Summary

Phytonematodes use a stylet and secreted effectors to modify host cells and ingest nutrients to support their growth and development. The molecular function of nematode effectors is currently the subject of intense investigation. In this review, we summarize our current understanding of nematode effectors, with a particular focus on proteinaceous stylet-secreted effectors of sedentary endoparasitic phytonematodes, for which a wealth of information has surfaced in the past 10 yr. We provide an update on the effector repertoires of several of the most economically important genera of phytonematodes and discuss current approaches to dissecting their function. Lastly, we highlight the latest breakthroughs in effector discovery that promise to shed new light on effector diversity and function across the phylum Nematoda.

I. Introduction

Phytonematodes are unique among the plant pathogens because they are animals (microscopic roundworms) that develop an intimate and sustained obligate parasitic relationship with their host plants. When compared with the microbial-feeding nematode Caenorhabditis elegans, a premier biological and genetic model (Jones et al., 2011; Yook et al., 2012), the two most striking adaptations for parasitism in phytonematodes (Hussey, 1989; Baldwin et al., 2004; Davis et al., 2004) include the development of a stylet and elaborate specialized secretory gland cells within the nematode esophagus (Fig. 1a,b). The stylet is a protrusible hollow mouth spear in the nematode head that is used to pierce host plant cell walls to access host cell contents for ingestion. The stylet also serves as a structure to deliver the secreted effectors produced in the nematode esophageal gland cells (Hussey, 1989; Davis et al., 2008) to modify host cells for a permanent source of nutrients.
The definition of effectors offered by Hogenhout et al. (2009) as 'all pathogen proteins and small molecules that alter host-cell structure and function' provides a broad palette that is inclusive of all current investigations of phytonematode effectors. Research on avirulence (avr) genes of microbial pathogens has emphasized the role of secreted pathogen effectors (avr gene products) with respect to their functions in incompatible (resistance gene-mediated) host–microbe interactions (Desveaux et al., 2006). By contrast, most studies of phytonematode effectors have focused primarily on their roles in promoting compatible (susceptible) parasitic interactions with their hosts (Davis et al., 2008; Rosso et al., 2012). Emerging evidence from functional investigations of pathogen effectors (Deslandes & Rivas, 2012; Feng & Zhou, 2012; Rafiqi et al., 2012; Win et al., 2012), including effectors of phytonematodes (Gheysen & Mitchum, 2011; Haegeman et al., 2012; Rosso et al., 2012; Hewezi & Baum, 2013), suggests roles not only in conditioning the host defense response but also in the augmentation of multiple processes of host cells during the course of parasitism.

Parasitic strategies differ among species of phytonematodes, from those that only pierce and ingest cellular contents as they migrate among host cells to species that induce elaborate modifications in plant cells selected to serve as the permanent feeding site for sedentary life stages of the nematode (Hussey & Grundler, 1998). The feeding sites formed in host plant tissues (Fig. 2) by root-knot (Meloidogyne spp.), cyst (Heterodera and Globodera spp.), and reniform (Rotylenchulus reniformis) nematodes have received particular attention in research for the dramatic changes in host cell morphology (Jones, 1981) and gene expression (Escobar et al., 2011) that are induced and the resultant plant damage that culminates in billions of dollars of annual global crop losses (Chitwood, 2003). Plant response to nematode parasitism in either susceptible host genotypes or resistant crop cultivars occurs in the feeding sites that surround the nematode head (Jones, 1981; Williamson & Kumar, 2006), suggesting that effectors are secreted from where the stylet and amphid (labial sensory neuron) openings exist.

Decades of research demonstrating the differential secretory activity of phytonematode esophageal gland cells throughout plant parasitism have identified these glands as the primary source of secreted effectors (summarized in Hussey, 1989; Davis et al., 2000, 2008; Davis et al., 2008; Rosso et al., 2012). Analyses of esophageal gland cell transcription coupled with bioinformatics have identified numerous parasitism genes that encode different putative effector proteins in cyst and root-knot nematodes (Davis et al., 2000, 2008; Haegeman et al., 2012; Rosso et al., 2012; Hewezi & Baum, 2013). More recent genomic and transcriptional analyses have extended effector
identification to other phytonematode species including some migratory plant parasites (Rosso et al., 2009, 2012; Abad & McCarter, 2011; Gheysen & Mitchum, 2011; Jacob & Mitreva, 2011). While exciting discoveries such as nematode cell wall-modifying enzymes of ancient microbial origin (via horizontal gene transfer; Smant et al., 1998; Yan et al., 1998; Scholl et al., 2003; Jones et al., 2005; Mitreva et al., 2009; Danchin et al., 2010; Kikuchi et al., 2011) and secreted nematode effectors that mimic endogenous plant signaling peptides (Wang et al., 2005, 2010; Yan et al., 2011; Mitchum et al., 2008, 2012; Lu et al., 2009) have been described, a majority of putative nematode effector proteins encode novel proteins (Davis et al., 2008; Rosso et al., 2012). The advances in our knowledge of the identities and functions of phytonematode effector proteins have been extraordinary and are beginning to paint a complex picture of nematode regulation of the parasitic process (Gheysen & Mitchum, 2011; Haegeman et al., 2012; Rosso et al., 2012; Hewezi & Baum, 2013), yet we have just begun to scratch the surface in our understanding of these unique host–parasite interactions.

II. Nematode effector regulation and delivery into host cells

As already noted, the three large specialized secretory gland cells, one dorsal and two subventral, in the nematode’s esophagus are the principal sources of effectors essential for phytonematodes to parasitize plants (Fig. 1a,b; Hussey, 1989). The complexity and secretory function of these unique unicellular glands are revealed in ultrastructural studies (Endo, 1984; Hussey & Mims, 1990). Each gland contains a large lobed nucleus with a prominent nucleolus and abundant Golgi complexes, rough endoplasmic reticulum, and other organelles typical of secretory cells. Effector proteins are synthesized in the nuclear region of the gland cells and N-terminal signal peptides direct them to the secretory pathway where they become packaged in membrane-enclosed spherical secretory granules (dense-core vesicles) formed by budding from the trans-Golgi network (Fig. 1c). The secretory granules are transported forward by a microtubule network in the glands’ cytoplasmic extensions to distal elaborate valves where the effector proteins are released into the valve’s end sac by exocytosis (Fig. 1d) and subsequently secreted into host tissues through the nematode’s stylet. During the parasitic cycle of sedentary endoparasitic nematodes, developmental changes occur in the activity of the gland cells and the effector proteins they produce. The subventral glands function primarily but not exclusively in the penetration and migration phases and the dorsal gland, which enlarges after the onset of parasitism, has a principal role in the formation and maintenance of the feeding cells.

While recent advances in molecular biology have enabled significant progress to be made in the identification and functional characterization of nematode effector proteins, little is known about the actual process of effector secretion. Although effectors have been shown to be developmentally expressed, how they are packaged in the granule matrix or what regulates the synthesis and secretion of different effectors throughout the parasitic process remains elusive. Nonetheless, the effector proteins appear to be synthesized in response to specific signals, because different effectors are required to be produced in time and space during the parasitic process. The secretion of effectors appears to occur via a regulated exocytosis pathway in response to external stimuli (Miller & Moore, 1990; Burgoyne & Morgan, 2003; Kim et al., 2006). The observations of nerve processes and neurosecretory cells apposed to the gland cell cytoplasmic extensions and ampulla also indicate that effector secretion is probably regulated by the nematode’s nervous system (Endo, 1984; Hussey & Mims, 1990).

Given the large number of different types of effector proteins that are produced in both types of esophageal gland cell, how the effectors are packaged in the secretory granules is unclear (Fig. 3).

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**Fig. 2** Feeding cells formed by sedentary endoparasitic phytonematodes. (a) A cartoon depicting feeding cells formed by cyst (Heterodera and Globodera spp.), root-knot (Meloidogyne spp.), and reniform (Rotylenchulus spp.) nematodes. (b, c) Sections through infected roots revealing the multinucleate giant cells (GC) and a multinucleate syncytium (S) formed adjacent to the nematode (N) head. Reproduced from Mitchum et al. (2008).
As effector production is developmentally regulated, the gland cells must contain secretory granules comprising a mixture of different effector proteins and/or a mixture of secretory granules containing individually packaged effector proteins, at any particular time. Co-immunolocalization studies to localize multiple effectors simultaneously within the esophageal gland secretory granules will aid in our understanding of effector packaging.

How and what triggers the secretion of specific effectors in different host tissues and cells at critical time-points in the parasitic process remains a mystery. For example, some effectors (e.g. endoglucanases (ENGs)) are clearly secreted into the apoplast during nematode intra- or intercellular migration in the root (Wang et al., 1999), whereas other effectors, shown to directly interact with specific cytoplasmic proteins or targeted to the nucleus, must enter the host cell to function in parasitism (Hewezi & Baum, 2013). Intuitively, the stylet must have evolved for the purpose of penetrating plant cell walls so the parasite could deliver effectors directly into the host cell cytoplasm as well as for ingesting nutrients from the cell during feeding. Indeed, ultrastructural studies of nematodes feeding on host cells reveal that a small pore is formed in the plasma membrane at the stylet orifice, which would be consistent with the parasite being able to secrete effectors directly into and feed from the cytoplasm of host cells (Figs 4, 5; Rebois, 1980; Hussey et al., 1992a). And new molecular evidence is emerging that supports the notion that effector proteins are secreted directly into the cytoplasm of the parasitized host cell. For example, a nematode effector protein that functions as a peptide mimic of plant CLAVATA3 (CLV3)/endosperm surrounding region (ESR) (CLE)-like peptides, which normally function in the apoplast by binding to extracellular receptors, is first delivered to the cytoplasm of host cells. The nematode protein then functions as a ligand mimic in the apoplast after being retargeted by a trafficking domain embedded within the secreted effector protein (Wang et al., 2010a,b). Additional support for functioning of nematode effectors inside plant cells comes from numerous studies that identify intracellular host proteins that directly interact with nematode-secreted effectors and the subcellular compartments targeted by different effectors (Huang et al., 2006a; Elling et al., 2007a; Patel et al., 2010; Lee et al., 2011; Jaouannet et al., 2012). Furthermore, feeding tubes formed within the cytoplasm of feeding cells (Fig 5; Rebois, 1980; Hussey & Mims, 1991) probably develop from nematode stylet secretions produced in the dorsal gland cell. Development of feeding tubes occurs quickly at the basal end near the stylet following its insertion into a host cell (Wyss & Zunke, 1986). In fact, accumulation of a crystalline secretory component similar in ultrastructure to the feeding tube wall was detected in an open dorsal gland valve in a female root-knot nematode (Hussey & Mims, 1990). Identifying the composition of feeding tubes presents an exciting challenge for the future.

Although the preponderance of the evidence supports the delivery of effectors directly into the cytoplasm, some effector proteins, including those that modify cell walls, have been found to accumulate in host extracellular spaces during feeding cell development and maintenance. Immunolocalization studies have...
shown that effectors from the amphids and dorsal gland cells of sedentary stages of root-knot nematodes accumulate at the wall of a feeding cell (Vieira et al., 2011). While the possibility exists that effectors could be deposited externally in the apoplast by the nematode and then translocated via the plasma membrane into the host cell cytoplasm, as determined for oomycete effector proteins (Birch et al., 2008), no signature translocation motifs have been identified in nematode effector proteins (Hewezi & Baum, 2013). Clearly we have only a rudimentary understanding of effector synthesis and secretion by phytonematodes. In the future, determining the environmental triggers for the synthesis and secretion of different effectors throughout the parasitic cycle and where they function in recipient host cells will be required for a complete understanding of nematode parasitism of plants. If the plant signals are first perceived by the amphids to regulate secretion, silencing of known amphid chemosensory genes or mutants in the plant signaling molecules may provide clues to the triggers of differential secretion in phytonematodes.

III. Nematode effectors as probes of plant cell biology

Phytonematodes that gain entry into plant tissues vary in their migration patterns. Some nematodes, such as root-knot nematode, are more stealthy as they migrate intercellularly. By contrast, the forceful intracellular migratory pattern of most endoparasites triggers a path of destruction that elicits a robust plant response. Recent studies have begun to document the changes and differences in gene expression in host roots in response to these different infection strategies (Kynadt et al., 2012). Presumably, the plant’s first line of defense against the invading nematode is triggered in response to damage-associated molecular patterns (DAMPs) or conserved pathogen-associated molecular patterns (PAMPs; Win et al., 2012), although these have yet to be identified for phytonematodes. Thus, many of the effectors secreted during the migratory phase of infection facilitate nematode penetration and migration by degrading components of the plant cell wall, as well as enable nematodes to dampen down the plant’s immune system (Smant & Jones, 2011).

Sedentary endoparasites eventually settle down to feed near the vasculature. Successful establishment and maintenance of feeding cells most certainly requires sustained suppression of plant immune responses. Transcriptional profiles of feeding cells has identified downregulation of defense gene and hormone signaling as a
common theme underlying feeding cell formation by sedentary endoparasites (Barcala et al., 2010; Damiani et al., 2012). Thus, effector proteins secreted during the sedentary phase of parasitism probably coordinately regulate plant defense suppression with cellular reprogramming to form a metabolically highly active feeding cell upon which it will depend for weeks to sustain its growth and development; that is, unless the plant carries genes conferring resistance to the nematode, in which case the interaction culminates in HR-like cell death of the feeding cell and ultimately death of the nematode (Williamson & Kumar, 2006; Kaloshian et al., 2011).

Histological studies provided the first glimpse into these unique feeding cells and detail the distinct morphological features characteristic of each plant–nematode interaction during compatible and incompatible interactions (Endo, 1965; Jones, 1981; Wyss, 1997). The feeding cells induced by root-knot (giant-cell) and cyst (syncytium) nematodes have received the most attention (Gheysen & Mitchum, 2011). Giant cells can be 100 times the size of a normal root cell and undergo repeated acytokinetic mitosis to become multinucleate (Fig. 2b). By contrast, a syncytium is a complex of hundreds of cells coalesced from adjacent wall dissolution to form a multinucleate feeding site (Fig. 2c). Some of the cellular changes leading to feeding cell establishment include alterations to the nuclei, cytoskeleton, hormone status, and metabolism of the selected cell. This is paralleled by extensive changes in cell wall architecture and may involve differing degrees of wall loosening and expansion, dissolution, and ultimately biogenesis, the latter leading to the formation of cell wall ingrowths to facilitate solute uptake typical of transfer cells. Conversely, these processes appear to be disrupted during incompatible interactions. More recently, transcriptome and metabolome analyses of feeding cells have provided insight into the massive changes in gene expression to reveal a complex network of molecular events leading to their formation in susceptible host plants (Ithal et al., 2007; Szakasits et al., 2009; Barcala et al., 2010; Hofmann et al., 2010) or their degeneration in plants resistant to the nematode (Kandoth et al., 2011).

While mechanisms underlying the development or demise of feeding sites for the most part remain a mystery, the end result is unquestionably under the direct influence of nematode stylet-secreted effectors. How do phytonematodes get away with such elaborate modifications of host cells? As we describe below (Section V), some of these effectors are common among the various phytonematodes, whereas others appear to be lifestyle-specific (summarized in Haegeman et al., 2012). The varied host responses and different ontogenies of feeding cells among the various types of sedentary nematodes appear to be driven by the unique combination of effectors delivered to host cells. Consequently, nematode effectors should prove useful as molecular probes to study plant cellular processes such as the cell cycle, the cytoskeleton, cell wall architecture, and cellular metabolism, among other processes.

1. Cell cycle and cytoskeleton

Cell cycle activation is required for feeding cell formation (de Almeida Engler et al., 1999). The enlarged nuclei of giant cells and syncytia are the consequence of aberrant cell cycles leading to genome amplification through endoreduplication, and several endocyte activators involved in this process have been identified (de Almeida Engler et al., 2012; de Almeida Engler & Gheysen, 2013). Both endoreduplication and mitotic activity are required for feeding cells to progress to maturity. Cell cycle aberrations are paralleled by dynamic cytoskeletal rearrangements in syncytia and giant cells (de Almeida Engler et al., 2004). Multiple points of disruption to the cytoskeleton have been described (Caillaud et al., 2008; Clement et al., 2009) that could be influenced by the nematode. While nematode effectors directly targeting these cellular processes have not yet been identified, the apparent hijacking of components of the cell cycle and cytoskeleton is materializing as a point of convergence for effectors from a wide range of pathogens (Wildermuth, 2010; de Almeida Engler et al., 2012) and is expected to come into view in future studies of effector function.

2. Cell wall architecture

A host response pivotal to feeding cell formation is controlled cell wall modifications. The recent use of glycan-specific antibodies and fluorescence imaging is beginning to reveal the distinct molecular architecture of feeding cell walls (Davies et al., 2012). Contributing to these changes are numerous classes of plant proteins involved in cell wall modification and biogenesis, such as expansins, ENGs, extensins, cellulose synthases and UDP-glucose dehydrogenases (Goellner et al., 2001; Wieczorek et al., 2006; Sobczak et al., 2011; Siddique et al., 2012). However, how these are coordinately regulated in time and space remains unclear. These modifications appear, in part, to be mediated cooperatively with nematode effectors, such as secreted cellulose-binding proteins (CBPs) found to interact with plant pectinmethylesterase (Hewezi et al., 2008a). Hence, the nematode may have more direct control over feeding cell wall modifications than originally anticipated.

3. Metabolism

As feeding cells develop, the large central vacuole is reduced to several small vacuoles and is replaced by dense cytoplasm reflecting one of the most impressive features of nematode feeding cells – increased metabolism. The abundant changes in plant genes involved in primary metabolism are well documented from transcriptome studies (Ithal et al., 2007; Szakasits et al., 2009; Barcala et al., 2010), and these are now being coupled with metabolic profiling to reveal the complex metabolic reprogramming within feeding cells (Hofmann et al., 2010). Certainly, this altered metabolism is under the direct influence of the nematode, but how much of this is being driven by nematode secretions? Changes to the phytohormone status of selected cells is crucial to feeding cell establishment (Goverse et al., 2000; Wubben et al., 2001; Grunwald et al., 2009a,b), and evidence is accumulating that nematode effectors may indeed drive these processes by directly targeting hormone transport proteins (Lee et al., 2011). Another effector, chorismate mutase, identified from a wide variety of phytonematodes to date (Lambert et al., 1999; Gao et al., 2003; Jones et al., 2003; Lu et al., 2008; Vanholme et al., 2009a; Haegeman et al., 2011; Yu et al., 2011) and more recently the
suffred fungal pathogen (Djamei et al., 2011), appears to be a key regulator of metabolic priming to promote pathogenesis by altering the plant’s shikimic acid pathway, although the exact mechanism of action remains to be elucidated. The shikimic acid pathway produces aromatic amino acids providing precursors for a variety of metabolites that may influence the plant–nematode interaction, including auxin, salicylic acid, and a variety of other phenolic compounds.

IV. Functional characterization of effectors

We currently only have a rudimentary understanding of the regulatory networks controlling feeding cell formation. A clearer picture is expected to emerge as the functions of more nematode effectors come into view. Functional analyses of the effector proteins encoded by phytonematode parasitism genes remain a challenge because these nematodes are obligate parasites with limitations in forward genetic analyses and a current lack of a genetic transformation system (Davis et al., 2008; Rosso et al., 2012). However, the gene silencing assays and protein interaction studies highlighted below have proved invaluable to assess nematode effector function (Rosso et al., 2009, 2012; Hewezi & Baum, 2013).

1. Silencing

The discovery and development of target gene silencing in *C. elegans* (Fire et al., 1998) using RNA interference (RNAi) has provided a technology to specifically and transiently silence target phytonematode genes, including the parasitism genes that encode secreted effector proteins (Gheysen & Vanholme, 2007; Davis et al., 2008; Rosso et al., 2009; Dalzell et al., 2011; Lilley et al., 2012). Ingestion of double-stranded RNA (dsRNA) complementary to the gene to be silenced is critical to RNAi efficacy for most target genes in nematodes (Timmons & Fire, 1998; Bakhetia et al., 2007; Rosso et al., 2009) and was first successfully demonstrated in phytonematodes by soaking infective cyst nematode juveniles in a dsRNA solution with a feeding stimulant (Urwin et al., 2002). Subsequent studies have demonstrated decreased plant infectivity, reduced target transcript abundance, and altered developmental potential of phytonematodes in RNAi soaking experiments that have targeted parasitism genes (summarized in Lilley et al., 2012; Rosso et al., 2009, 2012). Plant transformation vectors that express target gene dsRNA within transgenic plants have been utilized for host-derived delivery of dsRNA for ingestion by nematodes and subsequent silencing of target nematode genes expressed during the parasitic process (Gheysen & Vanholme, 2007; Davis et al., 2008; Rosso et al., 2009; Lilley et al., 2012). When an apparently essential phytonematode parasitism gene is silenced by plant host-derived RNAi technology, a relatively drastic reduction in nematode parasitic success has been observed (Huang et al., 2006b; Hamamouch et al., 2012; Chronis et al., 2013; Jaouannet et al., 2013; Xue et al., 2013) – results that suggest the functional significance of the encoded effector protein in the parasitic process. Moderate effects on parasitism of RNAi silencing of other phytonematode parasitism genes have also been observed (summarized in Gheysen & Vanholme, 2007; Rosso et al., 2009, 2012; Lilley et al., 2012), suggesting potential functional redundancies among some nematode effector proteins or potential technical difficulties in host expression, delivery, or processing of the dsRNA construct. The design of more effective RNAi constructs and enlistment of microRNA (miRNA) expressed in nematode feeding sites (Hewezi et al., 2008b) as tools for efficient host-derived gene silencing remain as key strategies for functional analyses of phytonematode effector proteins and the production of transgenic plants for future nematode control strategies.

2. Host target identification

Methods employed to identify the host targets of phytonematode effectors have proved successful to begin to elucidate the network of interactions and functions of effector proteins within recipient plant host cells. A wide range of plant subcellular compartments, including the apoplast, cell wall, cytoplasm, and nucleus, are targeted, which is indicative of a wide range of physiological functions in parasitism. Targets of nematode effectors suggest differential roles in host cell transcriptional, developmental, and metabolic reprogramming, protein degradation, and phytohormone transport and accumulation (Gheysen & Mitchum, 2011). These reported effector functions are consistent with the molecular and morphological responses observed in the complex feeding cells induced by sedentary species of phytonematodes (Jones, 1981; Escobar et al., 2011). Host proteins targeted by nematode effectors identified to date include putative SCARECROW-like (SCL) transcription factors (Huang et al., 2006a), a pectinmethylesterase (Hewezi et al., 2008b), a coiled-coiled nucleotide binding leucine-rich repeat (CC-NB-LRR) protein (Rehman et al., 2009a), an oxidoeductase (Patel et al., 2010), a spermidine synthase (Hewezi et al., 2010), an auxin influx transporter (Lee et al., 2011), several leucine-rich repeat receptor-like proteins (LRR-RLPs; Guo et al., 2011), a papain-like cysteine protease (Lozano-Torres et al., 2012), a β-1,3-endoglucanase (Hamamouch et al., 2012), and an aquaporin tonoplast intrinsic protein (Xue et al., 2013). In all cases, except for the LRR-RLPs, yeast two-hybrid analysis was the method of choice to identify the host-interacting proteins. In the majority of cases, these interactions were confirmed using either *in vitro* pull-down assays or *in planta* bimolecular fluorescence complementation (also known as BiFC) by bombardment into onion epidermal cells. In the case of nematode-secreted CLE effectors, radiolabeled peptides were tested for direct interaction with selected plant receptor peptides transiently expressed in *Nicotiana benthamiana* leaves (Guo et al., 2010, 2011).

The list of host cell targets and processes will greatly expand as existing and new putative phytonematode effectors are characterized.

V. An update on nematode effectors by genus

1. Heteroderda

Cyst nematode species of the genus *Heteroderda* are well known for their distinct host ranges. In susceptible hosts, these nematodes use...
a stylet to penetrate the cell wall of a selected root cell and secrete effector proteins to induce the formation of a syncytium. While the mechanisms underlying and driving the differences in host range among species are unknown, a role for nematode effectors in this process is anticipated. To date, effector discovery in *Heterodera* has focused on two species, *Heterodera schachtii* and *Heterodera glycines*. Unlike other cyst nematodes, the sugar beet (*Beta vulgaris*) cyst nematode *H. schachtii* infects *Arabidopsis thaliana*, and was adopted as a model system for studies of plant–nematode interactions nearly 20 yr ago (Sijmons et al., 1991; Gheysen & Fenoll, 2011). *Heterodera glycines*, the soybean (*Glycine max*) cyst nematode, has demonstrated utility as a genetic model system with which to study parasitism (Opperman & Bird, 1998; Niblack et al., 2006), but functional genomic approaches targeting identification of stylet-secreted effector proteins have accelerated the process. The earliest approaches focused on affinity purification of proteins using monoclonal antibodies to antigens in nematode stylet secretions (De Boer et al., 1996). This approach was successful in identifying the first nematode parasitism genes, encoding β-1,4-endoglucanases, in *H. glycines* and *Globodera rostochiensis* (Smant et al., 1998). The search for a higher throughput approach resulted in the identification of over 50 candidate parasitism genes encoding putative secreted effectors of *H. glycines* from cDNA libraries constructed from RNA isolated from cytoplasm microaspirated from esophageal gland cells of parasitic life stages (Gao et al., 2001a, 2003; Wang et al., 2001). A microarray-based expression atlas of the parasitism genes across *H. glycines* life stages followed (Elling et al., 2007b, 2009). The identification of orthologous *H. glycines* parasitism genes in the closely related *H. schachtii* has facilitated studies to acquire insight into the biological function of several nematode effectors using *A. thaliana* as a model system (Wang et al., 2005, 2010a; Hewezi et al., 2008a, 2010; Patel et al., 2010; Lee et al., 2011; Hamamouch et al., 2012). Additionally, approaches such as direct sequencing of *in vitro*-produced esophageal gland secretions and mining of expressed sequence tags (ESTs) generated from infective juveniles have identified a number of putative secreted effector proteins from *H. schachtii* (De Meutter et al., 2001; Vanholme et al., 2006).

To date, 70% of all effectors identified from *Heterodera* are novel proteins, which presents a considerable challenge in ascribing function. Among those identified from *H. glycines* and *H. schachtii* with sequence similarity to known proteins is an elaborate suite of cell wall-modifying proteins (CWMPs), including ENGs (Smant et al., 1998; De Meutter et al., 2001; Gao et al., 2002a, 2004a; Vanholme et al., 2006), CBPs (Gao et al., 2004b; Vanholme et al., 2006), pectate lyases (PELs; Vanholme et al., 2007), and an arabinogalactan endo-1,4-β-galactosidase (Vanholme et al., 2009b). These nematodes also produce secreted effector proteins with homology to chitinase (CHI; Gao et al., 2002b), chorismate mutase (CM; Bekal et al., 2003; Gao et al., 2003), ubiquitin (Gao et al., 2003; Tytgat et al., 2004), and venom allergen proteins (VAPs; Gao et al., 2001b, 2003), although their functions in nematode parasitism remain unknown. The first nematode-secreted peptide hormones sharing homology with plant CLE peptides were identified from *H. glycines* (Wang et al., 2001; Gao et al., 2003; Olsen & Shriver, 2003; Mitchum et al., 2012), and more recently from *H. schachtii* (Patel et al., 2008; Wang et al., 2011). These peptides were shown to functionally mimic the effects of plant CLE peptides in exogenous peptide assays and through complementation of the *A. thaliana clavata*3 mutant (Wang et al., 2005, 2010a, 2011). Although the biological function of these peptide mimics in feeding cell formation is unclear at present, they were recently shown to signal through an *A. thaliana* LRR-RLPs CLV1, CLV2, and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2; Repogle et al., 2011, 2013), probably triggering developmental changes necessary for syncytium formation. Another nematode-secreted effector identified from *H. glycines* and *H. schachtii* that may mimic endogenous host proteins is annexin. Annexins are calcium- and membrane-binding proteins with wide-ranging cellular functions. Nematode annexin was able to complement the reduced germination phenotype of a plant annexin mutant under high salt stress and was found to interact with an *A. thaliana* oxido-reductase, possibly promoting parasitism by modulating host stress and defense responses (Patel et al., 2010). Despite the challenges, tremendous progress has been made to elucidate the function of several novel effector proteins, including 10A06, 19C07, and 30C02, identified from both *H. glycines* (Gao et al., 2003) and *H. schachtii* (Hewezi et al., 2010; Lee et al., 2011; Hamamouch et al., 2012). The 10A06 and 30C02 effector proteins were found to interact with spermidine synthase (Hewezi et al., 2010) and β-1,3-endoglucanase (Hamamouch et al., 2012), respectively, and functional studies have suggested a role for these proteins in modulating host defense responses. The 19C07 effector protein was found to interact with an auxin influx transporter, LAX3, presumably to regulate auxin balance during the early stages of feeding cell formation (Lee et al., 2011).

Despite the considerable progress in nematode effector discovery in *Heterodera* species, we are just breaking the surface in our understanding of effector function.

2. *Globodera*

Species of nematodes belonging to the genus *Globodera* feed on plants of the Solanaceae family. The majority of effector studies to date have focused on the potato (*Solanum tuberosum*) cyst nematodes (PCNs; *G. rostochiensis* and *Globodera pallida*), internationally recognized quarantine pests that infect a number of important agricultural crops such as potato, tomato (*Solanum lycopersicum*), and eggplant (*Solanum melongena*). Like *Heterodera* species, these sedentary endoparasitic nematodes secrete a battery of CWMPs during their invasion of plant roots. Genes encoding ENGs, PELs, CBPs, and EXPs (expansins) that were found to be expressed within the subventral esophageal gland cells have been cloned from both PCN species (Smant et al., 1998; Popeijus et al., 2000; Qin et al., 2004; Kudla et al., 2007; Jones et al., 2009; Rehman et al., 2009b). Knocking down of *Gr*-eng genes by RNAi reduced nematode infectivity, probably as a result of impaired root penetration at the onset of parasitism (Chen et al., 2005; Rehman et al., 2009b). *Gr-Exp* genes were demonstrated to have cell-wall-extension activity in planta (Qin et al., 2004; Kudla et al., 2005). Predicted structural differences between *GrPEL1* and *GrPEL2* suggested that these enzymes may target different pectic
polysaccharides (Kudla et al., 2007). These nematode-secreted CWMPs appear to work in concert to weaken cell walls to facilitate nematode penetration and intracellular migration through the root tissue.

The two largest families of effector proteins identified from PCNs include the CLE-like proteins and secreted SP1a and Ryanodine receptor (SPRY) domain (SPRYSEC) proteins produced within the nematode’s dorsal esophageal gland cell (Jones et al., 2009; Lu et al., 2009; Rehman et al., 2009a). Unlike A. thaliana and Heterodera CLE genes, which encode proteins with a single C-terminal CLE motif, many Globodera CLE genes encode proteins with multiple CLE motifs that vary in their sequence. Ectopic overexpression of the multidomain GrCLE proteins in A. thaliana produces a wide range of plant phenotypes (Lu et al., 2009). The three identical CLE motifs of GrCLE1 were shown to be processed into an active 12-amino acid CLE peptide by plant proteases (Guo et al., 2011). These studies suggest that, once secreted in planta, the multidomain Globodera CLEs may give rise to an assortment of functional CLE peptides potentially acting in different plant CLE signaling pathways to facilitate nematode parasitism. The search for receptors in potato that perceive Globodera CLE peptides is actively being pursued. Multiple SPRYSEC genes have been cloned from PCNs and the genome of G. pallida is predicted to contain > 200 different SPRYSEC genes (Jones et al., 2009; Rehman et al., 2009a; Postma et al., 2012). The SPRYSEC family may target many different host proteins through the hypervariable regions residing in the SPRY domain (Rehman et al., 2009a) and were recently found to play roles in activation and suppression of effector-triggered immunity (ETI). Coexpression of GpRBP1 (RBP: Ran-binding protein), a member of the SPRYSEC family from G. pallida, with the Gpa2 (Globodera pallida 2) resistance protein in potato induced programmed cell death in N. benthamiana leaves, indicating that GpRBP1 triggers Gpa2-mediated nematode resistance (Sacco et al., 2009). The effector SPRYSEC-19 of G. rostochiensis was demonstrated to bind directly to SW5-F, a CC-NB-LRR type of disease resistance protein, in planta (Postma et al., 2012), but this association did not activate ETI in host plants. Instead, a role for GrSPRYSEC19 in suppressing ETI and disease resistance was recently demonstrated (Postma et al., 2012).

Ubiquitin carboxyl extension proteins (UBCEPs), VAPs, and CMs are also expressed in the two PCN species (Jones et al., 2003, 2009; Lu et al., 2008; Lozano-Torres et al., 2012; Chronis et al., 2013). GrUBCEP12, which is expressed exclusively within the dorsal gland cell of G. rostochiensis, was found to be processed into free ubiquitin and a 12-amino acid peptide (GrCERP12) in planta and the released GrCERP12 peptide could suppress plant immune responses. Once secreted into plant cells, GrUBCEP12 becomes two functional peptides that may act in different host cellular pathways to promote nematode parasitism (Chronis et al., 2013). GrVAP1, expressed within the subventral gland cells of G. rostochiensis, was recently shown to specifically interact with the papain-like cysteine protease Rcr3
dim (Rcr3 Solanum pimpinellifolium) in tomato, and this interaction could perturb the protein active site, resulting in increased plant susceptibility to the nematode (Lozano-Torres et al., 2012). Interestingly, GrVAP1 could also trigger Cladosporium fulvum 2 (Cf-2)-mediated nematode resistance, probably through perturbation of Rcr3
dim (Lozano-Torres et al., 2012). A unique finding for Globodera CM genes is that they undergo alternative splicing (Lu et al., 2008; Yu et al., 2011). Alternative splicing may be an important mechanism regulating CM activity and other nematode effector functions during nematode parasitism.

Putative novel effectors, such as transthyretin-like proteins and proteases, as well as homologs of a large number of novel effectors from Heterodera species, were identified from both PCN species through mining of nematode EST sequences (Jones et al., 2009; X. Wang et al., unpublished). The genome sequences of the two PCN species will become available soon, which will reveal a complete catalog of effectors used by Globodera nematodes in plant parasitism.

3. Meloidogyne

Among all phytonematodes discussed here, root-knot nematodes (Meloidogyne spp.) clearly hold an elevated status. Most Meloidogyne spp. have a remarkably wide host range and it is safe to say that there is no higher plant that is not host to at least one root-knot nematode species. Why are Meloidogyne spp. able to infect such an enormous variety of plants, whereas cyst nematodes have very narrow host ranges? The secret probably lies in Meloidogyne stylet-secreted effectors. Apart from cell wall-degrading and -modifying enzymes such as ENGs (Rosso et al., 1999), PELs (Doyle & Lambert, 2002) and CBPs (Ding et al., 1998), or enzymes that target the plant shikimate pathway (i.e. CMs; Lamb et al., 1999), there is little overlap between the battery of effectors we currently know in root-knot and cyst nematodes. This is especially true for those effector genes that are considered novel and that do not share similarity with known genes in other organisms (Huang et al., 2003, 2004; Davis et al., 2004; Bellafiore et al., 2008). Even 16D10, a 13-amino acid Meloidogyne peptide effector that has some homology to the C-terminal CLE motif of plant CLE peptides, functions differently from CLE-like effectors in cyst nematodes. Whereas the cyst nematode CLE-like effector HgCLE was able to rescue clv3 mutant phenotypes in A. thaliana (Wang et al., 2005, 2010a), 16D10 failed to do so (Huang et al., 2006a). Interestingly, 16D10 interacts with plant SCL transcription factors SCL6 and SCL21 (Huang et al., 2003, 2004), which suggests a high-level upstream regulatory role for this effector. Even though both cyst and root-knot nematodes probably target similar plant processes to initiate and maintain feeding sites, they clearly have different molecular means to accomplish their goals by using distinct sets of effectors.

Recent studies are beginning to untangle some of the complex molecular interactions between effectors of Meloidogyne spp. and their host plants. For example, the amphid-secreted Meloidogyne amphid protein 1 (MAP-1) effector, which was found by screening near-isogenic nematode populations that were avirulent or virulent in plants carrying the Meloidogyne incognita resistance gene Mi, seems to be involved in ETI (Semblat et al., 2001). Recent analyses indicate that highly variable tandem repeats in the MAP-1 protein correlate with nematode (a)virulence and that map-1 is part of a
gene family (Castagnone-Sereno et al., 2009; Tomalova et al., 2012). In addition to a classic role in ETI, it is conceivable that Meloidogyne effectors are responsible for defense suppression and the massive modifications of the host plant’s physiology to induce and maintain giant cells. Secreted CMs exist in several variants in a number of Meloidogyne spp. and seem to play an important role in the molecular interaction with the host plant (Doyle & Lambert, 2003; Huang et al., 2005; Long et al., 2006). Meloidogyne CM genes are highly upregulated 2 d after infection, which suggests a role in early events related to parasitism (Painter & Lambert, 2003). Overexpression of MjCM-1 in planta lowered the concentration of indole-3-acetic acid, presumably by competing for chorismate and thereby altering chorismate-derived metabolism, including salicylic acid-mediated plant defense reactions (Doyle & Lambert, 2003). Mi-CRT (CRT: calreticulin) from M. incognita was demonstrated to suppress PAMP-triggered immunity (PTI; Jaouannet et al., 2013). Transgenic A. thaliana overexpressing Mi-CRT showed increased susceptibility to M. incognita and the induction of defense-related genes and callose deposition after treatment with the PAMP elf18 (N-terminal 18 amino acids of Elongation factor Tu). Similarly, overexpression of effectors MJ-NULG1a (Meloidogyne javanica nuclear-localized gene 1a) (Lin et al., 2013) and 7E12 (Souza et al., 2011) in planta enhanced Meloidogyne infection, but the exact role of these effectors remains elusive. Interestingly, 7E12 overexpression also accelerated feeding site formation and vacuole size increase in giant cells (Souza et al., 2011). Meloidogyne giant cells are metabolic sinks and are speculated to function as highly specialized phloem transfer cells. The M. incognita effector Mi8D05 interacts with plant aquaporin tonoplast intrinsic protein 2 (TIP2), which supports this idea and suggests that effectors regulate solute and water transport in giant cells. Interestingly, Mi8D05 also induced accelerated shoot growth and early flowering when overexpressed in A. thaliana, but had no discernible phenotypic effect in roots (Xue et al., 2013).

Even though the current state of our knowledge of how exactly Meloidogyne effectors enable plant infection still resembles an incomplete jigsaw puzzle with an unknown number of pieces, promising progress in recent years suggests that we are beginning to put the pieces together and will eventually be able to fully decipher the molecular dialog between plant and nematode.

4. Rotylenchulus

The genus Rotylenchulus contains 10 known species that thrive in the tropical and subtropical parts of the world. All species are sedentary endo-endoparasites, which means that the posterior portion of their body is exposed on the root surface. Upon maturation, the adult female attains the characteristic reniform (i.e. kidney) shape. Rotylenchulus reniformis, the reniform nematode, is the most damaging species, having a broad host range that includes both dicot and monocot plants from 77 families (Robinson et al., 1997). Economically important hosts for R. reniformis include upland cotton (Gossypium hirsutum), soybean, pineapple (Ananas comosus), and sweet potato (Ipomoea batatas) (Robinson et al., 1997). The infective life stage (young adult female) of the reniform nematode establishes a feeding site within the root similar to the syncytium formed by Heterodera and Globodera spp. Rotylenchulus reniformis syncytia usually initiate from an endodermal cell and expand via partial cell wall dissolution (Rebois et al., 1975). A preliminary assessment of ESTs derived from R. reniformis sedentary females identified a number of sequences homologous to cyst nematode effectors including cellulase, SEC-2, G22C12, G16B09 and one EST that shared similarity with the HgCLE peptide (Wubben et al., 2010). Dorsal esophageal gland-specific expression of the R. reniformis CLE homolog has been demonstrated in situ hybridization (L. Gavilano et al., unpublished). A much larger transcriptome survey of sedentary parasitic R. reniformis females using next-generation sequencing has identified additional potential effector homologs, including homologs of H. glycines CM, annexin, UBCEP, VAP, PEL, and CHI (K. Showmaker et al., unpublished). While homology-based approaches to effector discovery can quickly identify candidates for further study, effectors that are either specific to R. reniformis or sufficiently divergent from other species will go unnoticed. Thus, employing approaches for comprehensive effector gene discovery (Maier et al., 2013) is warranted.

VI. New approaches for effector identification

Despite concerted efforts for more than two decades, we can safely say that we probably have not discovered the full effector spectrum of any plant or animal parasitic nematode. The fact that effectors appear for the large part synthesized in the esophageal gland cells has provided a clearly defined target for effector identification and led to the intense focus of research efforts on the esophageal glands.

The identification of effectors in phytomonatodes undoubtedly has been aided by the availability of genome sequences of root-knot nematodes (Abad et al., 2008; Opperman et al., 2008). Bioinformatic genome mining identified large panels of effector candidates with certain protein similarities and the presence of N-terminal signal peptides. Similarly, the impending release of genome sequences of other nematodes, notably potato cyst nematodes, clearly will aid effector identification in additional taxa. But even so, genome sequences alone will not allow the identification of complete effector repertoires and will miss effector types not fitting a preconceived notion of what effectors should look like. Rather, combinations of genome mining with other avenues of inquiry will be required. In particular, transcriptomic investigations of the esophageal glands combined with genome studies stand a great chance of truly expanding our understanding of effector repertoires and their variability among genera, species and pathotypes.

Focus on the esophageal gland cells as the immediate target opens up the possibility of unravelling a complete effector spectrum of an individual nematode species. Over the years, different methods to target this cell type have been used, and most recently culminated in a method that allows the actual direct isolation of these gland cells (Maier et al., 2013). This new gland isolation technique will allow the identification of effectors from nematodes with different modes of parasitism. So far the research community has generated a catalog of cyst nematode and root-knot nematode effectors and found surprisingly few that overlap. But what about other sedentary nematodes, such as R. reniformis or Tylenchulus...
semipenetrans, or even Cactodera or Meloidodera isolates? But that’s not all — what about migratory nematodes and then, of course, those nematodes that are in between — such as ring nematodes (Criconematid spp.), which retain their ability to move about between feedings but have prolonged their feeding sessions considerably and have adopted the ability to profoundly alter their host between feedings but have prolonged their feeding sessions considerably and have adopted the ability to profoundly alter their host feeding cells (Hussey et al., 1992b; Westcott & Hussey, 1992)? Exploring the effector spectra in these taxa should prove to be fascinating. As a consequence of such studies, evolutionary relationships may have to be rewritten, the origin of certain effectors may become clear, and exciting revelations about the mechanisms that govern host range or virulence will be made.

In addition to merely searching for protein sequence similarities, more in-depth future analyses will identify other criteria that define effectors. For example, it is very possible that certain small domains of conserved amino acids or conserved protein structures will be identified as hallmarks of nematode effector groups, and such discoveries will allow additional queries into available genomes and transcriptomes.

One of the challenges of identifying the complete effector repertoire of a single nematode species lies in the fact that not all effectors are released through the stylet. Clearly, nematodes are most likely, or already have been shown, to release bioactive proteins from their amphids and their hypoderms along the body. Furthermore, release of compounds from the excretory/secretory system or the cloaca is a possible avenue of effector delivery. While stylet delivery has all the hallmarks of being the route through which the feeding cells are manipulated, the other avenues could function in suppression of defenses during migration as well as during the disruptive growth of the sedentary adult female stages as they expand. Similarly, not all effectors are released from the nematode through a signal peptide-dependent secretory pathway. Several effector candidates released from nematodes without a signal peptide have been reported (Bellafore et al., 2008), and it remains to be seen how many additional such proteins will be uncovered in the future. In addition to the transcriptomic and genome sequence mining of nematodes, proteomic analyses of plant tissues or cells that have been in contact with parasitizing nematodes provide a potential means to identify proteins that are released by nematodes in planta. While sensitivity limitations currently exist for available technologies, it is expected that technical advances will open up new opportunities.

The picture becomes considerably more daunting when taking into account that, while the research community has focused on proteins as effectors, it is hard to imagine that the successful group of phytonematodes would have limited itself to only one class of effector molecules. Instead, nonproteinaceous molecules, potentially derived from diverse secondary metabolites, may also play important roles in parasitism. Indeed, there are literature reports that support this notion. De Meutter et al. (2003) described plant hormones being released from phytonematodes. Also, considering that rhizobial bacteria use carbohydrates as nodulation (NOD) factors and that McCarter et al. (2003) have reported nod gene homologs in the RKN genome, carbohydrate effectors may not be that unexpected. Interestingly, Weerasinghe et al. (2005) also observed root hair curling in advance of infective root-knot nematode juveniles, supporting a NOD factor-like function of an unknown nematode secretion.

**VII. A global perspective of nematode effector function**

In addition to the identification of all effectors, the characterization of their individual and integrative functions dwarfs all other challenges for a complete understanding of plant–nematode interactions. The current approach of overexpression of effector genes in plants, target gene silencing via RNAi, and identification of target host proteins, while successful (Hewezi et al., 2008a, 2010; Lee et al., 2011; Hamamouch et al., 2012), allows analyses of only a single effector at a time. Future investigations must consider how multiple nematode effectors function in concert during the parasitic process — as they do very successfully in nature (often to the detriment of agriculture). How many effectors (and which types) are required to form a feeding cell (or trigger host defense)? A fundamental change in approach is needed, or better, a bigger picture view. Interdisciplinary approaches, capitalizing on potential synergisms between experts in different fields, appear to be the most promising way to make comprehensive progress. What are some of the options that present themselves?

High-throughput assays clearly are urgently needed. For example, high-throughput assays for the identification of defense suppression activities of effectors are available and should be applied to nematode effectors. Similarly, high-throughput in planta effector expression approaches such as viral expression systems (Zhang et al., 2010) will allow the phenotypic analyses of a large number of effectors when introduced into plants. Moreover, the use of other pathogen effector delivery systems, most notably bacterial type III secretion systems (Sohn et al., 2007), to introduce nematode effectors into plants has significant promise for exploring effector traits in planta.

Approaches that allow high-throughput interactor screens, such as robotic yeast two-hybrid analyses, are particularly useful, and comprehensive interactome maps are realizable (Arabidopsis Interactome Mapping Consortium, 2011; Mukhtar et al., 2011; Van Leene et al., 2011; Hu, 2013; Mongiovì & Sharan, 2013). The prospect of identifying effectors interacting with other effectors also exists. While effector–effector interaction studies with yeast two-hybrid analyses may reveal such interactions, methods to identify multivalent protein interactions, such as techniques based on affinity purification of protein complexes followed by proteomics analyses, are highly desirable. Some effectors probably function in concert while interacting with host factors. For example, assembly of the feeding tube (Hussey & Mims, 1991) might involve a multi-effector/plant protein interaction, as the current mining of effector candidates has not yet revealed a clear candidate for an auto-assembling single effector feeding tube protein.

The localization of some nematode effectors to the plant host cell nucleus (Elling et al., 2007a; Jaouannet et al., 2012) and the demonstration of an interaction with plant transcription factors (Huang et al., 2006a) suggest potential regulatory roles at the transcriptional level. A related type of interaction is the direct binding of pathogen effectors to nucleic acids. Although discovered
in other pathosystems (Römer et al., 2007; Boch et al., 2009), DNA-binding activity and direct transcriptional regulatory function of nematode effectors have not yet been reported, but have the potential to exist. In that case, high-throughput yeast one-hybrid analyses to identify DNA-binding activities of effectors will be very desirable.

Computational approaches to reveal integrative effector functions should play a prime role in future strategies. Prediction of protein interaction sites or protein structures with known functions undoubtedly will become available for analyses of novel nematode effector proteins. While analyses of plant response to single effectors has utility, a contrasting approach may be to remove (silence) a single nematode effector and monitor the plant response (i.e. like using transcriptomics or, histology) in its absence upon host infection. Systems biology approaches (Lucas et al., 2011; Collakova et al., 2012; Benfey & Scheres, 2013) to elucidating global effector functions, that is, the integration of several ‘omics’ data (such as metabolomics, proteomics, and transcriptomics) will ultimately play a key role. By focusing on the plant response at the infection site with high resolution coupled with similar assays of effector function, systems biology holds tremendous promise in separating true effects from experimental noise. Data from the types of analyses presented above can provide the raw materials for integrative approaches to model the collective host cell pathways and hubs targeted by nematode effectors to orchestrate these fascinating parasitic interactions. Undoubtedly, the prospects are exciting and bright for more fully revealing the role of nematode effector proteins in this new emerging paradigm of parasitism.

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References


