Phytopathogenic Nematodes

Principles of Plant-Microbe Interactions

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Chapter 11
Phytopathogenic Nematodes

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Abstract Soil is teeming with life, and rhizosphere soil is even more densely inhabited than bulk soil. In terms of biomass, bacteria and fungi are dominant groups, whereas nematodes (roundworms) are the most abundant Metazoans. Bulk soil, soil not directly affected by living plant roots, typically harbours around 2000–4000 nematodes per 100 g, while in the rhizosphere these numbers should be multiplied by a factor 3–5. This difference is not only explained by a higher density of plant parasites, as also bacterivorous and fungivorous nematodes benefit from the local boost of the bacterial and fungal community. Most nematodes feeding on higher plants are obligatory parasites. In this chapter four independent lineages of plant-parasitic nematodes are discussed. Facultative plant parasites often occupy basal positions

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Fig. 11.1 Schematic overview of a plant-parasitic nematode. Amphids and phasmids are chemosensory organs. The stylet is a protrusible, hollow puncturing device, that is used to penetrate plant cell walls. Figure courtesy of Shinya et al. (2013)

within a lineage. Most, but not all, economically high impact plant parasites such a root knot, cyst and lesion nematodes belong to the most distal nematode clade (Clade 12; Holterman et al. Mol Biol Evol 23:1792–1800, 2006). In this chapter, some of the latest insights on the evolution, the ecology and the biology of phytopathogenic nematodes will be covered.

11.1 Nematodes

Nematodes are small (mostly between 0.2 and 2.5 mm in length), worm-shaped animals that together constitute the phylum Nematoda. This phylum, which is thought to have arisen during early phases of the Cambrian explosion (≈ 550 million years ago) in marine habitats, belongs to the superphylum Ecdysozoa that encompasses all moulting animals (Aguinaldo et al. 1997). Nematodes are not only present in terrestrial systems, but also in freshwater and marine habitats (Bongers and Ferris 1999). Next to their ubiquity, nematodes are abundant and can reach densities of up to millions per square meter in soil, residing in the water films attached to soil particles or e.g. in or around plant roots. Due to their size and their mobility, nematodes are easily extractable from soil as compared to protozoans, fungi and bacteria. The phylum Nematoda show a high trophic diversity; nematodes may feed upon bacteria, fungi, protozoa, algae, other nematodes, a combination of the aforementioned food sources (omnivores), or they may be facultative or obligate parasites of plants or animals (Yeates et al. 1993). Due to this diversity in feeding habits, nematodes can be found at all three levels of the soil food web (Ferris et al. 2001). Plant parasites, in fact herbivores, reside in the first trophic level as they feed directly on roots of higher plants. Bacterivores and fungivores are found in the second trophic level as they feed on primary decomposers. Carnivorous nematodes, also referred to as predators, feed mainly on other nematodes and are therefore positioned at the third trophic level. It should be noted that plant-parasites (see Fig. 11.1) usually constitute a minority within the nematode community, both in terms of number of individuals as well as of species.

11.2   Use of an SSU rDNA Framework to Reconstruct the Evolution of Nematodes

Nematodes are morphologically highly conserved; even for experts, the ability to identify certain species depends on the life stage or sex of the present individuals (Floyd et al. 2002). Considering the age of this phylum, the saying 'never change a winning team' is certainly applicable. As the number of informative morphological characters is limited, identification of nematodes on the basis of such characters is challenging and requires a considerable amount of time, experience and expertise.

Keeping the economical relevance of this animal phylum in mind, it is remarkable to see that nematode systematics is far from established. It has a long history of constant revision, and over a dozen general schemes for nematode classification have been proposed. One of the first phylum-wide classifications was proposed by Chitwood and Chitwood (1933). They divided the phylum into two classes, the Phasmidia and Aphasmidia, later renamed to Secernentea and Adenophorea respectively. This was based mainly on the fact that the Secernentea share several characters, including the presence of phasmids, small sensory organs on the tail. Although it was already recognized at the time that the Adenophorea did not form a natural group, the division of the Nematoda into these two groups persisted for a long time. The first person to apply cladistic principles to nematode systematics was Lorenzen (Lorenzen 1981). He also recognized that the Adenophorea were not a monophyletic group, but could not provide an alternative. Also at lower taxonomic levels (order, family and genus level), systematics were far from stable (De Ley and Blaxter 2002). Despite their ecological and physiological diversity, their conserved morphology and small size resulted in a paucity of observable, phylogenetically informative characters. Furthermore, many characters show a convergent evolution. In recent years DNA sequence data have brought a revival to the field of systematics. The first major classification to incorporate both morphological and molecular phylogenetic information was presented by De Ley and Blaxter (2002).

Appreciating that nematodes arose early in animal evolution, it would be conceivable that even relatively conserved genes such as the ribosomal RNA-encoding genes could offer us insights in the evolution of plant parasitism within this phylum. Especially among invertebrates, the small subunit ribosomal DNA (SSU rDNA) gene (coding for SSU rRNA) is frequently used to deduce deep phylogenetic relationships. Because of their vital role in the assembly of proteins in the ribosomes, there is a strong selection on the SSU and LSU ribosomal DNA genes (LSU: large subunit). As a consequence these genes—at least parts thereof—are very conserved. Among the ribosomal RNA encoding genes, the SSU rDNA is most conserved. Ribosomal DNA genes are usually present in multiple copies (the *Caenorhabditis elegans* genome harbours ≈ 55 copies; Ellis et al. 1986) and this implies that a relatively small quantity of starting material (e.g. a single nematode corresponding to ≈ 0.2 ng DNA) is sufficient for a polymerase chain reaction (PCR)-based amplification. Normally it would not be advisable to use a multicopy gene in phylogenetics because there could be the risk of comparing paralogs instead of orthologous gene copies.

However, intrachromosomal homogenization ensures that mutations in a copy of a SSU rDNA gene are either removed or taken over in the other SSU rDNA copies (Dover et al. 1993).

For some time, SSU rDNA in nematodes was suggested to have a substitution rate 2–3 times higher than in most other Metazoa. In a study on the phylogenetic position of the arthropods, Aguinaldo and co-workers carefully selected nematode species with relatively low SSU rDNA substitution rates (Aguinaldo et al. 1997). The SSU rDNA substitution rates within the phylum Nematoda appeared to be relatively variable. The model organism *Caenorhabditis elegans* and the animal parasite *Strongyloides* showed remarkably high substitution rates; respectively 0.192 and 0.187 substitutions per site, whereas another, more basal animal parasite—*Trichinella*—was shown to have a significantly lower rate of change (0.110 substitutions per site). Related Arthropods have values ranging from 0.040 to 0.080 (Aguinaldo et al. 1997). Higher rDNA substitution rates as observed for nematodes with a short life cycle and/or an advanced parasitic life style could result in unexpected resolution at lower phylogenetic levels.

The small subunit ribosomal DNA gene (≈ 1700 bp) has been used successfully for the generation of molecular frameworks throughout the animal kingdom. The SSU rDNA harbours a few regions that are almost fully conserved among eukaryotes and this allowed the design of so-called "universal primers" for the amplification of this particular gene from a wide range of Metazoans. In fact SSU rDNA has—in terms of diversity—a mosaic structure of very conserved regions interspersed by more variable elements. Amplifiability from unknown samples in combination with the presence of multiple regions with ample phylogenetic signals explains why this gene became popular in nematode phylogenetics. A first key paper by Blaxter et al. (1998) (53 nematode taxa), was followed by Holterman et al. (2006) (360 taxa), and Van Megen et al. (2009) (1250 taxa). Currently, the SSU rDNA molecular framework of nematodes at the Laboratory of Nematology, Wageningen University, includes approximately 2800 taxa. Bayesian inference based analysis of 360 nematode taxa by Holterman et al. (2006) gave rise to a phylogenetic framework characterized by a backbone consisting of 12 consecutive bifurcations. Based on these bifurcations, 12 clades were defined within the phylum Nematoda. From this analysis it was clear that trophic specialisations such as plant, mammal or insect parasitism all have arisen multiple times during evolution. It should be noted that due to the absence of good fossil records it is not possible to label nodes with a proper assessment of the era in which such a hypothetical common ancestor has lived.

11.3 The Evolution of Plant Parasitism Among Nematodes

From molecular analyses of a wide diversity of representatives of the phylum Nematoda, it became clear that plant parasitism has arisen at least four times independently. In all lineages plant parasites are equipped with a protrusible, hollow, injection
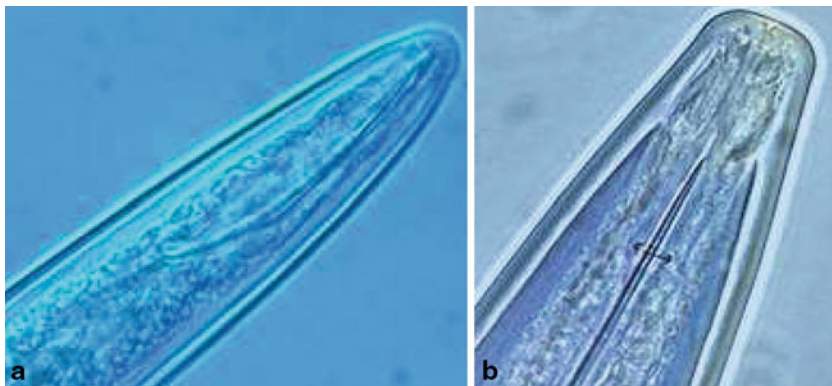
Fig. 11.2 Head regions of representatives of two distinct lineages of plant-parasitic nematodes. *Left*: *Trichodorus primitivus* (Triplonchida, Clade 1) and *right*: *Longidorus intermedius* (Dorylaimida, Clade 2). Pictures were taken at a 1000 times magnification. (Source: Hanny van Megen, Wageningen University, Laboratory of Nematology, The Netherlands)
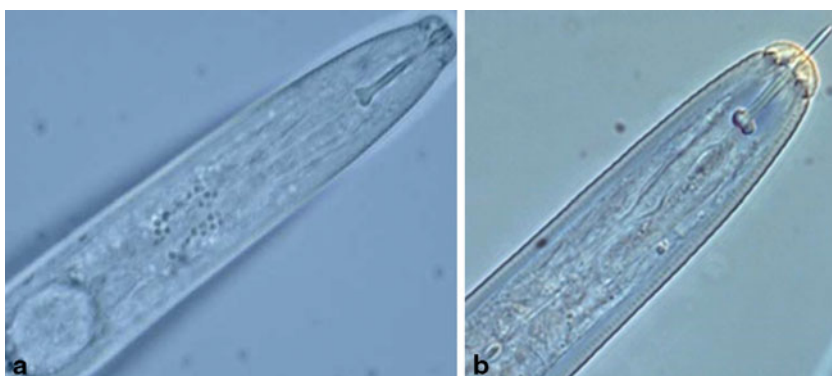
Fig. 11.3 Head regions of representatives of two distinct lineages of plant-parasitic nematodes. *Left*: *Aphelenchoides subtenuis* (Aphelenchida, Clade 10) and *right*: *Globodera rostochiensis* (Tylenchida, Clade 12). Pictures taken at a 1000 times magnification. (Source: Hanny van Megen, Wageningen University, Laboratory of Nematology, The Netherlands)

needle-like device that is used to puncture the plant cell wall, to deliver pathogenicity-related secretions in the plan root, and to take up nutrients from the plant (see Figs. 11.1, 11.2, and 11.3). Another common denominator among plant parasitic nematodes is the large size of the pharyngeal gland cells (usually a single dorsal and multiple subventral glands (Fig. 11.1), and the high activity of these glands just before and during plant parasitism.

Within Clade 1 (most basal clade of this phylum), obligatory plant parasites can be found within the order Triplonchida, in the family Trichodoridae. Plant-parasitic representatives within this family are generally known as 'stubby root nematodes'. These nematodes are ectoparasites, and they use a curved protrusible onchiostyle to

puncture rhizodermis cells (Fig. 11.2). Nematode secretions that are thought to be involved in root cell penetration and manipulation are produced in the pharyngeal glands located in the basal bulb. Stubby root nematodes harbour five pharyngeal glands cells: one dorsal gland, two posterior and two anterior ventrosublateral glands. These glands open into the lumen of the nematode, and produce pathogenicity related-proteins. It is so far unclear whether these five glands act during distinct phases of plant feeding (Karanastasi et al. 2003).

The second lineage can be found in Clade 2, in a family embedded in the order Dorylaimida called Longidoridae. The common name of this category of plant parasites is 'dagger nematodes'. Dagger nematodes are equipped with an odontostyle, a protrusible hollow spear (Fig. 11.2) that is used to puncture the plant cell wall, to secrete pathogenicity-related proteins and to take up food from the plant cell. As compared to stubby root nematodes, members of the family Longidoridae tend to feed on deeper cell layers (e.g. cortical instead of rhizodermal cells). The terminal bulb in the Dorylaimida contains five pharyncheal gland cells, but in case of the family Longidoridae, two ventrosublateral glands seems to be degenerated (or fused) as the nuclei of this second pair have disappeared (although the five gland orifices are still intact) (Loof and Coomans 1972). Also in this respect, the plant parasitic Longidoridae are distinct from the more basal plant-parasitic members of the order Triplonchida. It might be worth noting that both a number of representatives of both the Trichodoridae and the Longidoridae are transmitters of plant viruses.

The plant-parasitic members of the third lineage are mainly (or even exclusively) facultative plant parasites; as an alternative food source they can feed on fungi as well. They are members of the families Aphelenchoididae and Parasitaphelenchidae, and the most notorious representatives of these groups are the causal agent of white tip in rice, *Aphelenchoides besseyi*, and the pine wood nematode *Bursaphelenchus xylophilus.* Currently these two families are positioned in Clade 10, but this clade positioning is not robust, as the GC contents of SSU rDNAs of these families is relatively low as compared to related families (Holterman et al. 2006). Members of these two families are equipped with a stomatostylet (Fig. 11.3), a device functionally comparable with the onchiostyle and the odontostyle mentioned above. However, as suggested by their names, the origins of these injection needle-like devices are fundamentally distinct.

The fourth, and economically most important group of plant parasitic nematodes can be found in the most distal nematode clade (Clade 12). This clade roughly corresponds to the order Tylenchida, with the exception of the suborder Hexatylina, a branch that mainly comprises insect parasitic nematodes. The other three suborders, the Hoplolaimina, the Criconematina, and the Tylenchina, harbour plant-parasitic nematode species. Contrary to the first two suborders, numerous members of the Tylenchina are facultative parasites of higher plants, being able to use lower plants, fungi and oomycetes as alternative food sources. The most well-known and best-studied plant parasites such as the root knot (*Meloidogyne* sp.), cyst (*Heterodera* and *Globodera* sp.) and lesion (*Pratylenchus* sp.) nematodes all belong to the suborder Hoplolaimina. Tylenchida are equipped with a stomatostylet (Fig. 11.3), and at least of part of the secretory components involved in plant parasitism are produced in

the dorsal and in the two subventral pharyngeal glands. It should be noted that the subventral glands are most active in pre-parasitic second stage juveniles, and their activity slows down rapidly in parasitic second and third stage juveniles. The peak of dorsal gland activity is observed in parasitic life stages, and thus lags behind the peak of subventral gland activity.

11.4 Some Biological Characteristics of Migratory and Endoparasitic Plant Parasites

Root-knot, lesion and cyst nematodes pose a serious threat to main agricultural crops such as potato, sugar beet, and soybean. As such these distal representatives of the order Tylenchida constitute the economically most detrimental group of plant parasitic nematodes. Among the three genera mentioned above, root-knot nematodes such as *Meloidogyne incognita*, *M. hapla*, and *M. chitwoodi*, are most polyphagous, being able to infect almost all domesticated plants worldwide (Trudgill and Blok 2001). Lesion nematodes are members of a species-rich genus named *Pratylenchus* (*ca* 70 valid species), and species such as *P. penetrans, P. neglectus*, and *P. thornei* are notorious parasites in crops such as potato and tomat as well as a range of cereals and legumes. Lesion nematodes are migratory endoparasites (see below), and as such provide other opportunistic soil bacteria and fungi access to the plant root. The invasion of plant roots by root-knot and cyst nematodes leads to the formation of nematode feeding sites. In case of sedentary endoparasites, establishment of so called 'giant cells' or 'syncytia' (root-knot and cyst nematodes, respectively) is considered as one of the most sophisticated adaptations of plant parasitism. The syncytium, a conglomerate of plant cells fused by partial degradation of plant cell walls, functions as a metabolic sink that transfers plant assimilates from the conductive tissues in the vascular cylinder to the sedentary nematode. In case of rot knot nematodes, the formation of five to seven giant-cells grouped around the head region is induced. Upon the injection of nematode secretions, parasitized cells rapidly become larger, hypertrophied and multinucleate as nuclear division occurs in the absence of cell wall formation. Feeding sites, giant cells or syncytia, are used till the end of the nematode life cycle and serve as sink tissues to which nutrients are imported in a symplastic and/or apoplastic manner (Hoth et al. 2008).

On the other hand, migratory endoparasites, such as *Pratylenchus* species, exhibit feeding strategies that may be considered as less refined, but they are no less successful keeping their proliferation and host range in mind. In various life stages, lesion nematodes move freely through the root to feed and reproduce, creating numerous local tissue lesions, which are used as an entrance by the secondary pathogens such as bacteria or fungi. The feeding takes place mostly in the root cortex, but root hair feeding is observed for younger life stages as these are unable to perforate thicker epidermal cell wall (Zunke 1990). The parasitic success of the mentioned groups of nematodes is undoubtedly a result of their unusual ability to overcome the barrier of the plant cell wall. This biological obstacle is mechanically and chemically

weakened by the stylet, spear or onchiostyle-driven puncturing of the cell wall in combination with the local release of cell wall-degrading and modifying proteins.

11.5 The Role of Lateral Gene Transfer in the Evolution of Plant Parasitism

Contrary to vertical gene transfer, the transmission of genes from parents to their off-spring, lateral gene transfer (LGT) is about the stable acquisition of (parts of) genes in a manner other than traditional reproduction. Such non-conventional transfer of genetic material requires, among other things, close physical contact between the between donor and recipient. LGT could for instance occur between organisms with a trophic relationship. As an example, Doolittle described the transfer of genes to early eukaryotes from the bacteria taken by them as food (Doolittle 1998). In the same year, Smant and co-workers (Smant et al. 1998) discovered that the potato and soybean cyst nematodes (*G. rostochiensis and H. glycines* (order Tylenchida) produce and secrete β-1,4-endoglucanase (cellulases). Till this discovery, animals were thought to be incapable degrading plant cell walls; herbivores use microbial endosymbionts for the degradation of these recalcitrant polymers.

This finding constituted the starting point of a series of papers reporting a range of cell wall-degrading enzymes (CWDE) from plant-parasitic nematodes, including pectate lyases (Popeijus et al. 2000), exo-polygalacturonase (Jaubert et al. 2002), xylanases (Mitreva-Dautova et al. 2006) and expansins (Qin et al. 2004). It should be noted that CWDEs are produced in the subventral glands of plant parasitic nematodes during the early phases of infection. These enzymes are unlikely to be involved in feeding site formation in the plant root.

The discovery of a set of cell wall-degrading enzymes (CWDE) is remarkable. Nematodes are devoid of plant cell wall-like structures, and hence it seems safe to state that plant cell wall penetration by parasitic nematodes is the result of mechanical weakening and local depolymerization. Remarkably these plant cell wall-degrading enzymes produced by nematodes were far more similar to their bacterial equivalents than to orthologs from other eukaryotes such as higher plants, fungi or oomycetes. This prompted Keen and Roberts (1998) in a commentary paper to the hypothesize that ancestral bacterivorous nematodes could have acquired a pathogenicity island with multiple plant parasitism-related genes by the ingestion of (plant-parasitic) soil bacteria. Such an acquisition could have enabled them to penetrate a plant cell wall, and exploit a food source that was till that time inaccessible.

If it is true that the evolution of plant parasitism among nematodes was facilitated by the acquisition of CWDEs of bacterial origin, one might wonder whether the four independent lineages as described in 10.3 harbour distinct or similar core sets of cell wall-degrading enzymes. Here we will use cellulases as an example as this is so far the best-characterized CWDE among plant parasitic nematodes. As indicated above, β-1,4-endoglucanases (cellulases) were first discovered in two cyst nematode species *Globodera rostochiensis* and *Heterodera glycines* (Smant et al. 1998).

They are encoded by a multi-copy gene family, which is expressed in the subventral esophageal glands of the infective second stage juveniles (J_2). The biochemical activity of these enzymes is determined as a hydrolysis of β-1,4 glycosidic bonds of cellulose microfibrils. According to their biochemical characteristics, cellulases are represented in various glycoside hydrolase (GH) families. All members of the order Tylenchida (lineage 4 in this chapter) investigated so far (even the insect parasite *Delandenus siridicola*) harbour cellulases belonging to family 5 (GHF5; glutamic acid (Glu) residues essential for catalysis).

The GHF5 genes in Tylenchida comprise at least a catalytic domain, and occasionally this is connected to a linker and/or a type II cellulose-binding domain (CBDII). Due to the slight differences in intron-exon composition and noticeable phylogenetic distance, those cellulases are thought to belong to at least two distinct lineages (Kyndt et al. 2008). These lineages are referred to as *CelI* and *CelII* by (Rehman et al. 2009a), and in a more recent study these are similar to catalytic domains type B and type C respectively (Rybarczyk-Mydłowska et al. 2012). Phylogenetic analysis of nematode GHF5 cellulases suggests for an early acquisition of this category of plant cell wall-degrading enzymes, maybe even by the common ancestor of the order Tylenchida and the family Aphelenchidae (subfamilies Aphelenchinae and Paraphelenchinae) (Rybarczyk-Mydłowska et al. 2012).

A facultative plant parasite belonging to lineage 3, the pinewood nematode *Bursaphelenchus xylophilus*, was shown to produce cellulases from glycoside hydrolase family 45 (GHF 45; aspartic acid (Asp) residues essential for catalysis) (Kikuchi et al. 2004). *Bursaphelenchus* belongs to the family Parasitaphelenchidae, and recently a cellulase from its sister family, Aphelenchoididae, was isolated and characterized. A small-scale analysis of the transcriptome of the foliar nematode Aphelenchoides besseyi resulted in the discovery of another GHF45 cellulase that is produced and presumably secreted (as the core protein is preceded by a predicted signal peptide for secretion) by this nematode species (Kikuchi et al. 2014). So far each plant parasite lineage was thought to be characterized by the production of cellulases from a single GH family. Therefore the finding of a GHF5 cellulase from another facultative plant parasite within this lineage, *Aphelenchoides fragariae*, by Fu et al. (2012) was remarkable Notably, this finding was the result of a directed search as the authors used degenerated GHF5 primers to screen for the presence of cellulases in A. *fragariae*. The GHF5 cellulases described from A. *fragariae* seemed to have some unusual features. For example, it was not possible to detect genomic copies of this sequence in nematodes reared on fungi. Until the finding of this GHF5 cellulase in this foliar nematodes species is confirmed by more detailed research, we tend to state that the presence of GHF45 cellulases is a typical and unique characteristic of this lineage of plant parasitic nematodes.

As compared to the two most distal lineages of plant parasites, the two remaining families within the more basal clade 1 and 2 are poorly characterized. Within the family Longidoridae, a cellulase was identified from *Xiphinema index*, and this enzyme belonged to yet another GH family (Jones and Helder, unpublished results). Based on this fragmentary information, we hypothesize that individual lineages of

plant-parasitic nematodes are characterized by the presence of a single, lineage-dependent kind of cellulase.

11.6   Plant Manipulation by Parasitic Nematodes

Even plant-parasitic nematode species residing in the most basal lineage, ectoparasites belonging the family Trichodoridae, do not show a hit-and-run strategy, i.e. a strategy by which the nematode would just insert its puncturing device into a plant cell, and take up the cytosol, and move on to the next plant cell. In case of the stubby root nematode species *Paratrichodorus anemones* the delivery of nematode secretion into the rhizodermis cell resulted in the redistribution of cytoplasm from all areas of the cell towards the penetration site (Karanastasi et al. 2003). In the interaction of *Ficus carica* seedlings with a representative of the second lineages of plant parasites, the dagger nematode *Xiphinema index,* hypertrophied, multinucleate cells were induced. Most likely the re-differentiation of plant cells is induced by saliva proteins produced by this ectoparasite (Wyss et al. 1980). Hence, plant cell re-differentiation as an essential process linked to parasitism is not a unique characteristic for the most distal nematode taxa in Clade 12, and also this trait seems to have arisen multiple times. Most likely proteins produced in de dorsal glands are responsible for plant cell re-differentiation, but so far the underlying mechanism is unknown. Recently, a number of effector proteins has been identified that are produced in these glands in infectious life stages. It should be noted that the examples given below are all from cyst and root knot nematodes, representatives of the most distal lineages. Indications for local auxin manipulation as an early step in feeding site formation (Goverse et al. 2000) were recently confirmed by the identification of an effector protein from the soybean cyst nematode *Heterodera glycines*, called 19C07, that was shown to interact with an auxin influx transporter (LAX3) in Arabidopsis (Lee et al. 2011). Another category of dorsal gland proteins, the so-called SPRYSECs (secreted proteins containing a SPRY domain) have been identified from the potato cyst nematodes *Globodera rostochiensis* and *G. pallida,* close relatives of the soybean cyst nematode. As compared to e.g. *Caenorhabditis elegans,* the pinewood nematode *Bursaphelenchus xylophilus* and the tropical root knot nematode *Meloidogyne incognita*, this protein family has expanded enormously in cyst nematodes; both potato cyst nematodes were shown to harbor dozens of secreted SPRY proteins (Rehman et al. 2009b, Cotton et al. 2014). Although the function of these proteins in the interaction with the host plant is unknown for most members of this family, one member, SPRYSEC-19, was demonstrated to suppress the CC-NB-LRR disease resistance response in host plants (Postma et al. 2012).

Hence, plant parasitism within the phylum Nematoda is characterized by ample convergent evolution. As we have seen, all of the plant-parasitic lineages developed similar morphological adaptations that allow them to overcome the plant cell wall, a major physical barrier. A similar picture starts to arise for the non-morphological

characteristics of these lineages. Although the origin and the exact blend of cell wall-degrading enzymes might be unique for individual taxa, a roughly similar pallet of enzymes is likely to be produced by all obligatory plant parasites. It will probably become clear in the next years whether or not convergent evolution can be observed in the mechanisms underlying host cell manipulation and the suppression of resistance responses.

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