps were also identified as secreted proteins (ASP) in the animal-parasitic nematode, *Ancylostoma caninum*. Secretory proteins encoded by genes expressed in the esophageal gland cells of phytonematodes have key roles in nematode parasitism of plants (Gao et al. 2001b). Two venom allergen-like protein cDNAs (designated hg-vap-1 and hg-vap-2) were isolated from *Heterodera glycines* gland cell cDNA libraries. Both cDNAs hybridized to genomic DNA of *H. glycines* in Southern blots. The hg-vap-1 cDNA contained an open reading frame encoding 215 amino acids with the first 25 amino acids being a putative secretion signal. The hg-vap-2 cDNA contained an open reading frame encoding 212 amino acids with the first 19 amino acids being a putative secretion signal. Genes of hg-vap-1 and hg-vap-2 contained four introns, which ranged in size from 44 to 574 bp, and five exons ranging in size from 43 to 279 bp. In situ hybridization analyses showed that mRNAs of both vap genes accumulated specifically in the subventral gland cells of *H. glycines* during parasitism. The gland cell-specific expression and the presence of predicted secretion signal peptides in both VAPs suggest that these proteins are secreted from the nematode and may play a role in the infection of host plants by this parasite.

Secretions from the esophageal glands of *Bursaphelenchus xylophilus* play an important

role in pathogenicity (Shifeng Lin et al. 2011). A cluster of three venom allergen-like protein genes and one pseudogene, BxVap-1, BxVap-2, and BxVap-3 and BxVap-P, were identified within a 3.7-kb region. Additionally, three putative modification, transport, and regulatory protein genes were also detected in the same flanking region of the BxVap gene cluster. Genes vap-1, vap-2, and vap-3 were functional and encoded three major allelic variants of PWN venom allergen-like proteins. But BxVap-P was an untranscribed pseudogene. Genes vap-1, vap-2, and vap-3 produced predicted products of 204, 206, and 203 amino acid residues, respectively, including the putative signal peptide sequence at the amino termini. In situ mRNA hybridization analysis showed that the transcripts of genes vap-1, vap-2, and vap-3 accumulated exclusively within the esophageal gland cells of *B. xylophilus*.

Of the three genes encoding the venom allergen-like protein in *B. xylophilus*, BxVap-1 showed the highest transcript levels at the pine-grown propagative stage (Kang et al. 2012). In addition, Western blot and immunohistochemical analyses using anti-BxVap-1 polyclonal antibody verified a specific increase in BxVap-1 expression levels at the pine-grown propagative stage. Using immunohistochemistry, BxVap-1 was detected around the putative esophageal glands and metacarpus, suggesting that BxVap-1 is secreted into the host pine tree and is involved in the parasitic mechanism. To explain the parasitic role of BxVap-1, the migration rate inside pine seedlings of *B. xylophilus* was measured either with or without BxVap-1 knockdown by RNA interference. BxVap-1 knockdown resulted in a significantly lower migration rate in the >6-cm region compared with the control *B. xylophilus*. These results suggest that BxVap-1 is involved in *B. xylophilus* migration, perhaps by suppressing the pine tree defense mechanism.

Venom allergen-like proteins are members of the SCP/Tpx-1/Ag5/PR-1/Sc7 family of eukaryotic secreted proteins. Lu Shunwen et al. (2013) identified a VAP gene (designated GrVAP-1) from *Globodera rostochiensis*. The GrVAP-1 gene contains an open reading frame (660 bp) encoding a putative secreted protein that contains a SCP-like domain and a cysteine-rich C-terminus. Southern blot analysis indicated the presence of multiple copies of the GrVAP-1 gene in the *G. rostochiensis* genome. The GrVAP-1 genomic DNA contains three introns with sizes ranging from 48 to149 bp. In situ mRNA hybridization showed the transcript of GrVAP-1 accumulated exclusively within the subventral esophageal gland cells of both preparasitic second-stage juvenile and parasitic stages of *G. rostochiensis*. RT-PCR analysis revealed that the GrVAP-1 gene was highly expressed in both preparasitic J2 and parasitic stages, but its expression was low in the egg stage.

Secretory proteins encoded by genes expressed in the esophageal gland cells of phytonematodes play key roles in nematode parasitism of plants. Two venom allergen-like protein cDNAs (designated hg-vap-1 and hg-vap-2) were isolated from Heterodera glycines gland cell cDNA libraries (Gao et al. 2001a). Both cDNAs hybridized to genomic DNA of *H. glycines* in Southern blots. The hg-vap-1 cDNA contained an open reading frame encoding 215 amino acids with the first 25 amino acids being a putative secretion signal. The hg-vap-2 cDNA contained an open reading frame encoding 212 amino acids with the first 19 amino acids being a putative secretion signal. Genes of hg-vap-1 and hg-vap-2 contained four introns, which ranged in size from 44 to 574 bp, and five exons ranging in size from 43 to 279 bp. In situ hybridization analyses showed that mRNAs of both vap genes accumulated specifically in the subventral gland cells of *H. glycines* during parasitism.

10.8.6 Calreticulin

Calreticulin, also known as calregulin, CRP55, CaBP3, calsequestrin-like protein, and endoplasmic reticulum resident protein 60 (ERp60), is a protein that binds to misfolded proteins and prevents them from being exported from the endoplasmic reticulum to the Golgi bodies. Esophageal secretions from endoparasitic sedentary nematodes have key roles throughout plant parasitism, in particular, during the invasion of the root tissue

and the initiation and maintenance of the nematode feeding site essential for nematode development (Jaubert et al. 2005). Calreticulin-like proteins are secreted from other parasitic nematodes and, therefore, are good candidates for being involved in parasite–host interactions. A calreticulin-like protein preceded by a signal peptide was identified as being produced in the subventral glands of a root-knot nematode.

Root-knot nematodes are obligate biotrophic parasites that settle close to the vascular tissues in roots, where they induce the differentiation of specialized feeding cells and maintain a compatible interaction for 3–8 weeks (Jaouannet et al. 2013). Transcriptome analyses of the plant response to parasitic infection showed that plant defenses are strictly controlled during the interaction. This suggests that, similar to other pathogens, this nematode secretes effectors that suppress host defenses. It was showed that Mi-CRT, a calreticulin (CRT) secreted by the nematode into the apoplasm of infected tissues, played an important role in infection success, because Mi-CRT knockdown by RNA interference affected the ability of the nematodes to infect plants. Stably transformed *Arabidopsis thaliana* plants producing the secreted form of Mi-CRT were more susceptible to nematode infection than wild-type plants. They were also more susceptible to infection with another root pathogen, the oomycete, *Phytophthora parasitica*. Mi-CRT overexpression in *A. thaliana* suppressed the induction of defense marker genes and callose deposition after treatment with the pathogen-associated molecular pattern elf18. These findings showed that Mi-CRT secreted in the apoplasm by the nematode has a role in the suppression of plant basal defenses during the interaction.

The secretion in plants of esophageal cell wall-degrading enzymes by migratory juveniles has been shown, suggesting a role for these enzymes in the invasion phase. Nevertheless, the secretion of an esophageal gland protein into the nematode feeding site by nematode sedentary stages has never been demonstrated. The calreticulin Mi-CRT is a protein synthesized in the esophageal glands of the root-knot nematode *Meloidogyne incognita*. After three-dimensional modeling of the Mi-CRT protein, a surface peptide was selected to raise specific antibodies. In plants, immunolocalization showed that Mi-CRT is secreted by migratory and sedentary stage nematodes, suggesting a role for Mi-CRT throughout parasitism. During the maintenance of the nematode feeding site, the secreted Mi-CRT was localized outside the nematode at the tip of the stylet. In addition, Mi-CRT accumulation was observed along the cell wall of the giant cells that compose the feeding site, providing evidence for a nematode esophageal protein secretion into the nematode feeding site.

10.8.7 Annexin

Annexin is a common name for a group of cellular proteins. The annexins are a class of calcium-dependent, phospholipid-binding proteins that are presumed to underlie a number of calcium-regulated activities on membrane surfaces or between interacting membranes. The mRNA for a secretory isoform of an annexin-like protein was identified as being expressed in the dorsal gland of the soybean cyst nematode. Annexin genes represent a large family coding for calcium-dependent phospholipid-binding proteins with a wide range of reported functions. An annexin gene also had been identified from the potato cyst nematode *G. pallida*. This gene coded for a protein that was immunodetected in the excretory/secretory products of this nematode despite the fact that the protein did not contain a signal peptide and was not present in the esophageal glands.

Nematode parasitism genes encode secreted effector proteins that play a role in host infection (Patel et al. 2010). A homologue of the expressed Hg4F01 gene of *Heterodera glycines,* encoding an annexin-like effector, was isolated by these authors in the related *Heterodera schachtii* to facilitate the use of *Arabidopsis thaliana* as a model host. Hs4F01 and its protein product were exclusively expressed within the dorsal esophageal gland secretory cell in the parasitic stages of *H. schachtii.* Hs4F01 had a 41 % predicted amino acid sequence identity to the nex-1 annexin of *C.*

elegans and 33 % identity to annexin-1 (annAt1) of Arabidopsis, it contained four conserved domains typical of the annexin family of calcium- and phospholipid-binding proteins, and it had a predicted signal peptide for secretion that was present in nematode annexins of only *Heterodera* spp. Constitutive expression of Hs4F01 in wild-type *Arabidopsis* promoted hypersusceptibility to *H. schachtii* infection. Complementation of an AnnAt1 mutant by constitutive expression of Hs4F01 reverted mutant sensitivity to 75-mM NaCl, suggesting a similar function of the Hs4F01 annexin-like effector in the stress response by plant cells. Yeast two-hybrid assays confirmed a specific interaction between Hs4F01 and an *Arabidopsis* oxidoreductase member of the 2OG-Fe(II) oxygenase family, a type of plant enzyme demonstrated to promote susceptibility to oomycete pathogens. RNA interference assays that expressed double-stranded RNA complementary to Hs4F01 in transgenic *Arabidopsis* specifically decreased parasitic nematode Hs4F01 transcript levels and significantly reduced nematode infection levels. The combined data suggested that nematode secretion of an Hs4F01 annexin-like effector into host root cells may mimic plant annexin function during the parasitic interaction.

The recent characterization of an annexin, nex-1, from the nematode, *C. elegans,* suggested that this annexin was associated with collagen secretion and/or deposition, membrane trafficking during autophagocytosis of yolk granules, and with the coordinated folding and unfolding of cell surface membranes during the opening and closing of the spermathecal valve (Creutz et al. 1996). These activities parallel a number of activities the annexins have been postulated to underlie in mammalian cells, such as cartilage formation and mineralization, and the chaperoning of membrane interactions in endocytosis and exocytosis. However, the initial isolation and localization of the major nematode annexin left several important issues unresolved (Daigle and Creutz 1999). First, progress in the sequencing of the nematode genome had revealed the presence of two additional nematode annexin genes (nex-2 and nex-3; Creutz et al. 1996), and more recently, a fourth has been encountered for which we propose the name nex-4 (GenBank acquisition number U88315). Since only a single annexin protein, nex-1, was isolated, it has not been clear whether the other annexin genes are expressed. It is important to know whether these genes are active in order to design and interpret annexin gene knockout experiments since the different annexins may be redundant in function. Evidence was provided for active transcription of mRNA from the nex-2 and nex-3 genes.

A second area that was not addressed in the initial description of the nematode nex-1 annexin was the characterization of this protein as a lipid-binding and aggregating protein regulated by calcium. Different members of the mammalian annexin protein family have characteristic calcium sensitivities and abilities to promote membrane aggregation. Since the nex-1 annexin is 39–42 % identical in sequence to all mammalian annexins, it is not possible to speculate to which mammalian annexin the nex-1 annexin is most closely related on the basis of sequence data alone. They demonstrated that recombinant nex-1 protein produced in a yeast expression system can bind and aggregate biological membranes in a calcium-dependent manner. A third important unresolved issue concerns the identity of the annexin(s) that was localized previously in the nematode using an antiserum directed against nex-1. Because of sequence similarities among the annexins, it is possible that the antiserum reacted with other annexins as well. The specific nex-1 promoter was used to drive the expression of GFP to permit localization of cells that actively express the nex-1 gene. This has led to the discovery of a new site of nex-1 expression, the hypodermal cells of the body wall. A fourth outstanding issue was whether the nex-1 annexin is an intracellular or an extracellular protein.

Although often presumed to be intracellular proteins as a family, some annexins are also released from cells. In some areas of the nematode, particularly the grinder and the spermathecal valve, the nex-1 annexin is highly enriched on the cell surface membrane. However, even at the electron microscope level, it was not possible to resolve whether the protein may be partially

exposed to the extracellular space (Creutz et al. 1996). Daigle and Creutz (1999) injected fluorescently labeled antibodies into non-permeabilized nematodes to determine if any nex-1 is exposed on the extracellular surfaces of nematode tissues.

The nematode, like higher animals and green plants, expresses a diverse family of annexins (Daigle and Creutz 1999). The major annexin, nex-1, is localized to an array of different cell types and may underlie a multiplicity of functions or a common function in an assortment of cells. The most evocative localization is on the intracellular faces of the folds of the membranes in the spermathecal valve where it is likely the annexin functions in the coordinated, calcium-regulated folding of these membranes. Whether this will prove to be a representative model for the role of other annexins in membrane trafficking events will hopefully be revealed by annexin gene knockout experiments in this and other model organisms.

10.8.8 Avirulence Genes

Single pathogen genes that are required for R gene-mediated resistance have been identified in bacteria, viruses, and fungi. There is genetic evidence for avirulence genes in *Globodera rostochiensis* that correspond to the resistance gene *H1*. Genetic analyses of inbred strains of soybean cyst nematodes have identified dominant and recessive determinants of parasitism on different soybean lines. The root-knot species against which *Mi* is effective does not reproduce sexually, making Mendelian analysis of its avirulence and pathogenicity genes impossible. Nearly isogenic strains of root-knot nematodes that differ in virulence in the presence of *Mi* have been used to investigate pathogenicity. Differential-marker analysis identified a polymorphic band that was present in avirulent strains but absent from closely related virulent strains of *M. incognita.* The corresponding gene, *Meloidogyne* avirulence protein-1 (map-1), was cloned and found to encode a protein that localized to nematode amphidial secretions. Secretions from the virulent and avirulent nematodes were not compared, however, and functional analysis of map-1 has not yet been carried out. A transcript that is present in avirulent but lacking in virulent *Meloidogyne javanica* has also been identified. However, this gene does not resemble map-1, suggesting that there may be more than one gene that can mediate nematode recognition in tomato plants that have the *Mi* gene.

Although it is currently not possible to predict the number of members of the parasitome of plant-parasitic nematodes, only a small fraction of the estimated 15,000–20,000 genes (based on the ~19,000 genes of *Caenorhabditis elegans*) of a plant-parasitic nematode should be expected to encode proteins that have a direct role in parasitism. The first members of a parasitome to be cloned from plant-parasitic nematodes were ß-1,4-endoglucanases (cellulases) developmentally expressed in the two subventral gland cells of *Heterodera glycines* and *Globodera rostochiensis* (Yan et al. 1998). Two cellulase cDNAs in each cyst nematode species (Hg-eng-1 and Gr-eng-1) encode a predicted secretion signal peptide, cellulase catalytic domain, small peptide linker, and a cellulose-binding domain (CBD). A smaller cellulase cDNA in *G. rostochiensis* (Gr-eng-2) lacks the CBD, and one (Hg-eng-2) from *H. glycines* is missing both the peptide linker and CBD. The presence of a CBD presumably enhances cellulase activity toward crystalline cellulose. mRNA in situ hybridization and immunolocalization with anti-ENG polyclonal sera confirmed that eng-1 and eng-2 were expressed exclusively within the subventral esophageal gland cells of both nematode species (Smant et al. 1998).

Expression of Mj-cm-1 is localized within the subventral esophageal gland cells of parasitic *M. javanica* by mRNA in situ hybridization and with antisera generated to the product of Mj-cm-1 (Hussey et al. 2002a, b). Chorismate mutase initiates the conversion of chorismate, the end product of the shikimate pathway, to the aromatic amino acids, phenylalanine and tyrosine. The secretion of Mj-cm-1 into the cytosol of a plant cell could potentially alter the spectrum

of chorismate-dependent compounds, which, among other functions, are involved in cell wall formation, hormone biosynthesis, and synthesis of defense compounds in plants. Alternatively, these compounds (tyrosine) could be used by the nematode in cuticle formation.

The most evolutionary advanced adaptations for plant parasitism by nematodes are the products of parasitism genes expressed in their esophageal gland cells and secreted through their stylet into the host tissue to control the complex process of parasitism. Molecular analyses of nematode parasitism genes are revealing the complexity of the tools a nematode possesses that enable it to attack plants (Baum et al. 2007). RNA fingerprinting has been used to analyze differential gene expression between preparasitic and parasitic stages of *M. incognita*. A cDNA encoding for a secretory cellulose-binding protein (Mi-cbp-1) was isolated using this method (Ding et al. 1998).

Nematode esophageal gland cell secretions are released through valves within ampulla for transport out of the stylet (feeding spear) into host tissues. Cell wall-modifying proteins (endoglucanases, pectinases, hemicellulases, and expansin) may be secreted to aid the migration of infective juveniles through host plant tissues. Other nematode gland cell secretions might have multiple roles in the formation of specialized feeding cells by the nematode, including effects on host cell metabolism by secreted chorismate mutase; signaling by secreted nematode peptides such as homologues to plant CLAVATA/ESR-related peptides; selective degradation of host proteins through the ubiquitin–proteasome pathway by UBQ, S-phase kinase-associated protein 1 (Skp-1), and RING-H2 secreted from the nematode; and potential effects of secreted nematode proteins that contain nuclear localization signals within the host cell nucleus.

The first members of a parasitome to be cloned from plant-parasitic nematodes were ß-1,4-endoglucanases (cellulases) in *Heterodera glycines* and *Globodera rostochiensis* (Dong and Opperman 1997). Parasitism gene may be active in any or all part of the parasitic cycle. The ability of nematode to live on plant hosts involves multiple parasitism genes. Root-knot and cyst nematodes have evolved to alter gene expression in specific root cell to modify them into specialized cells. Genes for resistance or parasitism are altered by modifier genes present in each genome and are influenced by various environmental factors, which may be biotic or abiotic.

With *C. elegans*, the phylum Nematoda contains one of the best-studied model organisms for genetics and with this an excellent baseline for comparative genetic studies. Genetic work requires methods to induce, isolate, cross, and characterize mutants and, as probably the most challenging element, ways of physically identifying the genes that carry the mutations isolated based on their phenotypes. In *C. elegans*, this has been traditionally achieved by a process called positional cloning. This approach requires a dense, high-quality genetic map for accurate genetic mapping and a physical map, ideally a full genome sequence that is highly interlinked with the genetic map. In addition, transgenic technology is required to narrow down genomic regions during mapping and for gene verification after final identification.

A genetic analysis of parasitic ability in *Heterodera glycines* was performed by Ke Dong and Charles H. Opperman (1997). To identify and characterize genes involved in parasitism, they developed three highly inbred *H. glycines* lines, OP20, OP25, and OP50, for use as parents for controlled crosses. Through these crosses, they have identified genes obtained in the inbred parents that control the reproduction of the nematode on hosts that carry resistance genes. These genes, designated as ror-* for reproduction on a resistant host, segregate in a normal Mendelian fashion as independent loci. Host range tests of F(1) generation progeny indicated that at least one parasitism gene in both the OP20 and OP50 lines for host PI 88788 was dominant. Parasitism genes in OP50 for hosts “Peking” and PI 90763 were recessive. Two types of single female descent populations, a single backcrossed BC(1) F(2) derived and a double backcrossed BC(2) F(1) derived, were established on the susceptible

soybean cultivar "Lee 68." Host range tests for parasitism in these lines demonstrated the presence of two independent genes in OP50, one for host PI 88788 designated ror-1 and one for host PI 90763 designated ror-2. OP20 carries two independent genes for parasitism on PI 88788, designated as alleles kr3 and kr4.

One of the most surprising and interesting findings in the discovery of nematode parasitism genes is the large number of candidate parasitism genes that encode novel proteins (Hussey et al. 2002a, b). Remarkably, over 70 % of the parasitism genes have no homology with functionally annotated genes in the databases. These pioneer parasitism genes seem to represent genes unique for nematode parasitism of plants, a hypothesis supported by the unique and complex interactions that sedentary endoparasites have with their host plants. These parasitism genes may have evolved from "basal" nematode genes, while other parasitism genes, e.g., cell wall-degrading enzymes and chorismate mutase, may have been acquired by horizontal gene transfer from prokaryotic microbes. Identifying the complete profile of parasitism genes expressed throughout the parasitic cycle of a nematode is the key to understanding the molecular basis of nematode parasitism of plants and defining what makes a nematode a plant parasite. The nematode parasitism genes being discovered are revealing the complexity of the tools a nematode possesses that enable it to attack plants and paint a more elaborate picture of host cellular events under specific control by the parasite than previously hypothesized. Interspecific and intraspecific comparison of the structure of parasitism genes encoding stylet secretions that induce feeding cell formation will also provide the knowledge that should lead to establishing a genetic basis for host range specificity among nematode species or races. Understanding this genetic variability will have an important positive effect on the development and deployment of sustainable nematode management strategies.

Full tool sets for the isolation and systematic study of mutations in known genes generated by forward and reverse genetics are currently available only for two nematode species other than *C. elegans*, namely *Caenorhabditis briggsae* and *P. pacificus*. For both species, relatively dense genetic maps, which are well anchored in the genome, allow the positional cloning of genes (Koboldt et al. 2010). Mutations in molecularly defined genes have been isolated by polymerase chain reaction-based screening for small deletions. Finally, transgenic techniques are available for both species (Schlager et al. 2009). Comparative genetic work in these species has concentrated on developmental processes that are very well understood in *C. elegans*. These studies have offered interesting insights into how the genetic control of development can change during evolution. The induction of the vulva in *C. elegans* is one of the best-studied genetic processes in animal development.

The switch of the signaling system involves a novel regulatory linkage of Wnt signaling, which is unknown from other organisms (Wang and Sommer 2011). In *C. elegans* vulva development, Wnt signaling is also required but for different processes. It acts prior to induction to maintain the competence of vulval precursor cells to respond to the inductive signal, and it is used again after the induction for the correct specification of tissue polarity. Secretory proteins encoded by genes expressed in the esophageal gland cells of plant-parasitic nematodes have key roles in nematode parasitism of plants (Gao et al. 2001a). Two venom allergen-like protein cDNAs (designated hg-vap-1 and hg-vap-2) were isolated from *Heterodera glycines* gland cell cDNA libraries. Both cDNAs hybridized to genomic DNA of *H. glycines* in Southern blots. The hg-vap-1 cDNA contained an open reading frame encoding 215 amino acids with the first 25 amino acids being a putative secretion signal. The hg-vap-2 cDNA contained an open reading frame encoding 212 amino acids with the first 19 amino acids being a putative secretion signal. Genes of hgvap- 1 and hg-vap-2 contained four introns, which ranged in size from 44 to 574 bp, and five exons ranging in size from 43 to 279 bp. In situ hybridization analyses showed that mRNAs of both vap genes accumulated specifically in the subventral gland cells of *H. glycines* during parasitism. The gland cell-specific expression and presence of predicted

secretion signal peptides in both VAPs suggest that these proteins are secreted from the nematode and may play a role in the infection of host plants by this parasite.

Root-knot nematodes alter plant cell growth and development by inducing the formation of giant cells for feeding. Nematodes inject secretions from their esophageal glands through their stylet and into plant cells to induce giant cell formation. *Meloidogyne javanica* chorismate mutase 1 (Mj-cm-1) is one such esophageal gland protein likely to be secreted from the nematode as giant cells form (Doyle and Lambert 2003). Mj-cm-1 has two domains, an N-terminal chorismate mutase (CM) domain and a C-terminal region of unknown function. It is the N-terminal CM domain of the protein that is the predominant form produced in root-knot nematodes. Transgenic expression of Mj-cm-1 in soybean hairy roots resulted in a phenotype of reduced and aborted lateral roots. Histological studies demonstrated the absence of vascular tissue in hairy roots expressing Mj-cm-1. The phenotype of Mj-cm-1 expressed at low levels can be rescued by the addition of indole-3-acetic acid (IAA), indicating Mj-cm-1 overexpression reduces IAA biosynthesis. It was proposed that Mj-cm-1 lowers IAA by causing a competition for chorismate, resulting in an alteration of chorismate-derived metabolites and, ultimately, in plant cell development. It was hypothesized that Mj-cm-1 was involved in allowing nematodes to establish a parasitic relationship with the host plant.

Genetic variation in *Meloidogyne incognita* virulence against the tomato *Mi* resistance gene was investigated by Castagnone-Sereno et al. (1994). Resistance to the parthenogenetic *Meloidogyne incognita* is controlled in tomato by the single dominant gene *Mi,* against which virulent pathotypes are able to develop. Isofemale lines (i.e., families) were established from a natural avirulent isolate of *M. incognita* in order to study the genetic variability and inheritance of the nematode virulence. From the progeny of individual females, the production of egg masses on the root system of the Mi-resistant tomato "Piersol" was analyzed in artificial selection experiments. A family analysis revealed, after two successive generations, a strongly significant variation between the 63 isofemale lines tested, and the results obtained for the mothers and their daughters were also significantly correlated. These results together clearly demonstrated the existence of a genetic variability and inheritance for this character. In a second experiment, a four-generation selection was performed on 31 other isofemale lines. The results revealed a significant response to selection apparently limited only to the two families able to produce, in first generation, a significant minimal egg mass number on the resistant cultivar.

Richard Janssen et al. (1991) crossed a virulent and an avirulent inbred line of *G. rostochiensis* to determine the genetics of virulence to the resistance gene HI of *Solanum tuberosum* ssp. *andigena* CPC 1673. The 3:1 segregation in avirulent and virulent larvae of the FZ generation, obtained by selfing the FI, showed that virulence to the HI gene is controlled by a single major recessive gene. The virulence percentages of the FI generations agreed with this finding. Reciprocal crosses showed no evidence of sex-linked inheritance of virulence. The cloning parasitism genes encoding secretory proteins expressed in the esophageal gland cells are the key to understanding the molecular basis of nematode parasitism of plants (Gao et al. 2001b). Suppression subtractive hybridization (SSH) with the microaspirated contents from *Heterodera glycines* esophageal gland cells and intestinal region was used to isolate genes expressed preferentially in the gland cells of parasitic stages. Twenty-three unique cDNA sequences from a SSH cDNA library were identified and hybridized to the genomic DNA of *H. glycines* in Southern blots. Full-length cDNAs of 21 clones were obtained by screening a gland cell long-distance polymerase chain reaction cDNA library. Deduced proteins of ten clones were preceded by a signal peptide for secretion, and PSORT II computer analysis predicted eight proteins as extracellular, one as nuclear, and one as plasmalemma localized. In situ hybridization showed that four of the predicted extracellular clones were expressed specifically in the dorsal gland cell, one in the subventral gland cells and three in the intestine in *H. glycines*. The predicted

nuclear clone and the plasmalemma-localized clone were expressed in the subventral gland cells and the dorsal gland cell, respectively. SSH is an efficient method for cloning putative parasitism genes encoding esophageal gland cell secretory proteins that may have a role in *H. glycines* parasitism of soybean.

Several resistance genes have been identified and genetically mapped for *H. glycines*; however, resistance levels in many soybean cultivars are not durable. Some older cultivars are no longer resistant to certain *H. glycines* populations in many production areas, especially if a soybean monoculture has been practiced. Past soybean registration reports showed that all resistant cultivars developed in public institutions from the mid-1960s to the present were derived from five plant introductions. This narrow genetic background is fragile. To further complicate the issue, soybean–*H. glycines* genetic interactions are complex and poorly understood (Re doong et al. 1997). Studies to identify soybean resistance genes sometimes have overlapped, and the same genes may have been reported several times and designated by different names. Nevertheless, many potential resistance genes in existing germplasm resources have not yet been characterized. Clearly, it is necessary to identify new resistance genes, develop more precise selection methods, and integrate these resistance genes into new cultivars. Rational deployment of resistant cultivars is critical to future sustained soybean production.

Within the genus *Caenorhabditis,* hermaphroditism has evolved multiple times. Baldi et al. (2009) showed that reducing the activity of only two genes is required to transform *C. remanei* females into self-fertile hermaphrodites. Lowering, but not eliminating, the activity of *tra-2*, a key component of somatic and germ line sex determination in *C. elegans*, was sufficient to allow spermatogenesis to occur in addition to oogenesis in *C. remanei*. However, the sperm formed was not activated. Sperm activation and subsequent self-fertilization were achieved by reducing the activity of *swm-1*, a gene known to prevent premature sperm activation in *C. elegans*.

The genetic analysis of parasitic nematodes, so far, is rather rudimentary because of technical constrains. Usually, the sexually reproducing worms are within their hosts, rendering them difficult to access and manipulate, except for a few cases where in addition to parasitic adults, free-living adults also occur, e.g., *Strongyloides* spp. (Grant et al. 2006). Nevertheless, efforts to make parasitic nematodes amenable to genetic analysis have been made and some groundwork laid out. Strategies to perform controlled crosses between defined isolates or even individuals have been developed for a few parasitic nematodes and molecular genetic markers were isolated (Eberhardt et al. 2007). Although genetic maps are published for three phytonematodes, viz., *Meloidogyne hapla* and *Heterodera glycines* (Atibalentja et al. 2005) and *Globodera rostochiensis* (Rouppe van der Voort et al. 1999), these methods and tools are not yet sufficient for mutational analysis and positional cloning of genes but have already been used successfully to elucidate modes of inheritance and to characterize reproductive strategies.

Protocols for the experimental induction of mutations have been reported for very few parasitic or parasitoid nematodes (Viney et al. 2002; Zioni Cohen-Nissan et al. 1992). In addition, spontaneous mutants were found and characterized genetically in several parasitic nematodes, i.e., worms that are virulent for otherwise resistant hosts or resistant against certain nematicidal drugs and pose an enormous economic and medical problem. The main reason why more effort has not so far been made to isolate mutants is probably because there was no straightforward way to identify the gene in which the mutation occurred made to isolate mutants in parasitic nematodes.

Amplified fragment length polymorphism fingerprinting of three pairs of *Meloidogyne incognita* near-isogenic lines was used to identify markers differential between nematode genotypes avirulent or virulent against the tomato *Mi* resistance gene (Semblat et al. 2001). One of these sequences, present only in the avirulent lines, was used as a probe to screen a cDNA library from second-stage juveniles and allowed cloning of a cDNA encoding a secretory protein. The putative full-length cDNA, named *map-1*, encoded a 458-amino acid protein containing a

predictive N-terminal secretion signal peptide. The MAP-1 sequence did not show any significant similarity to proteins deposited in databases. The internal part of the protein, however, was characterized by highly conserved repetitive motives of 58 or 13 aa. Reverse transcription-polymerase chain reaction experiments confirmed that *map-1* expression was different between avirulent and virulent near-isogenic lines. In PCR reactions, *map-1*-related sequences were amplified only in nematode populations belonging to the three species against which the *Mi* gene confers resistance: *M. arenaria*, *M. incognita*, and *M. javanica.* Polyclonal antibodies raised against a synthetic peptide deduced from the MAP-1 sequence strongly labeled J2 amphidial secretions in immunofluorescence microscopy assays, suggesting that MAP-1 may be involved in the early steps of recognition between (resistant) plants and (avirulent) nematodes.

10.9 Defense Signaling

Complex defense signaling pathways, controlled by different hormones, are involved in the reaction of plants to a wide range of biotic and abiotic stress factors. Kamrun Nahar et al. (2011) studied the ability of salicylic acid, jasmonate (JA), and ethylene (ET) to induce systemic defense in rice (*Oryza sativa*) against *Meloidogyne graminicola*. Exogenous ET (ethephon) and JA (methyl jasmonate) supply on the shoots induced a strong systemic defense response in the roots, exemplified by a major upregulation of pathogenesis-related genes *OsPR1a* and*OsPR1b*, while the salicylic acid analog BTH (benzo-1,2,3-thiadiazole-7-carbothioic acid *S*-methyl ester) was a less potent systemic defense inducer from shoot to root. Experiments with JA biosynthesis mutants and ET-insensitive transgenics showed that ET-induced defense requires an intact JA pathway, while JA-induced defense was still functional when ET signaling was impaired. Pharmacological inhibition of JA and ET biosynthesis confirmed that JA biosynthesis is needed for ET-induced systemic defense, and quantitative real-time reverse transcription-polymerase chain reaction data revealed that ET application onto the shoots strongly activates JA biosynthesis and signaling genes in the roots. It was observed that the JA pathway plays a pivotal role in rice defense against root-knot nematodes. The expression of defense-related genes was monitored in root galls caused by *M. graminicola*. Different analyzed defense genes were attenuated in root galls caused by the nematode at early time points after infection. However, when the exogenous defense inducers ethephon and methyl jasmonate were supplied to the plant, the nematode was less effective in counteracting root defense pathways, hence making the plant more resistant to nematode infection.

Interaction between the Avr gene product and the R gene product triggers a series of signaling responses. These result in the biosynthesis of salicylic acid which acts as a central signaling intermediate in plant defense. Salicylic acid in turn triggers both local and systemic responses. These include programmed cell death at the site of infection, local resistance to the pathogen. Salicylic acid is part of the Mi-1-mediated defense response to root-knot nematode in tomato. Mi-1 gene of tomato confers resistance against three species of root-knot nematode in tomato. Transformation of tomato carrying Mi-1 with a construct expressing NahG, which encodes salicylate hydroxylase, a bacterial enzyme that degrades salicylic acid (SA) to catechol, results in partial loss of resistance to root-knot nematodes. These results indicate that SA is an important component of the signaling that leads to nematode resistance and the associated hypersensitive response (Branch et al. 2004).

10.10 Molecular Basis for Nematode Resistance

According to gene-for-gene model, for each resistance gene in the host, there is a corresponding gene for avirulence in the pathogen, and for each virulence in the pathogen, there is a gene for susceptibility in host plant. A loss or alteration to either the plant resistance (R) gene or the pathogen avirulence (Avr) gene leads to disease

(compatibility) interactions involved in R gene and Avr gene incompatibility.

10.10.1 Gene-for-Gene Hypothesis

For resistance (incompatibility) to occur, complementary pairs of dominant genes must be present in the host and pathogen. These genes are referred to as resistance (host) and avirulence (pathogen). Altering either of these genes leads to compatibility (disease). The mechanisms of resistance most likely involve interaction between the Avr protein (an elicitor) and the R gene product (the receptor). This theory proposes that HR will occur when product of plant resistance gene (*R*) interacts with product of pathogen virulence or avirulence gene (*Avr)*.

10.10.2 Steps Involved

Major steps include the following: pathogen enters plant cell via wounds or connection with infected cells; protein and other molecules are released by the pathogen; R gene products bind to certain molecules from pathogens (Avr gene products); binding activates R gene product and triggers protective hypersensitivity response; and when R and Avr gene products do not match, no hypersensitivity response occurs and plant is susceptible to disease.

Disease resistance requires a dominant resistance (R) gene in the plant and a corresponding avirulence (Avr) gene in the pathogen. R genes are presumed to enable plants to detect Avr gene-specified pathogen molecules or initiate signal transduction to activate defenses and possess the capacity to evolve new R gene specificities rapidly.

10.11 Identification of Resistance (R) Genes

Most R genes are dominant, as are their cognate pathogen avirulence (Avr) genes. Plants possess many R genes active against many different pathogens. R genes are often found clustered on chromosomal loci. Plant breeders have successfully introduced resistance through introgression of foreign R genes.

In general, plant disease R genes include one or more of the following structural motifs, i.e., nucleotide-binding site (NBS), leucine-rich repeat (LRRs), or serine/threonine protein kinase domains. At present the largest number of known resistance proteins belongs to a class that contains both NBS and LRR motifs. Members of this group confer resistance to a number of nematodes (Ellis and Jones 1998). Most encode proteins that carry a structural motif with a repeating pattern of 20–30 amino acids called a leucine-rich repeat (LRR). LRR motifs participate in protein–protein interactions in a wide range of organisms (Kobe and Deisenhofer 1995).

The tomato *Mi*-1 gene confers resistance against root-knot nematodes and a biotype of the potato aphid (*Macrosiphum euphorbiae*). Four mutagenized Mi-1/Mi-1 tomato populations were generated and screened for altered root-knot nematode resistance. Four independent mutants belonging to two phenotypic classes were isolated. One mutant was chosen for further analyses; rme1 (for resistance to *Meloidogyne*) exhibited levels of infection comparable with those found on susceptible controls. Molecular and genetic data confirmed that rme1 has a single recessive mutation in a locus different from Mi-1. Cross sections through galls formed by feeding nematodes on rme1 roots were identical to sections from galls of susceptible tomato roots. In addition to nematode susceptibility, infestation of rme1 plants with the potato aphid showed that this mutation also abolished aphid resistance. A study was conducted to determine whether Rme1 functions in a general disease-resistance pathway, the response against *Fusarium oxysporum f.* sp. *lycopersici* race 2, mediated by the I-2 resistance gene (De Ilarduya et al. 2001). Both rme1 and the wild-type plants were equally resistant to the fungal pathogen. These results indicated that Rme1 does not play a general role in disease resistance but may be specific for *Mi*-1-mediated resistance.

Mi was introduced into cultivated tomato, *Lycopersicon esculentum*, from its wild relative *L. peruvianum* in the early 1940s (Smith 1944). With the assistance of linked markers, beginning with the isozyme marker *Aps-1* and more recently with DNA markers such as *Rex-1*, *Mi* has been incorporated into many modern tomato cultivars (Williamson et al. 1994).

10.12 Identified R Genes Against Nematodes

Gene from the wild relative of sugar beet, *Beta procumbens*, has been used in the isolation of resistant genes. The $Hs1^{pro\text{-}1}$ locus confers resistance to the beet cyst nematode (*Heterodera schachtii*), a major pest in the cultivation of sugar beet (*Beta vulgaris*). Gpa-2 gene confers resistance against *Globodera pallida.* The $Hs1^{pro\text{-}1}$ gene was cloned with the use of genome-specific satellite markers and chromosomal markers (Cai et al. 1997). Rk gene of wild species of cowpea is resistant against *Meloidogyne arenaria* and *M. incognita*, with moderate resistance against *M. javanica*. Multiple sites in broad-based resistance would increase the effectiveness of host plant resistance. Improved cultivars are being developed that carry the broad-based resistance gene Rk2 (Ehlers et al. 2000).

Root-knot nematode resistance of F1 progeny of an intraspecific hybrid (*Lycopersicon peruvianum* var. *glandulosum* Acc. No. 126443 × *L. peruvianum* Acc. No. 270435), *L. esculentum* cv. Piersol (possessing resistance gene *Mi*), and *L. esculentum* cv. St. Pierre (susceptible) was compared. Resistance to (1) isolates of two *Meloidogyne incognita* populations artificially selected for parasitism on tomato plants possessing the *Mi* gene, (2) the wild-type parent populations, (3) four naturally occurring resistance (*Mi* gene)-breaking populations of *M. incognita* and *M. arenaria* and two undesignated *Meloidogyne* spp., and (4) a population of *M. hapla* was indexed by numbers of egg masses produced on root systems in a greenhouse experiment. Artificially selected *M. incognita* isolates reproduced abundantly on Piersol, but not ($P = 0.01$) on resistant F1 hybrids. Thus, the gene(s) for resistance in the F1 hybrid differs from the *Mi* gene in Piersol. Four naturally occurring resistance-breaking populations reproduced extensively on Piersol and on the F1 hybrid, demonstrating ability to circumvent both types of resistance. *Meloidogyne hapla* reproduced on F1 hybrid plants, but at significantly ($P = 0.01$) lower levels than on Piersol (Roberts et al. 1990).

The gene of wild species of tomato, *Solanum lycopersicum*, has been exploited for the resistance. *Mi-1* confers resistance to root-knot nematodes (*Meloidogyne* spp.) and also confers resistance to sweet potato whitefly (*Bemisia tabaci*). Allele *Sgt1, Rar1*, and *Hsp90* are known to participate early in resistance gene signaling pathways (Kishor et al. 2007). Resistance can be either broad (effective against several nematode species) or narrow (controlling only specific biotypes of a species, also variously referred to as races or pathotypes). For other sources of resistance, inheritance may be controlled by single gene called monogenic (vertical resistance) or controlled by many genes called polygenic (horizontal resistance). Several dominant or semidominant resistance genes have been identified and mapped to chromosomal locations or linkage groups (Tables 10.3 and 10.4) (Williamson and Kumar 2006).

10.13 Origin of Parasitism Genes

The origin of parasitism genes is from nematode ancestors. Genes evolved from nematode ancestors of contemporary species are of one likely origin of nematode parasitism genes. In this regard, the value of the information generated in the *C. elegans* genome sequencing project to identify genes basic to the biology of plant-parasitic nematodes cannot be overemphasized. It is already clear that some *C. elegans* genes match those identified in plant-parasitic nematodes (Davis 2000). In other instances, however, such as the cellulose genes of cyst and root-knot nematodes, no significant similarity to any *C. elegans* gene can be found.

Table 10.3 Mapped nematode resistance loci/genes

Crop	Species of origin	Locus	Nematode	Genetic location
Tomato	*L. peruvianum*	*Mi*	*M. incognita, M. javanica, M. arenaria*	Chromosome 6
Tomato	*L. peruvianum*	*Mi3*	*M. incognita, M. javanica*	Chromosome 12
Tomato	*L. pimpinellifolium*	*Hero*	*G. rostochiensis*	Chromosome 4
Potato	*S. tuberosum* spp. *andigena*	*H1*	*G. rostochiensis* pathotypes *Ro1* and *Ro4*	Chromosome 5
Potato	*S. spegazzinii*	*Gro1*	*G. rostochiensis, pathotypes Ro1* and *Ro5*	Chromosome 7
Potato	*S. spegazzinii*	*Gpa*	*G. pallida, pathotypes Pa2* and *Pa3*	Chromosome 5
Potato	*S. vernei*	*GroV1*	*G. rostochiensis, pathotype Ro1*	Chromosome 5
Potato	*S. bulbocastanum*	$R_{Mc\text{-}1}$	*M. chitwoodi*	Chromosome 11
Sugar beet	*B. patellaris*	$Hs1^{pat\text{-}1}$	*H. schachtii*	Chromosome 1
Soybean	*Glycine max*	Rhg_4	*H. glycines, race 3*	Linkage group A
Wheat	*Triticum aestivum*	*Cre*	*H. avenae*	Long arm of chromosome 2B
Wheat	*T. tauschii*	*Cre3*	*H. avenae*	Long arm of chromosome 2D

Table 10.4 Parasitism genes of major phytonematodes

Plant-parasitic nematodes	Parasitism genes
Heterodera glycines	*Hg-eng-1* and *ror-1,2,3*
Globodera rostochiensis	*Gr-eng-1*
Meloidogyne javanica	*Mj-cm-1*
Meloidogyne incognita	*Mi-cbp-1* and *mi-msp1*

Table 10.5 Cyst and root-knot nematode parasitism genes with predicted functions

Nematode secretions	Cyst nematode	Root-knot nematode
I. Cell wall-degrading enzymes		
1. β-1,4-endoglucanase	+	+
2. Pectate lyase	+	+
3. Polygalacturonase	–	+
4. Expansin	+	+
5. Xylanase	–	+
6. Cellulose-binding domain	+	+
II. Calreticulin	–	+
III. Chorismate mutase	+	+
IV. Ran BPM	+	–
V. Ubiquitin extension	+	–
VI. CLAVATA3 or other peptides	+	+
VII. Phytohormones	–	+

Plant parasitism is believed to have evolved at least three times independently. The genes that were evolved from nematode ancestors of contemporary species are one likely possible mechanism for the origin of nematode parasitism genes and the other mechanism may be horizontal gene transfer (Table 10.5). It was reported that those genes expressed in the esophageal gland cells of plant-parasitic nematodes show strongest similarities to the bacterial genes which strengthened the existing hypothesis that parasitism genes in plant nematodes may have been acquired, at least in part, by horizontal gene transfer from bacteria and other microorganisms that inhabit the same parasitic environment. The genes *Mj-cm-1*and *Mi-cbp-1* show strongest similarities to the genes of bacteria. The complementation of a bacterial mutant with *Mj-cm-1* was also used to provide functional analysis of the gene. Most of the parasitism genes are found to be highly similar to bacterial sequences, thereby suggesting that these parasitism genes could have been acquired from bacteria through horizontal gene transfer. For example, the nematode endo-1,4-β-glucanases from the Tylenchomorpha, which belong to glycosyl hydrolase family (GHF5), show less similarity to plant endoglucanases but show resemblance to the bacteria.

The genes encoding the cellulase enzymes of both nematode and bacteria may have evolved from an ancient cellulase of a common ancestor of both the bacteria and nematodes. The endoglu-

canases from nematode show the highest similarity with the bacterial one, which also points to a horizontal gene transfer from bacteria to an ancestor of the cyst nematode. However, it is not possible and advisable to provide the conclusive evidence for a horizontal gene transfer from one organism to another organism germ line. There are examples of putative cases of horizontal gene transfer from eukaryote to prokaryote, from prokaryote to prokaryote, and from prokaryote to eukaryote. On the other hand, the presence of bacterial symbionts in nematode ancestors, such as the bacterium *Wolbachia* symbiont found in filarial nematodes, may also represent a source for transfer of genetic material from bacteria to nematodes (Ngangbam and Devi 2012).

Cellulase genes and several other genes cloned from nematode esophageal gland cells have striking similarities to microbial genes, suggesting that some nematode parasitism genes may have been acquired by ancient horizontal gene transfer. Horizontal gene transfer is a central process in shaping nematode genomes. High incidence of gene acquisition can happen through horizontal gene transfer. In nematodes, genes are gained and lost frequently. A major challenge in plant nematology is to identify the avirulence gene products of parasitic nematodes. Genes are also required for various major activities including egg hatching (*hch*), dauer formation (*daf*), and sex determination (*xol, her, and fem).*

10.14 Nuclear Localized Parasitism Proteins

A significant number of the proteins encoded by parasitism genes expressed in plant nematode esophageal gland cells (Gao et al. 2003; Huang et al. 2003) contain both a predicted secretion signal peptide and a motif encoding a putative nuclear localization signal (NLS). These data present the tempting hypothesis that the secreted products of some nematode parasitism genes become localized to the host cell nucleus. This is not without precedent since the antigens of secreted products from the animal-parasitic nematode *Trichinella spiralis* have been immunolocalized to the nucleus of host muscle cells. Expression of GFP- and GUS-tagged SCN parasitism proteins in plant cells has demonstrated that some of the predicted NLS domains do indeed function to import products into the plant cell nucleus. DNA-binding domains are also predicted in some of the NLS-containing proteins predicted to be secreted by plant nematodes, suggesting an extraordinary potential for regulatory control within the nucleus of host feeding cells if confirmed.

10.14.1 RanBPM

Ran-binding protein microtubule-organizing center (RanBPM) appears to function as a scaffolding protein in several signal transduction pathways. RanBPM is a crucial component of multiprotein complexes that regulate the cellular function by modulating and/or assembling with a wide range of proteins in different intracellular regions and thereby mediate diverse cellular functions. This suggests a role for RanBPM as a scaffolding protein. A homologue of a Ran-binding protein to microtubule (*RanBPM*) gene, initially known as IC5, was identified and characterized in second-stage juveniles (J2s) of *Globodera pallida* (Blanchard et al. 2005). The full-length cDNA (937 bp) was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE), and specific primers were designed to amplify the genomic sequences of 2,396 bp containing six introns. The ORF (798 bp) encodes a putative 265-amino acid sequence with a predicted SPRY domain and a signal peptide of 23 amino acids on the N-terminal part of the protein. In situ hybridization experiments showed that the transcript is located in the dorsal gland of the J2s, suggesting that the encoded protein has an extracellular function and can be involved in the late stages of parasitism such as feeding site establishment. This gene was specifically overexpressed in the juveniles before and during parasitism, but not in adult developmental stages. As this gene is presumed to be involved in plant–nematode interaction, particularly in the development and maintenance of the feeding structure that allows

the nematode to achieve parasitic development, homologous genes were sought in other cyst nematode species. One of them was cloned and sequenced in the closely related species *Globodera mexicana*.

Expressed genes encoding secretory proteins with high similarity to proteins that bind to the small G-protein Ran, so-called RanBPMs (Ran-binding protein in the microtubule-organizing center), were identified in the dorsal esophageal gland cell of cyst nematodes (Blanchard et al. 2005).

10.14.2 Ubiquitination/Proteasome

The proteasome is a protein-destroying apparatus involved in many essential cellular functions, such as the regulation of cell cycle, cell differentiation, signal transduction pathways, antigen processing for appropriate immune responses, stress signaling, inflammatory responses, and apoptosis. It is capable of degrading a variety of cellular proteins in a rapid and timely fashion, and most substrate proteins are modified by ubiquitin before their degradation by the proteasome. The proteasome is a large protein complex consisting of a proteolytic core called the 20S particle and ancillary factors that regulate its activity in various ways.

The most common form is the 26S proteasome containing one 20S core particle and two 19S regulatory particles that enable the proteasome to degrade ubiquitinated proteins by an ATPdependent mechanism. Another form is the immunoproteasome containing two 11S regulatory particles, PA28 alpha and PA28 beta, which are induced by interferon gamma under the conditions of intensified immune response. Other regulatory particles include PA28 gamma and PA200. Although PA28 gamma also belongs to a family of activators of the 20S proteasome, it is localized within the nucleus and forms a homoheptamer. PA28 gamma has been implicated in the regulation of cell cycle progression and apoptosis. PA200 has been identified as a large nuclear protein that stimulates proteasomal hydrolysis of peptides. Several parasitism genes expressed in cyst nematode esophageal gland cells encode secreted isotypes of cytoplasmic proteins involved in the ubiquitination pathway, namely, ubiquitin itself, along with proteins (i.e., RING-Zn-Finger-like and Skp1-like proteins) similar to those found in the host E3 ubiquitin protein ligase complex (Gao et al. 2003).

10.14.3 The 14-3-3 Protein Family

14-3-3 proteins constitute a family of eukaryotic proteins that are key regulators of a large number of processes ranging from mitosis to apoptosis. 14-3-3s function as dimers and bind to particular motifs in their target proteins. To date, 14-3-3s have been implicated in the regulation or stabilization of more than 35 different proteins. This number is probably only a fraction of the number of proteins that 14-3-3s bind to, as reports of new target proteins have become more frequent. An examination of 14-3-3 entries in the public databases reveals 153 isoforms, including alleloforms, reported in 48 different species. Two isoforms of a protein identified as a member of the 14-3-3 family were isolated from stylet secretions induced in vitro from second-stage juveniles of root-knot nematode (Jaubert et al. 2002). The expressed genes encoding each 14-3-3 were cloned, and in situ hybridization analysis indicated that one isoform (14-3-3a) was expressed in genital primordia and the other isoform (14-3-3b) was expressed within the dorsal esophageal gland of root-knot nematode juveniles.

To develop a better understanding of invertebrate anhydrobiosis, Kshamata Goyal et al. (2005) characterized dehydration-inducible genes and their proteins in anhydrobiotic nematodes and bdelloid rotifers. Initial work with the fungivorous nematode *Aphelenchus avenae* led to the identification of two genes, both of which were markedly induced on slow drying (90–98 % relative humidity, 24 h) and also by osmotic stress, but not by heat or cold or oxidative stresses. The first of these genes encodes a novel protein, anhydrin; it is a small, basic polypeptide, with no counterparts in sequence databases, which is predicted to be natively unstructured and highly hydrophilic. The second is a member of the group 3 LEA

protein family; this and other families of LEA proteins are widely described in plants, where they are most commonly associated with the acquisition of desiccation tolerance in maturing seeds. Like anhydrin, the nematode LEA protein, Aav-LEA-1, is highly hydrophilic, and a recombinant form has been shown to be unstructured in solution. In vitro functional studies suggested that Aav-LEA-1 was able to stabilize other proteins against desiccation-induced aggregation, which is in keeping with a role of LEA proteins in anhydrobiosis. In vivo, however, Aav-LEA-1 was apparently processed into smaller forms during desiccation. A processing activity was found in protein extracts of dehydrated, but not hydrated, nematodes; these shorter polypeptides are also active anti-aggregants, and it was hypothesized that processing LEA protein serves to increase the number of active molecules available to the dehydrating animal. Other LEA-like proteins were identified in nematodes, and it seems likely, therefore, that they play a major role in the molecular anhydrobiology of invertebrates, as they are thought to do in plants.

10.14.4 Surface Defense

Chemical composition, origin, and biological role of the surface coat of plant-parasitic nematodes are described by Spiegel and McClure (1995) and compared with those of animal-parasitic and free-living nematodes. The surface coat of the plant-parasitic nematodes is 5–30 nm thick and is characterized by a net negative charge. It consists, at least in part, of glycoproteins and proteins with various molecular weights, depending upon the nematode species. The lability of its components and the binding of human red blood cells to the surface of many tylenchid plant-parasitic nematodes, as well as the binding of several neoglycoproteins to the root-knot nematode *Meloidogyne*, suggest the presence of carbohydrate recognition domains for host plants and parasitic or predatory soil microorganisms (e.g., *Pasteuria penetrans* and *Dactylaria* spp.). These features may also assist in nematode adaptations to soil environments and to plant hosts with defense mechanisms that depend on reactions to nematode surfaces. Surface coat proteins can be species and race specific, a characteristic with promising diagnostic potential.

The hypodermis is a syncytial cell layer directly beneath the (nonliving) nematode cuticle that forms the new cuticle during molts and secretes a number of molecules for deposition on the cuticle surface of the nematode body. Several genes expressed within the hypodermis encode proteins deposited on the cuticle surface that are in direct contact with host cells during nematode invasion of plant tissues. Among the dynamic mixture of proteins at the cuticle surface are proteins with potential roles in mitigating host defense response. Peroxidase genes are expressed in the potato cyst nematode hypodermis (Jones et al. 2002), and the peroxidase proteins accumulate on the nematode body surface presumably to detoxify reactive oxygen species generated by the defense response of the host.

10.14.5 Stealth Signals

Several molecules from nematodes with observable effects on plant cells have been reported, but the nature and origins of these molecules from nematodes are unclear. The isolation of secreted cytokinins from root-knot nematode juveniles in the absence of plant hosts (De Meutter et al. 2003) confirmed earlier evidence suggesting that nematodes produce cytokinin endogenously (Dimalla and Van Staden 1977). It was suggested that cytokinins of nematode origin may be an excreted waste product of nucleic acid degradation. This hypothesis relates to the localized hyperplasia that is a hallmark of gall formation, cell cycle regulation in feeding sites, and other cytokinin effects stimulated in roots by root-knot nematodes (Lohar et al. 2004). The incubation of roots of the legume *Lotus japonicus* in the presence of viable juveniles of root-knot nematode prior to infection stimulated identical cytoskeletal activity in root hairs as observed by treatment with rhizobial Nod factors (Weerasinghe et al. 2005). The "NemF"

(Nem factor) from root-knot nematodes also provided a response identical to Nod factor in Nod-receptor mutants, and a similar response to NemF was observed in root hairs of tomato. The identity of NemF and its origins in root-knot nematode have not been reported (Davis et al. 2008).

10.14.6 Bioactive Peptides

Perhaps the most interesting group of parasitism genes are those that encode signaling peptides. Secretions collected and fractionated from hatched juveniles of the potato cyst nematode contained a peptide or peptides of less than 3 kDa that induced mitogenic activity in tobacco leaf protoplasts and human peripheral blood mononuclear cells (Goverse et al. 1999).

Below is a sequence/list of cyst and root-knot nematode parasitism genes with predicted functions:

- Peptide signaling – *H. glycines;* contained C-terminal domain similar to plant CLAVATA3/ESR-related (CLE) peptides
- Altered cellular metabolism – chorismate mutase *(CM)*
- Cell cycle augmentation – *RanBPMs*
- Nuclear localization *(NLS)*
- Cytokinins and protein degradation – ubiquitin proteasome pathway

Parasitism genes expressed in the esophageal gland cells of root-knot nematodes encode proteins that are secreted into host root cells to transform the recipient cells into enlarged multinucleate feeding cells called giant cells (Huang et al. 2006). Expression of a root-knot nematode parasitism gene which encodes a novel 13-amino acid secretory peptide in plant tissues stimulated root growth. Two scarecrow-like transcription factors of the GRAS protein family were identified as the putative targets for this bioactive nematode peptide in yeast two-hybrid analyses and confirmed by in vitro and in vivo co-immunoprecipitations. This discovery is the first demonstration of a direct interaction of a nematode-secreted parasitism peptide with a plant-regulatory protein, which may represent an early signaling event in the root-knot nematode–host interaction.

Meloidogyne hapla, a diploid root-knot nematode with a compact (54 Mbp) sequenced genome, has been established as a tractable model to study the genetic and biochemical basis for plant parasitism (Bird et al. 2012). The current annotation freeze (HapPep4) predicts 14,207 proteins, many of which have been confirmed by LC-MS^{E}. Comparative genomics, particularly with the sympatric species *M. chitwoodi* and the migratory species *Pratylenchus coffeae*, proved to be a powerful approach to deduce the evolution and mechanisms of parasitic ability. A current target of a functional analyses was genes encoding small proteins that exhibit sequence similarity to members of two classes of plant peptide hormones, viz., RAR (root architecture regulator) and CLE (clavata-like elements). In *M. hapla*, RAR mimics were encoded by a 12-member gene family, and 8 genes encode CLE peptide mimics. Ectopic exposure of roots to RAR peptides was sufficient to elicit galling even in the absence of rhizobacteria or RKN. Further genome analysis of these loci in *M. hapla* supported the model that these genes were acquired from an ancestral dicot via horizontal gene transfer. Bioassays were developed to study the biology of these and other ligands involved in parasitism and established methods for efficient isotopic labeling of nematode proteins to empower an MS-based approach to map plant and nematode proteins.

Plant-parasitic cyst nematodes are known to secrete proteins that mimic the function of plant CLE signaling peptides to promote a successful infection. However, the mechanistic details of this process have yet to be elucidated. Wang Wang Xiaohong (2011) identified and functionally characterized CLE-like genes from several cyst nematode species including the two species of potato cyst nematodes and demonstrated that nematode CLEs can be trafficked by the function of the variable domain to the place outside plant cells where they can act as plant CLE mimics by interacting with membrane-bound plant CLE receptors. A transient expression system was used to demonstrate the processing of nematode CLE in plants. The majority of the earlier research was focused on the identification of the

processed and bioactive forms of nematode CLE peptides that function in plant cells and the initial investigation of plant receptors that perceive nematode CLE signals. By overexpression of a nematode CLE protein in potato roots coupled with proteomic analyses, they determined that the bioactive forms of nematode CLEs share striking structural similarity to mature plant CLE peptides. These results provided the direct evidence that nematode CLEs, once being delivered into host root cells, can be recognized by host cellular machinery to become mimics of plant CLE signals. Using genetic and biochemical approaches, it was demonstrated that plant receptors of CLV2 and CRN were required for nematode CLE-mediated parasitism. They identified that BAM1 and BAM2 were additional host receptors that can bind to processed nematode CLE peptides. These results suggested that multiple host receptors might be involved in perceiving nematode CLE peptides to facilitate nematode parasitism. Mimicry of host plant signals was an extraordinary adaptation by a plant parasite to have evolved the ability to reprogram host plant cells for its own benefit.

10.14.7 Chorismate Mutase

Chorismate (or prephenate) is a precursor for a variety of compounds including cellular aromatic amino acids, phytohormone indole-3-acetic acid (IAA), plant defense-related salicylic acid, and a range of other secondary metabolites. These chorismate-derived compounds (CDCs) therefore play important roles in plant growth and development, in defense, and in interactions with other organisms. The activity of chorismate mutase is a key regulatory mechanism that determines the cellular balance of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Romero et al. 1995). Chorismate mutase (CM) catalyzes the pericyclic Claisen-like rearrangement of chorismate to prephenate in the last step of the shikimate pathway, which is a primary metabolic route found in plants and microorganisms (Huang et al. 2005).

Chorismate mutase is well characterized in microbes and plants and not described from any animals outside of phytonematodes. The first animal CM gene (Mj-cm-1) was cloned from the root-knot nematode *Meloidogyne javanica* and found to be expressed in the nematode esophageal gland cells (Lambert et al. 1999). Chorismate mutases are potentially involved in early development of the feeding sites induced by plant-parasitic nematodes, but how the nematode CMs alter the development of host plant cells is still not clear. Recently, CMs have been identified in soybean and potato cyst nematodes. The presence of CM homologues in different obligate, sedentary endoparasitic nematode species that induce elaborate feeding sites in roots suggests that they have key roles in plant–nematode interactions.

Some plant endoparasitic nematodes are biotrophic and induce remarkable changes in their hosts in order to ensure a continuous supply of food. Proteins secreted from esophageal gland cells have been implicated in this pathogenic process (Jones et al. 2003). A potentially secreted chorismate mutase has been isolated from *Globodera pallida.* The gene encoding this protein is expressed in the subventral esophageal gland cells of the nematode, and the mRNA derived from this gene is only present in the early parasitic stages. Sequence analysis of this gene showed that, like other genes involved in the host–parasite interaction of plant-parasitic nematodes, it is likely to have been acquired by horizontal gene transfer from bacteria. The presence of a signal peptide in the deduced amino acid sequence of the *G. pallida* chorismate mutase and its expression in the subventral esophageal gland cells suggested that it is secreted from the nematode, pointing to a role for the protein in the host–parasite interaction. The shikimate pathway, of which chorismate mutase is normally a part, is not found in animals but is present in plants and bacteria. In plants it gives rise to a variety of compounds which are important in amino acid synthesis and defense signaling pathways, as well as auxins, which have been implicated in the early development of nematode feeding sites.

Parasitism genes encoding secretory proteins expressed in the esophageal glands of phytoparasitic nematodes play critical roles in nematode invasion of host plants, establishment of feeding sites, and suppression of host defenses (Huang et al. 2005). Two chorismate mutase genes potentially having a role in one or more of these processes were identified from a *Meloidogyne incognita* esophageal gland cell subtractive cDNA library. These *M. incognita* enzymes (designated as Mi-cm-1 and Mi-cm-2), with amino-terminal signal peptides, were significantly similar to chorismate mutases in *M. javanica* and bacteria. The complementation of an *Escherichia coli* CM-deficient mutant by the expression of Mi-cm-1 or Mi-cm-2 confirmed their CM activity. In situ mRNA hybridization showed that the transcripts of Mi-cm-1 and Mi-cm-2 accumulated specifically in the two subventral esophageal gland cells of *M. incognita*. RT-PCR analysis confirmed that their transcript abundances were high in the early parasitic juvenile stages and low (Mi-cm-1) or undetectable (Mi-cm-2) in later parasitic stages of the nematode. Southern blot analysis revealed that these CM genes were members of a small multigene family in *Meloidogyne* species. The widespread presence of CMs in the specialized sedentary endoparasitic nematode species suggests that this multifunctional enzyme may be a key factor in modulating plant parasitism.

10.15 Genetic Analysis of Parasitism

The use of forward genetic strategies to add to our understanding of nematode parasitism and virulence has been complicated due to the obligate nature of phytonematodes, parthenogenetic mode of reproduction, and lack of tools for genetic mapping. A genetic linkage map for *H. glycines* has been constructed and its utility was demonstrated by mapping the *HG-Cm-1* gene. Of the four most damaging *Meloidogyne* spp., *M. hapla* has been found to reproduce by facultative meiotic parthenogenesis, so that both selfed and outcrossed progeny can be generated for classical genetic studies; hence it has been adopted as a model system. In addition to the *M. hapla* genome sequencing project, genetic mapping is also under construction and F2 mapping populations have been generated.

These new tools help researchers to identify the genes controlling various traits – nematode virulence and host range to understand pathogenesis. Molecular approaches to explore the altered gene expression in nematode feeding site (NFS) include differential screening and subtraction of cDNAs, promoter b-glucuronidase (GUS) fusions, mRNA in situ hybridization, reverse transcription-polymerase chain reaction (RT-PCR), Mabs, Pabs, ESTs, and microarray.

10.15.1 Molecular Analysis of Genes

These include cDNA RT-PCR, cDNA-AFLP, RNA fingerprinting, expressed sequence tag (EST) analysis, LD-PCR (long-distance PCR), PSORT II, and BLASTP analysis. Nematode parasitism genes are active in any or all parts of the parasitic cycle including preparasitic and parasitic life stages. Parasitism genes are expressed in their esophageal gland cells and secreted through their stylet into host tissue to control the complex process of parasitism. Sixty different proteins are secreted by the stylet during parasitism. Plant genes induced during a compatible plant–nematode interaction sequence include complex changes in plant gene expression. Pectate lyase genes are expressed in the early nematode developmental stages and their pectate lyase activities would help to degrade the pectin matrix and assist nematode migration between the cells in host roots.

10.15.1.1 *Hg-eng-1 and Gr-eng-1*

These genes have been isolated from *Heterodera glycines* and *Globodera rostochiensis.* These are produced in subventral glands. They play a role in the cell wall dissolution (cellulase) (Re Dong 1997). Two β-1,4-endoglucanases (EGases), Hg-eng-1 and Hg-eng-2, were cloned from the soybean cyst nematode, *Heterodera glycines,* and their expression was shown in the subventral esophageal glands of hatched second-stage juveniles (de Boer et al. 1999). They examined the

expression of these EGases in the subventral glands of all postembryonic life stages of *H. glycines* by in situ hybridization and immunolocalization. The first detectable accumulation of EGase mRNAs occurred in the subventral glands of unhatched J2. EGase transcripts remained detectable in J2 after hatching and during subsequent root invasion. However, in late parasitic J2 and third-stage juveniles (J3), the percentage of individuals that showed EGase transcripts decreased. In female fourth-stage juveniles and adult females, EGase transcripts were no longer detected in the subventral glands. EGase hybridization signal reappeared in unhatched males coiled within the J3 cuticle, and transcripts were also present in the subventral glands of migratory adult males. Immunofluorescence labeling showed that EGase translation products are most abundantly present in the subventral glands of preparasitic J2, migratory parasitic J2, and adult males. The presence of EGases predominantly in the migratory stages suggests that the enzymes are used by the nematodes to soften the walls of root cells during penetration and intracellular migration.

10.15.1.2 *Mj-cm-1 and Mi-cbp-1*

These genes are isolated from *Meloidogyne javanica* and *Meloidogyne incognita*. These are produced in subventral esophageal gland cells. The function of these genes is to convert chorismate mutase into aromatic amino acid and tyrosine. Those genes expressed in the esophageal gland cells of plant-parasitic nematodes show strongest similarities to the bacterial genes which strengthened the existing hypothesis that parasitism genes in plant nematodes may have been acquired, at least in part, by horizontal gene transfer from bacteria and other microorganisms that inhabit the same parasitic environment (Blaxter et al. 1998). The genes Mj-cm-1 and Mi-cbp-1 show strongest similarities to the genes of bacteria. The complementation of a bacterial mutant with Mj-cm-1 was also used to provide functional analysis of the gene. Most of the parasitism genes are found to be highly similar to bacterial sequences, thereby suggesting that these parasitism genes could have been acquired from bacteria through horizontal gene transfer.

In brief, the cloning and characterization of genes that promote nematode parasitism of plants is in its early stages, but already, some promising research directions and unexpected results have been realized. Genetic analyses will be merged with physical maps of plant-parasitic nematode genomes to isolate nematode (a) virulence genes, but it is unclear if these genes will represent a subset of modified parasitism genes or if they will have functions unrelated to plant parasitism. Direct molecular analyses of genes expressed in the nematode esophageal gland cells whose products are secreted into plant tissue during parasitism are proving to be a fruitful area of investigation.

10.16 Nematode Genomics

Combined with the extensive genetic and genomic analyses of *C. elegans,* the increase in genomic analyses of parasitic nematodes in recent years promises to provide an unprecedented understanding of nematode biology and pathogenesis. SPS of random clones from cDNA libraries to generate ESTs (expressed sequence tags) from different nematode life stages become a powerful tool for identifying genes important in nematode–host interactions, which contributed more than 400,000 publicly available parasitic nematode expressed sequences to databases enabling in identification of nematode-specific gene families.

To elucidate nematode genome organization, phytoparasitic nematode genome sequencing projects for both root-knot nematodes (*M. incognita* and *M. hapla*) and cyst (*H. glycines*) nematodes are currently underway and upon completion will provide genome-wide catalogues of nematode PGCs for both functional and comparative analyses. By utilizing this gene structure, organization and function can be compared across different genomes to facilitate evolutionary analyses within the phylum Nematoda.

A surprising result is that the nematode cellulase genes and several other genes cloned from nematode esophageal gland cells have striking similarities to microbial genes, suggesting that some nematode parasitism genes may have been acquired by ancient horizontal gene

transfer. The application of genomics to the study of nematode parasitism genes will help to address questions and allow the isolation of additional nematode parasitism genes to progress. The development of efficient assays for functional analysis of isolated parasitism genes will be of paramount importance to understanding the evolution and complexity of plant parasitism by nematodes.

Expanding genomic data on plant pathogens open new perspectives for the development of specific and environment friendly pest management strategies based on the inhibition of parasitism genes that are essential for the success of infection (Arguel et al. 2012). Identifying such genes relies on accurate reverse genetics tools and the screening of pathogen knockdown phenotypes. Root-knot nematodes are major cosmopolitan crop pests that feed on a wide range of host plants. Small interfering RNAs (siRNAs) would provide a powerful tool for reverse genetics of nematode parasitism genes provided that they could (1) target genes expressed in inner tissues of infective nematodes and (2) target genes expressed during parasitism. It was shown that siRNAs can access inner tissues of the infective juveniles during soaking and accumulate in the esophagus, amphidial pouches, and related neurons of the nematode. Evidence was provided that siRNAs could trigger knockdown of the parasitism gene *Mi-CRT*, a calreticulin gene expressed in the esophageal glands of *Meloidogyne incognita*. *Mi-CRT* knockdown in infective juveniles affected nematode virulence. However, *Mi-CRT* knockdown was not persistent after plant infection, indicating that siRNA-mediated RNAi is best suited for functional analysis of genes involved in preparasitic stages or in the early steps of infection.

10.17 RNA Interference in Phytonematodes

RNA interference (RNAi, also called RNA-mediated interference) is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid inhibits the expression of genes with complementary nucleotide sequences (Mehmet Karaka 2008). Conserved in most eukaryotic organisms, the RNAi pathway is thought to have evolved as a form of innate immunity against viruses and also plays a major role in regulating development and genome maintenance. RNAi has recently been demonstrated in phytonematodes. It is a potentially powerful investigative tool for the genome-wide identification of gene function that should help improve our understanding of plant-parasitic nematodes. RNAi helps to identify gene and, hence, protein targets for nematode management strategies.

There is accumulating evidence for the efficacy of RNAi in plant-parasitic nematodes. A range of genes have been targeted for silencing in cyst and root-knot nematode species and both the phenotypic and the molecular effects (Table 10.6). Nevertheless, the molecular detail of the RNAi process in plant parasitic nematodes has yet to be elucidated. Infective stages of plant-parasitic nematodes are sufficiently small to make their microinjection with dsRNA a major technical challenge. In addition, they do not normally ingest fluid until they have infected a host plant. However, RNAi effects have been achieved using octopamine to stimulate oral ingestion by preparasitic second-stage juveniles of cyst nematodes *H. glycines* and *G. pallida* (Urwin et al. 2002) and root-knot nematode *M. incognita* (Bakhetia et al. 2005). Resorcinol and serotonin also induce dsRNA uptake by second-stage juvenile of *M. incognita* and may be more effective than octopamine for this nematode.

Alterations to the original method, including the addition of spermidine to the soaking buffer and an extended incubation time, were reported to increase the efficiency of RNAi for *G. rostochiensis*. The genes targeted by RNAi to date are expressed in a range of different tissues and cell types. The ingested dsRNA can silence genes in the intestine and also in the female reproductive system, sperm, and both subventral and dorsal esophageal glands (Huang et al. 2006). Uptake of dsRNA from the gut is a proven route to systemic RNAi in *C. elegans*. The systemic nature of RNAi in plant-parasitic nematodes following ingestion of dsRNA sug-

Table 10.6 Plant-parasitic nematode genes targeted by RNAi

Nematode species	Gene function	RNAi effect	Site of gene expression
Meloidogyne incognita	Cysteine proteinase	Delayed development	Intestine
		Decreased number of established nematodes	
	Dual oxidase	Decreased number of established nematodes	Presumed role in extracellular matrix
		Decreased fecundity	
	Splicing factor	Reduced galling	Unknown
		Reduced number of females	
	Integrase	Reduced galling	Unknown
		Reduced number of females	
	Secreted peptide 16D 10	Reduced galling	Subventral pharyngeal glands
		Decreased number of established nematodes	
Heterodera glycines	Cysteine proteinase	Increased male:female ratio	Intestine
	C-type lectin	Decreased number of established nematodes	Hypodermis
	Major sperm protein	Reduction in mRNA	Sperm
		No phenotypic effect at 14 dpi	
	Aminopeptidase	Decreased number of established nematodes	Female reproductive system
		Increased male:female ratio	
	Beta I,4-endoglucanase	Decreased number of established nematodes	Subventral pharyngeal glands
	Pectate lyase	Increased male:female ratio	Subventral pharyngeal glands
	Chorismate mutase	Increased male:female ratio	Subventral pharyngeal glands
	Secreted peptide SYV 46	Decreased number of established nematodes	Dorsal esophageal gland
Globodera pallida	Cysteine proteinase	Increased male:female ratio	Intestine
	FMRFamide-like peptides	Mobility inhibition	Nervous system
M. artiellia	Chitin synthetase	Delayed egg hatch	Eggs
G. rostochiensis	Beta I,4-endoglucanase	Decreased number of established nematodes	Subventral pharyngeal glands
	Secreted amphid protein	Decreased ability to locate and penetrate roots	Amphids

gests they share similar uptake and dispersal pathways. Alternative routes to dsRNA uptake may exist for plant-parasitic nematodes. RNAi of a chitin synthase gene expressed in the eggs of root-knot nematode *Meloidogyne artiellia* was achieved by soaking intact eggs contained within their gelatinous matrix in a solution containing dsRNA. The enzyme plays a role in the synthesis of the chitinous layer in the eggshell. Depletion of its transcript by RNAi led to a reduction in stainable chitin in eggshells and a delay in hatching of juveniles from treated eggs. The results implied that the eggs of this nematode and possibly others are permeable to dsRNA.

10.18 Evolution of Parasitism

Parasitism is an important life history strategy in many metazoan taxa. This is particularly true of the phylum Nematoda, in which parasitism has evolved independently at least nine times. The apparent ease with which parasitism has evolved

among nematodes may, in part, be due to a feature of nematode development acting as a preadaptation for the transition from a free-living to a parasitic life history. One candidate preadaptive feature for evolution in terrestrial nematodes is the dauer larva, a developmentally arrested morph formed in response to environmental signals (Stasiuk et al. 2012). For this parasitic clade, and perhaps more widely in the phylum, the evolution of parasitism co-opted the dauer switch of a free-living ancestor. This lends direct support to the hypothesis that the switch to developmental arrest in the dauer larva acted as a preadaptation for the evolution of parasitism and suggests that the sensory transduction machinery downstream of the cue may have been similarly co-opted and modified.

Despite extraordinary diversity of free-living species, a comparatively small fraction of nematodes are parasites of plants (Baldwin et al. 2004). These parasites represent at least three disparate clades in the nematode tree of life, as inferred from rRNA sequences. Plant parasites share functional similarities regarding feeding, but many similarities in feeding structures result from convergent evolution and have fundamentally different developmental origins. Although Tylenchida rRNA phylogenies are not fully resolved, they strongly support convergent evolution of sedentary endoparasitism and plant nurse cells in cyst and root-knot nematodes. This result has critical implications for using model systems and genomics to identify and characterize parasitism genes for representatives of this clade. Phylogenetic studies reveal that plant parasites have rich and complex evolutionary histories that involve multiple transitions to plant parasitism and the possible use of genes obtained by horizontal transfer from prokaryotes. Developing a fuller understanding of plant parasitism will require integrating more comprehensive and resolved phylogenies with appropriate choices of model organisms and comparative evolutionary methods.

It is unlikely that a species switches from a fully free-living to a parasitic lifestyle in one step, and it is generally accepted that prior to the transition to parasitism, preadaptations must exist. Preadaptations are features that evolved for different reasons but facilitated the step toward parasitism (Dieterich and Sommer 2009). Indeed, many free-living nematodes can be found in association with other organisms without being parasitic. These associations range from short-term, rather unspecific, phoretic interactions to long-term associations that can be highly species specific. For example, dauer juveniles of different species of the genus *Pristionchus* associate in a species-specific manner with scarab beetles (Weller et al. 2010). The worms seem not to harm the beetle, but instead wait until the beetle dies to resume development on the rich microbial fauna that emerges on the carcass. There is empirical evidence that at least for some parasitic nematodes, the infective stages, which are crucial for entering the host, are homologous to the dauer juveniles in free-living nematodes (Wang et al. 2009). Hence, dauer juveniles and phoretic and necromenic interactions are likely candidates for the preadaptations that facilitated the evolution of parasitism by allowing a stepwise formation of tight and specific interactions. The closely related species with very different ecologies and very distantly related species with similar ecologies make nematodes an interesting system with which to investigate how genomes are shaped by the environment and by evolutionary descent.

10.19 Horizontal Gene Transfer (HGT) in Nematodes

Horizontal gene transfer (HGT), the transmission of a gene from one species to another by means other than direct vertical descent from a common ancestor, has been recognized as an important phenomenon in the evolutionary biology of prokaryotes (Danchin 2011). In eukaryotes, in contrast, the importance of HGT has long been overlooked, and its evolutionary significance has been considered to be mostly negligible. However, a series of genome analyses has now shown that HGTs not only do probably occur at a higher frequency than originally thought in eukaryotes, but recent examples have also shown that they have been subject

to natural selection, thus suggesting a significant role in the evolutionary history of the receiver species. Surprisingly, these examples are not from protists in which integration and fixation of foreign genes intuitively appear relatively straightforward, because there is no clear distinction between the germ line and the somatic genome. Instead, these examples are from nematodes, multicellular animals that do have distinct cells and tissues and do possess a separate germ line. Hence, the mechanisms of gene transfer appear in this case much more complicated.

One of the most unexpected findings from whole genome sequencing projects in nematodes is the widespread occurrence of HGT, which is the transmission of genes between organisms in a form other than vertical inheritance. Although HGT is frequent in prokaryotes, it was thought to be rare among eukaryotes with sexual reproduction (Andersson 2005). Recent genome and EST sequencing projects, however, provide strong evidence for HGT from bacteria, fungi, amoebozoa, or endosymbionts into various nematode genomes. Best characterized are examples of HGT in parasitic *Meloidogyne*, *Heterodera, Globodera*, and *Pratylenchus* groups, the fungivorous *Bursaphelenchus*, necromenic *Pristionchus* species, and the filarial parasite *B. malayi* (Dieterich and Sommer 2009).

Horizontal gene transfer implies the nonsexual exchange of genetic material between species, in some cases even across kingdoms. Although common among Bacteria and Archaea, HGTs from pro- to eukaryotes and between eukaryotes were thought to be extremely rare. Recent studies on intracellular bacteria and their hosts seriously question this view. Recipient organisms could benefit from HGT as new gene packages could allow them to broaden or change their diet, colonize new habitats, or survive conditions that previously would have been lethal (Mitreva et al. 2009). About a decade ago, plant-parasitic nematodes were shown to produce and secrete cellulases. Prior to this, animals were thought to fully depend on microbial symbionts for the breakdown of plant cell walls. It was hypothesized that the ability of nematodes to parasitize plants was acquired by HGT from soil bacteria to (ancestral) bacterivorous nematodes. Since the identification of the first nematode cellulases, many more plant cell wall-degrading enzymes (CWDE) have been identified in a range of plant-parasitic nematode species.

HGT requires close physical contact between donor and recipient, and this could be achieved in, for example, a symbiont–host or a trophic relationship. The former type of relationship was indeed shown to potentially result in the transfer of genetic material (e.g., *Brugia malayi* and *Wolbachia*). However, currently known endosymbionts of nematodes may not be the source of CWDEs. Remarkably, all cellulases discovered so far within the order Tylenchida belong to a single glycoside hydrolase family (GHF5). A range of soil bacteria harbors GHF5 cellulases, but of course, nothing can be said about the gene content of soil bacteria at the time HGT took place, if at all.

The characterization of cellulases/other CWDEs and their genomic organization in more basal (facultative) plant-parasitic Tylenchida are needed to find out if CWDEs were indeed acquired via HGT from bacteria. A more complete picture about the evolution of CWDEs among plant-parasitic Tylenchida needs a detailed characterization of two – so far – fully unexplored basal suborders, Tylenchina and Criconematina. The natural acquisition of novel genes from other organisms by horizontal or lateral gene transfer is well established for microorganisms. There is now growing evidence that horizontal gene transfer also plays important roles in the evolution of eukaryotes (Mayer et al. 2011). Genome sequencing and EST projects of plant and animal associated nematodes such as *Brugia*, *Meloidogyne*, *Bursaphelenchus*, and *Pristionchus* indicate horizontal gene transfer as a key adaptation toward parasitism and pathogenicity. However, little is known about the functional activity and evolutionary longevity of genes acquired by horizontal gene transfer and the mechanisms favoring such processes.

Werner Mayer et al. (2011) attempted to transfer cellulase genes to the free-living and beetle-associated nematode, *Pristionchus pacificus*, for

which detailed phylogenetic knowledge was available, to address predictions by evolutionary theory for successful gene transfer. They used transcriptomics in seven *Pristionchus* species and three other related diplogastrid nematodes with a well-defined phylogenetic framework to study the evolution of ancestral cellulase genes acquired by horizontal gene transfer. Intraspecific, interspecific, and intergenic analyses were performed by comparing the transcriptomes of these ten species and tested for cellulase activity in each species. Species with cellulase genes in their transcriptome always exhibited cellulase activity indicating functional integration into the host's genome and biology. The phylogenetic profile of cellulase genes was congruent with the species phylogeny demonstrating gene longevity. Cellulase genes showed notable turnover with elevated birth and death rates. Comparison by sequencing of three selected cellulase genes in 24 natural isolates of *Pristionchus pacificus* suggested that these high evolutionary dynamics are associated with copy number variations and positive selection.

Not surprisingly, nematodes have evolved to occupy diverse ecological niches as they are the most abundant and speciose metazoans and account for up to 80 % of the kingdom's members (Boucher and Lambshead 1994). Like the well-studied *C. elegans*, most are free living and graze on microbes or detritus, and, as such, have no obvious direct impact on humans. Others, however, are adapted as parasites and are responsible for such widespread problems as human disease, debilitation of livestock, and crop damage. Plant-parasitic forms are responsible for an estimated $100 billion in annual crop damage worldwide. The most damaging family (the *Heteroderidae*) includes the root-knot (*Meloidogyne* spp.) and the cyst (*Globodera* and *Heterodera* spp.) nematodes. Root-knot nematodes penetrate plant hosts and migrate between the cells in roots, where they induce the formation of large multinucleate cells called "giant cells." Galls form around the giant cells and the roots become distorted, often leading to compromised root function and retardation of plant growth. The origin of plant parasitism within the phylum Nematoda is intriguing. The ability to parasitize plants has originated independently at least three times during nematode evolution, and as more molecular data has emerged, it has become clear that multiple instances of horizontal gene transfer (HGT) from bacteria and fungi have played a crucial role in the nematode's adaptation to this new lifestyle (Jones and Danchin 2011). The first reported HGT cases in phytonematodes were genes encoding plant cell wall-degrading enzymes. Other putative examples of HGT were subsequently described, including genes that may be involved in the modulation of the plant's defense system, the establishment of a nematode feeding site, and the synthesis or processing of nutrients. Although, in many cases, it is difficult to pinpoint the donor organism, candidate donors are usually soil dwelling and are either plant-pathogenic or plant-associated microorganisms, hence occupying the same ecological niche as the nematodes. The exact mechanisms of transfer are unknown, although close contacts with donor microorganisms, such as symbiotic or trophic interactions, are a possibility. The widespread occurrence of horizontally transferred genes in evolutionarily independent plant-parasitic nematode lineages suggests that HGT may be a prerequisite for successful plant parasitism in nematodes.

Phylogenetic reconstruction of nematode cell wall-degrading enzymes strongly indicates the independent acquisition from distinct microbial donors (Danchin et al. 2010). For example, the characterized cellulases from plant-parasitic Tylenchida are from glycoside hydrolase family 5 (GHF5). A GHF5 gene cassette consisting of the catalytic domain and the carbohydrate-binding module 2 (CBM2) was acquired as an intronless ancestral gene from putative bacterial donors. In contrast, the pine wood nematode *Bursaphelenchus xylophilus*, which is part of the same clade as the Tylenchida, has independently acquired a different family of cellulases (GHF45) from fungi. Similar findings have been made for other families of cell wall-degrading enzymes of plant-parasitic nematodes by systematic investigations of the evolutionary history of the corresponding genes. These studies also suggested massive gene duplications after the ancestral

acquisition by HGT, a finding that has several evolutionary implications.

Horizontal gene transfer occurs frequently in prokaryotes, but seems to be rare in eukaryotes. For example, ~1 % of the gene repertoire in *Meloidogyne* probably originated by horizontal transfer (Scholl et al. 2003) compared to 1–5 % of single-copy genes and at least 22 % of gene duplicates in Y-proteobacteria *Meloidogyne hapla*, a plant-parasitic nematode, seems to have gained at least a dozen genes by horizontal gene transfer from bacteria that occupy similar niches in the soil and roots. Those genes gained are useful for the nematode's parasitic lifestyle, such as cellulases for digesting plant material, and signaling molecules that induce morphological changes in the plant, facilitating invasion. A distantly related plant parasite, *Bursaphelenchus xylophilus*, seems to have independently acquired a cellulase gene from a fungus. Perhaps horizontal transfer can spur the transition to parasitism. Several groups of parasitic nematodes, including *Brugia malayi*, live in symbiosis with specific bacteria carried by the nematodes. Some of these are extracellular symbionts, but others are intracellular, such as *Wolbachia* living in *B. malayi* and other filarial nematodes. The capture of the *Wolbachia* gene set seems to have been adaptive for filarial nematodes, since killing *Wolbachia* with antibiotics reduces the growth and fecundity of the nematodes.

Free-living nematodes may also have pinched genes from organisms that live nearby. Many nematodes use other animals, often arthropods and mollusks, as transport hosts. For example, *C. remanei* lives in close association with mollusks and isopods. Indeed, *C. elegans* has four genes, including an alcohol dehydrogenase, that have stronger sequence matches to fungi than to other animals. These *C. elegans* genes group with fungal genes in phylogenetic trees. Similar phylogenetic analyses will allow us to scan the eight new genomes for stolen genes.

It is not clear which genetic differences between the plant-parasitic and nonparasitic forms may be responsible for conferring parasitic ability. On the basis of phylogenetic analysis, it appears that plant parasitism arose independently at least three times over the course of nematode evolution. Consequently, one cannot be assured that any gene or set of genes that aid in the parasitic lifestyle in one nematode species will also exist in another. Conceptually, several mechanisms affecting evolution to parasitism can be envisioned. These include adaptation of preexisting genes to encode new functions, changes in genes regulating metabolic or developmental pathways, gene duplication, gene loss, and the acquisition of genes from other species, HGT. HGT has become a widely accepted mechanism of rapid evolution and diversification in prokaryotic populations (Ochman et al. 2001). Recent genome analyses of primitive eukaryotes, such as the sea squirt (*Ciona intestinalis*) and single-celled parasitic diplomonads, implicate HGT events in early eukaryotic evolution. In contrast, the extent of horizontal transfer involving higher eukaryotes has been controversial, with many cases of hypothesized horizontally transferred genes having been refuted by later studies.

On the basis of biochemical and immunological criteria, genes have been identified in *Globodera rostochiensis* and *Heterodera glycines* that allow these nematodes to endogenously produce enzymes that can degrade cellulose and pectin, the two major components of plant cell walls. A possible ancient bacterial origin of these genes has been theorized (Popeijus et al. 2000). A bacterial origin for a number of root-knot nematode genes also has been proposed, although their possible role in parasitism is less clear. Some, such as a gene encoding chorismate mutase, were likewise identified on the basis of biochemical properties, whereas others, including a polygalacturonase gene, were identified from expressed sequence tag (EST) data sets, the latter from our data using a keyword search. Veronico et al. (2001) isolated a presumed polyglutamate synthetase gene with bacterial homology by sequencing neighboring regions of the *M. artiellia* chitin synthetase locus. They were to determine whether other root-knot nematode genes might have been acquired by horizontal gene transfer, particularly as such genes might potentially be related to parasitism. Although bacteria-like *Meloidogyne*

genes that are not present in *C. elegans* and *Drosophila* comprise a preliminary pool of candidates, multiple gene loss may be responsible for the presence/absence pattern revealed by the filter. To test this more thoroughly, the authors established a screen to compare the now small pool of preliminary candidates with all other sequences in the public databases. The most parsimonious explanation to be drawn from candidates with no significant matches to any metazoan genes is that they arose by horizontal gene transfer from a non-metazoan pool, as opposed to multiple independent gene losses in the metazoan lineages. Candidates thus identified were subsequently validated through phylogenetic analysis of relationships between the most similar matches from our screening processes.

Elizabeth H. Scholl et al. (2003) carried out high-throughput genomic screening and found it as an effective way to identify horizontal gene transfer candidates. Transferred genes that have undergone amelioration of nucleotide composition and codon bias have been identified using this approach. Analysis of these horizontally transferred gene candidates suggests a link between horizontally transferred genes in *Meloidogyne* and parasitism.

10.20 Identifying "Parasitism Genes"

Parasitism of plants and animals has evolved independently at least nine times in the history of the nematodes (Dorris et al. 1999). Four of the nematodes whose genomes are being sequenced are parasites: *Haemonchus contortus*, *Meloidogyne hapla*, *Brugia malayi,* and *Trichinella spiralis*. The adoption of parasitism in nematodes probably required adaptation of genes present in their free-living ancestors (Blaxter 2003). For example, the modification of nutrient-acquisition genes found in *C. elegans*, such as digestive enzymes or secreted hydrolases, is likely to have been important for the evolution of parasitism. The ability of parasitic nematodes to survive immunological attack, some living in an infected individual for years, has long been a puzzle. The cuticle is the main site of interaction between a nematode and its environment, and many nematode genes so far implicated in evading host defenses are secreted or cuticle proteins.

Identifying parasitism genes encoding proteins secreted from a plant-parasitic nematode esophageal gland cells and injected through its stylet into plant tissue is the key to understanding the molecular basis of nematode parasitism of plants (Hussey et al. 2011). Parasitism genes have been cloned by directly microaspirating the cytoplasm from the esophageal gland cells of different parasitic stages of cyst or root-knot nematodes to provide mRNA to create a gland cell-specific cDNA library by long-distance reverse transcriptase-polymerase chain reaction. cDNA clones are sequenced and deduced protein sequences with a signal peptide for secretion are identified for high-throughput in situ hybridization to confirm gland-specific expression.

The main soluble surface glycoprotein of filarial nematodes, a secreted glutathione peroxidase (GPX-1), is hypothesized to have a role in immune evasion (Zvelebil et al. 1993). In viral, bacterial, and protozoan parasites, genes involved in host immune evasion or recognition are often under positive selection and so show patterns of rapid amino acid substitution. Indeed, *B. malayi* GPX-1 shows signs of positive selection. By scanning for *Haemonchus contortus* genes that have diverged sharply in sequence from their *Pristionchus* and *Caenorhabditis* orthologs and that bear secretory signals, it may be possible to identify *H. contortus* genes that have adapted for a parasitic lifestyle. Some genes essential for parasitism in worms may be novel genes. One possible source is gene duplication, which allows one duplicate to keep the original role and the other duplicate to take on a parasitic role. For example, the *alt* gene family of filarial nematodes, which has been implicated in establishing infection, has a single *C. elegans* ortholog. On the other hand, other novel genes adapted for parasitism may have been assembled de novo or have been gained by horizontal gene transfer. Plant-parasitic nematodes seem to have

acquired "parasitism genes" from bacteria in their environment.

Some "parasitism genes" may by identifiable by examining the expression pattern of their *C. elegans* orthologs. In *Haemonchus contortus* and *Brugia malayi*, the infective stage of the life cycle is the third larval developmental stage (Blaxter 2003). In *C. elegans* the larval developmental stage is an alternative developmental pathway adopted when food is scarce, called the dauer larva. Thus, identifying the orthologs of *C. elegans* genes expressed in the dauer larva (Wang and Kim 2003) may be a route to pinpointing *Brugia* and *Haemonchus* genes involved in infection. The ability of nematodes to live on plant hosts involves multiple parasitism genes. The most pronounced morphological adaptations of nematodes for plant parasitism include a hollow, protrusible stylet (feeding spear) connected to three enlarged esophageal gland cells that express products that are secreted into plant tissues through the stylet. Reverse genetic and expressed sequence tag (EST) approaches are being used to discover the parasitism genes expressed in nematode esophageal gland cells. Some genes cloned from root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes have homologues reported in genomic analyses of *Caenorhabditis elegans* and animal-parasitic nematodes. To date, however, the candidate parasitism genes endogenous to the esophageal glands of plant nematodes, such as the ß-1,4-endoglucanases, have their greatest similarity to microbial genes, prompting speculation that genes for plant parasitism by nematodes may have been acquired by horizontal gene transfer.

Nematode feeding sites are constructed from plant cells, modified by the nematode to feed itself. Powerful new techniques permit to begin to elucidate the molecular mechanisms that produce the ultrastructural features in nematode feeding cells (Gheysen and Fenoll 2002). Many plant genes that are expressed in feeding sites produced by different nematodes have been identified in several plant species. Nematode-responsive plant genes can now be grouped in categories related to plant developmental pathways, and their roles in the making of a feeding site can be illuminated. The black box of how nematodes bring about such elaborate cell differentiation in the plant is also starting to open. Although the information is far from complete, the groundwork is set so that the functions of the plant and nematode genes in feeding site development can begin to be assessed.

Secretory proteins encoded by genes expressed in the esophageal gland cells of plant-parasitic nematodes have key roles in nematode parasitism of plants (Gao et al. 2001a). Two venom allergen-like protein cDNAs (designated hg-vap-1 and hg-vap-2) were isolated from *Heterodera glycines* gland cell cDNA libraries. Both cDNAs hybridized to genomic DNA of *H. glycines* in Southern blots. The hg-vap-1 cDNA contained an open reading frame encoding 215 amino acids with the first 25 amino acids being a putative secretion signal. The hg-vap-2 cDNA contained an open reading frame encoding 212 amino acids with the first 19 amino acids being a putative secretion signal. Genes of hg-vap-1 and hg-vap-2 contained four introns, which ranged in size from 44 to 574 bp, and five exons ranging in size from 43 to 279 bp. In situ hybridization analyses showed that mRNAs of both vap genes accumulated specifically in the subventral gland cells of *H. glycines* during parasitism. In this study, the gland cell-specific expression and presence of predicted secretion signal peptides in both VAPs suggested that these proteins are secreted from the nematode and may play a role in the infection of host plants by this parasite.

10.21 Mechanisms Involved

With many of the resistance genes, mechanisms have been described in genetic basis of host–pathogen interactions, which include gene–gene relationship, localized necrosis (hypersensitive response), the degradation of feeding structures in the host (a sign of poor nutrition for nematode), changes in gene expression, and molecular responses.

10.21.1 Gene–Gene Relationship

Plants have a repertoire of resistance genes that protect them from many pathogens, including fungi, bacteria, nematodes, and viruses (Williamson 1999). In many cases, pathogen recognition by the host is mediated by single resistance genes (*R genes*) in the host and single gene in the pathogen called avirulence (*Avr*) genes. This relationship proposes that hypersensitive reaction occurs when the product of plant resistance gene (*R*) interacts with the product of pathogen virulence or avirulence gene (*Avr*). Disease resistance requires a dominant resistance (R) gene in the plant and a corresponding avirulence (Avr) gene in the pathogen. R genes are presumed to either enable plants to detect Avr gene-specified pathogen molecules or initiate signal transduction to activate defenses or possess the capacity to evolve new R gene specificities rapidly. The following steps are involved in plant pathogens:

- Pathogen entry into plant cell or connection with infected cells.
- Release of protein and other molecules.
- Binding of R gene products to certain molecules from pathogens (Avr gene products).
- Binding activates R gene product and triggers protective hypersensitivity response (HR).
- When R and Avr gene products do not match, no HR occurs and plant becomes susceptible to disease.

Recently, R genes have been cloned from several plant species. Most encode proteins that carry a structural motif with a repeating pattern of 20–30 amino acids called a leucine-rich repeat (LRR). A few have been cloned and a number of additional genes are likely to be cloned in the near future.

Mi-1: The *Mi* gene of tomato confers effective resistance against several root-knot nematode species. This gene was introduced into cultivated tomato, *Lycopersicon esculentum*, from the wild species *L. peruvianum* by embryo rescue of the interspecific cross. The Mi gene was isolated by positional cloning and its identity confirmed by complementation of function. Transgenic plants with *Mi* were also found to be resistant to the potato aphid, *Macrosiphum euphorbiae*, indicating that this gene confers resistance to the aphid as well as to root-knot nematodes.

Gpa2: Recently Gpa2, a gene that confers resistance against some isolates of the potato cyst nematode, *Globodera pallida*, was cloned by a positional cloning strategy. This gene is a member of the NBS-LRR gene family and contains a possible LZ near its amino terminus.

GroI: A gene mediating resistance against the potato cyst nematode, *Globodera rostochiensis.*

Hs1$^{pro\text{-}1}$: The first nematode resistance gene to be cloned was *Hs1$^{pro\text{-}1}$*, a gene from a wild relative of sugar beet that confers resistance against *Heterodera schachtii*.

Cre3: A gene mediating resistance against cereal cyst nematode (CCN). Using the sequences of NBS-LRR gene as a probe, homologues were detected in other regions of the wheat genome, some of which correspond to other CCN R gene loci. In addition, loci with homology to R genes have been found to be associated with CCN and aphid resistance in barley.

The recognition of pathogen by host initiates a cascade of defense responses, often including a hypersensitive response consisting of localized cell necrosis at the infection site. Feeding site becomes surrounded by necrosing tissues and eventually collapses. For example, *Mi*-mediated resistance is characterized by a localized necrosis of host cells near the invading nematode (Zacheo et al. 1993). Additional molecular changes occur rapidly after the infection of resistant plants. For instance, the activity levels of the enzymes phenylalanine ammonia lyase and anionic peroxidase are induced early in the resistance response in tomato to many pathogens including nematodes. With *Hi*-mediated resistance of potato to *G. rostochiensis*, the feeding site begins to develop and the nematode becomes sedentary. However, the developing feeding site becomes surrounded by necrotic tissues and collapses. The few nematodes that do develop on *H1* potato plants are mostly male, a sign of poor nutrition for the nematode.

10.21.2 Changes in Gene Expression

Changes in gene expression are suggestive of a stress or defense response, which have been observed after infection with either cyst or root-knot nematodes. For example, changes in gene expression in potato leaves after root infection by the cyst nematode *G. rostochiensis* include the induction of pathogenesis-related proteins; in tomato roots infected with root-knot nematodes, genes with homology to several known plant defense genes (including peroxidase, chitinase, lipoxygenase, and proteinase inhibitors) are induced locally within 12 h of inoculation; the induction of the family of genes encoding glycoproteins that form a major component of plant cell walls and are induced in plant defense responses is significantly increased in *M. javanica-induced* galls at 1 week after infection as well as in tomato root tips by 12 h after infection.

Molecular responses include wounding or stress caused by nematode infection as well as perturbations directed toward the initiation and maintenance of feeding sites. The complex morphological and physiological changes that occur during the establishment of feeding sites are reflected by altered gene expression in the host. Because many of the genes identified in the response are members of gene families with complex regulation, their regulation is difficult to interpret. Not surprisingly, phytohormone levels are also abnormal in root-knot nematode-infected roots, providing an additional level of complexity in understanding plant responses to nematode infection.

In total, intraspecific genetic variation in host range and response to specific resistance genes is high, especially for the sexually reproducing plant-parasitic nematode species. This heterogeneity has made it difficult to breed for nematode resistance in crops. There may be multiple genetic mechanisms by which a nematode can acquire the ability to circumvent resistance. Loss of a nematode gene product could result in failure of the plant to recognize the nematode. Hence, efforts to clone several nematode resistance genes are currently in progress. To fill the gap, a variety of strategies to engineer synthetic resistance are being developed. Strategies that combine one or more natural resistance genes with synthetic resistance may be the most effective.

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