ROOT-KNOT AND CYST NEMATODE PARASITISM GENES: THE MOLECULAR BASIS OF PLANT PARASITISM

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INTRODUCTION

Roundworms of the Nematoda comprise one of the largest animal phyla on Earth (1). They inhabit diverse terrestrial and aquatic niches through adaptations of a spectrum of trophic groups, including parasites that threaten human, animal and crop plant health. The most well-known nematode, *Caenorhabditis elegans*, is a native soil-dwelling microbivore that has emerged as a premier model for animal biology and genomics (2). While studies of *C. elegans* provide a blueprint of fundamental nematode biology, recent advances in molecular genetics of parasitic nematodes indicate specific divergence in adaptations of nematodes for obligate parasitism of an array of plant and animal host species (3-8). Identifications of the molecular tools enabling a particular mode of parasitism by nematodes are providing some intriguing discoveries about the nature of parasite evolution.

Plant-parasitism by nematodes can be distinguished by which plant part is parasitized and the length of time the nematode feeds from a plant cell. Plant-parasitic nematodes, or phytonematodes, are considerably larger than a host plant cell, so a single, unmodified plant cell cannot sustain nematode feeding throughout the parasite's life cycle. This critical host–parasite balance is manifested in the adaptation of two very different phytonematode groups: migratory parasites that feed while moving from plant cell to cell and sedentary parasites that first modify plant cells in order to be able to continuously feed in one location as their bodies enlarge and they become immobile. These two groups frequently are likened to primitive *versus* highly evolved forms of parasitism, respectively. While this nomenclature probably does not describe the two modes of parasitism adequately since all parasitism more than likely encompasses highly-evolved traits, this distinction serves well in describing the different levels of complexity of the two different parasitic modes. Emphasis is placed in this review on adaptations of sedentary phytonematodes that induce dramatic changes in host feeding cells to sustain parasitism in one location.

MAJOR SEDENTARY PHYTONEMATODES

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 (Figure 1), 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 (9). 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 (Figure 2A). 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 (Figure 2B). Nuclei of syncytial cells undergo endoreduplication of their DNA content but do
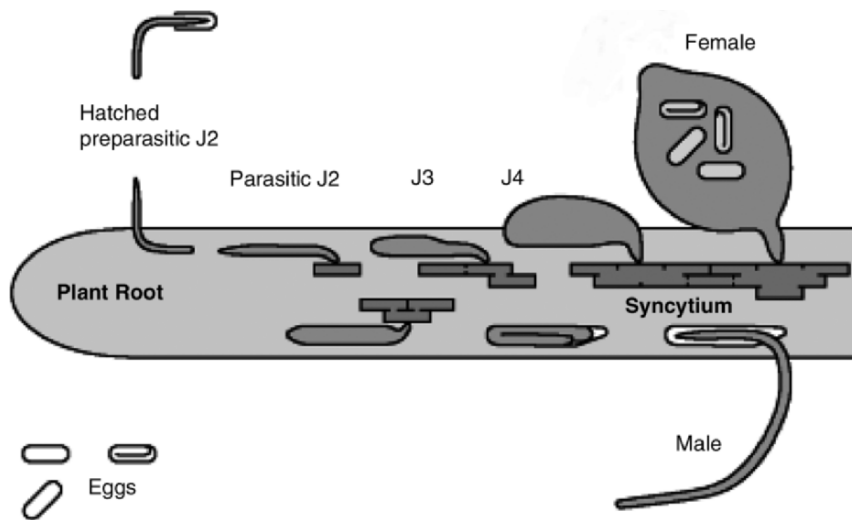
Figure 1. Cyst nematode life cycle. Second-stage juvenile (J2) cyst nematodes hatch from eggs in the soil and become parasitic by penetrating into the root of a host plant. Close to the root vascular tissue, parasitic J2 become sedentary and induce the formation of feeding sites called syncytia, which consist of fused root cells. Feeding from its syncytium, a nematode enlarges and matures through the third (J3) and fourth juvenile (J4) stage into either an adult female or a male nematode. Males regain mobility and exit the plant root to fertilize the still sedentary females (Drawing by J. de Boer).

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 (10). 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 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.

ADAPTATIONS FOR PLANT PARASITISM

Plant-parasitism is thought to have evolved at least three times independently (3), but morphological adaptations for plant parasitism are surprisingly similar among all plant-parasitic nematodes. Most notably, all plant-parasitic nematodes are equipped with a stylet (hollow mouth spear) 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
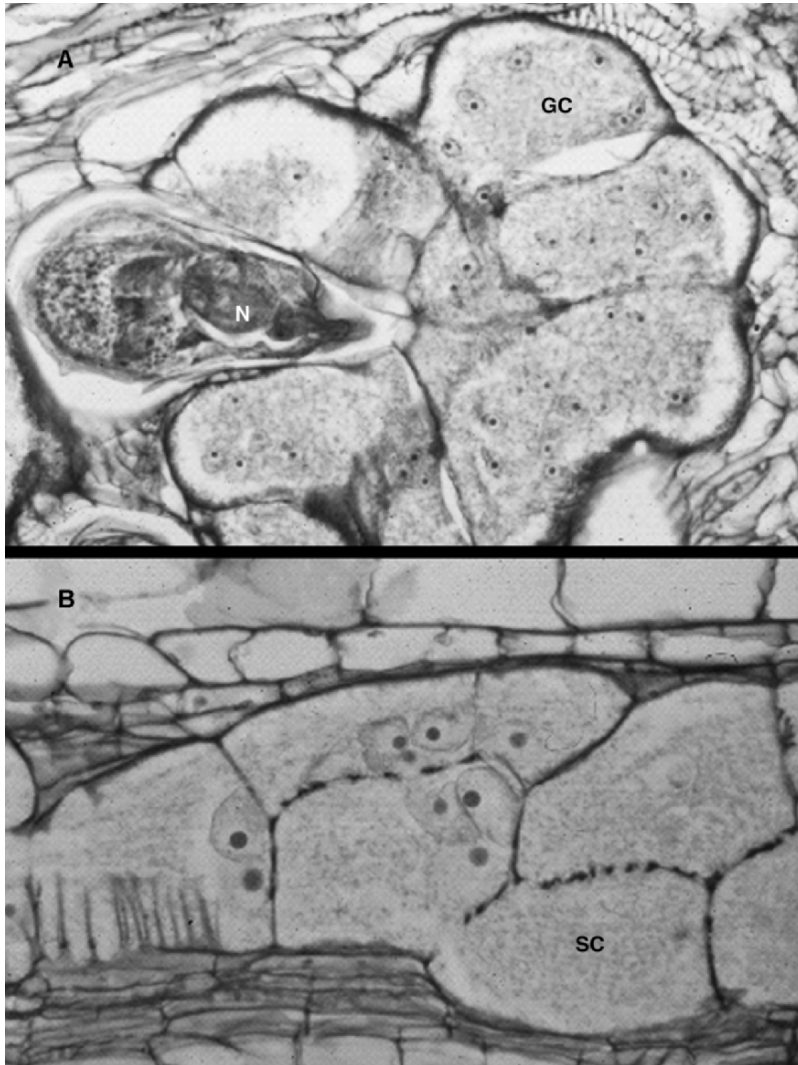
Figure 2. Feeding sites of root-knot and cyst nematodes. (A) Root-knot nematodes (N) induce the formation of giant-cells (GC) in the roots of their host plants. Each giant-cell contains multiple nuclei, which are visible in this figure (unknown source). (B) Cyst nematodes induce the formation of syncytia by fusion of individual syncytial cells (SC) through cell wall dissolution. Perforated cell wall remnants are clearly visible in this panel (Pictures by B. Endo).

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* (7). 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 (Figure 3). Even though these structures are called glands, they are de-facto single
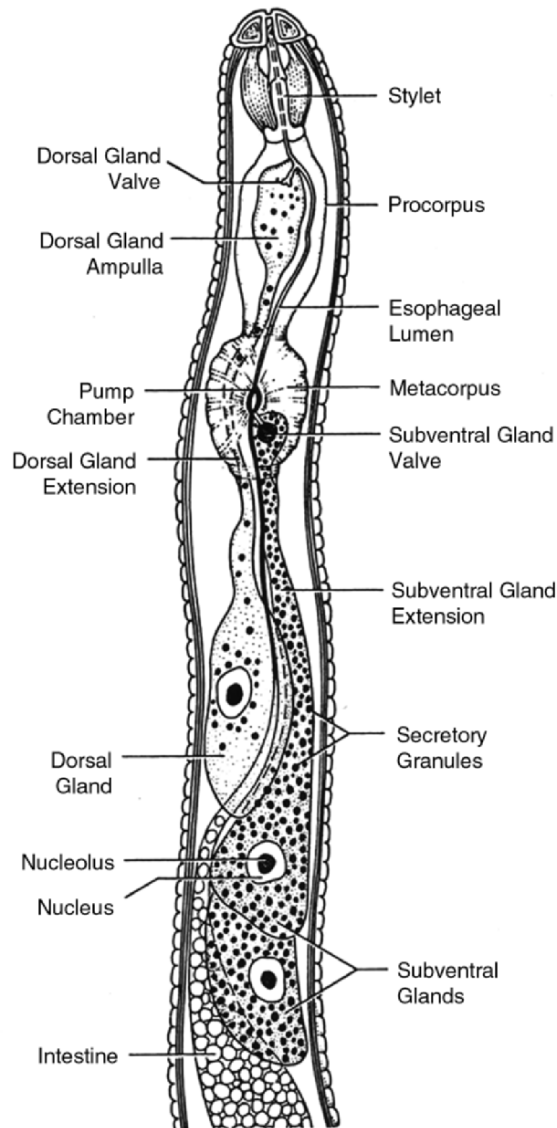
Figure 3. Anterior end of a second-stage juvenile cyst nematode. The anterior end of cyst nematodes harbors major adaptations for plant parasitism, particularly the stylet and the three esophageal glands (one dorsal gland and two subventral glands). This anatomy is completely shared by the root-knot nematodes (Drawing by R. Hussey).

cells, each having long cytoplasmic extensions that are connected through valves to the lumen of the esophagus (11). Secretory proteins are synthesized in these cells and packaged into membrane-bounded secretory granules. The granules move anteriorly through the gland extensions, and their contents are released into the esophageal lumen by exocytosis *via* the respective gland-cell valve. While in

root-knot and cyst nematodes the two subventral gland cell extensions open into the esophageal lumen immediately posterior to a muscular pump chamber in the median bulb, the dorsal gland cell extends anterior in the esophageal wall to empty through a valve into the esophageal lumen at the base of the stylet. This morphological difference implies different functions of the requisite glands, and this assumption is confirmed by the dramatically-different developmental appearance of the gland cells during the developmental cycle of the root-knot and cyst nematodes. As early as in fully-developed J2 in the egg, the extensions of the subventral glands of root-knot and cyst nematodes are packed with secretory granules whereas the dorsal gland extension is relatively empty. During the transition from host-root penetration to feeding site induction and maintenance, the subventral glands become smaller and less active while the dorsal gland enlarges and increases in activity for the remainder of the parasitic cycle. The movement of contents from both esophageal gland cell types for secretion through the stylet has been documented in elegant video-enhanced microscopy of plant-parasitic nematodes within roots (12-15). While there was initial conviction that only the dorsal gland, due to the opening of its cytoplasmic extension near the base of the stylet, has a function in parasitism, the subventral glands were thought to function only in secreting digestive proteins destined for the nematode intestine. This restricted role of the subventral glands has now been convincingly refuted, as will be discussed later on. The developmental changes in gland cell activity (and secretory proteins noted below) during different stages of parasitism, and the conduit to the parasitized host cell through the stylet, point to secretions from both gland types as direct adaptations to promote parasitism.

NEMATODE PARASITISM GENES AND THEIR PRODUCTS

Plant-parasitic nematodes are parasites that become pathogens only secondly, depending on the human perception of the severity of parasitism, i.e., whether the parasitism causes visible, economically-damaging symptoms. Therefore, the molecular mechanisms allowing a nematode to infect a plant are those mechanisms making a nematode a parasite—and not a pathogen. Hence, the genetic determinants that enable a nematode to infect plants are appropriately named parasitism genes. It is obviously of utmost interest to determine what makes a nematode a plant parasite, i.e., to determine which nematode genes are responsible for the ability to parasitize plants. In the widest sense, genes underlying morphological adaptations (e.g., the stylet), behaviors (e.g., host-finding or mating), or abilities (reproductive or survival strategies) that promote a successful parasitic lifestyle represent essential and often specific adaptations for parasitism. However, this global view, while academically interesting, does not focus on the direct molecular interactions between parasite and host, which are at the biochemical basis of plant-parasitism by nematodes. A more focused view of nematode parasitism genes targets those genes that code for proteins released from the nematode that directly interact with host molecules to promote the parasitic interaction. For reasons cited above, genes encoding secretions produced by nematode esophageal gland cells are prime candidates as nematode parasitism genes (Figure 4). Studies have confirmed that nematode stylet secretions
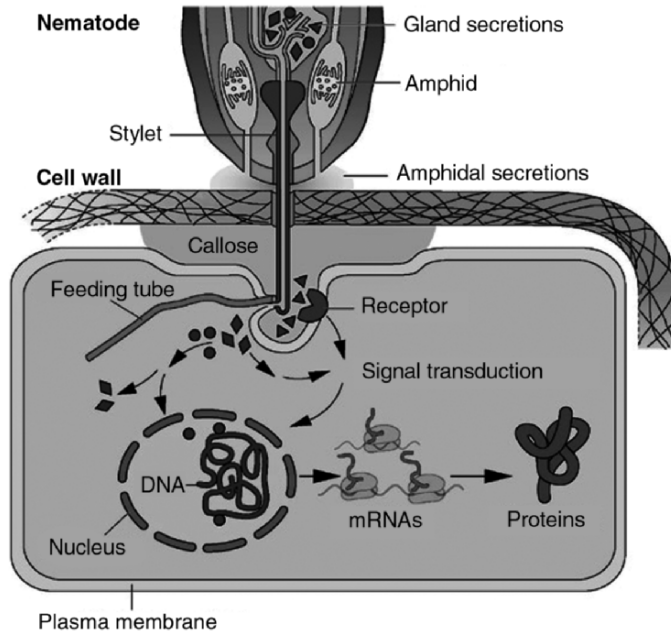
Figure 4. Parasitism gene functions. Parasitism genes in a narrow sense code for secretory proteins directly involved in the nematode-plant interaction. These parasitism proteins are secreted through the stylet into the parasitized plant where they have important functions during the induction of feeding cells like giant-cells and syncytia. Parasitism proteins may function as extracellular or intracellular ligands or signal transduction components, be imported into the nucleus, or act on cytoplasmic components, all of which could modify the recipient plant cell. Furthermore, parasitism proteins have functions during feeding like the formation of feeding tubes (Drawing by R. Hussey).

produced in the esophageal gland-cells are proteinaceous and not nucleic acids (16), suggesting that secretions are translated directly from parasitism gene transcripts. Molecules released or secreted from other nematode body regions could also be involved in parasitism, either as encoded proteins or as the products of metabolic pathways. There are examples of candidate parasitism proteins produced in the amphids (chemosensory organs found at the head of nematodes) or even the hypodermis (the inner living cell layer of the nematode's body wall). The best studied examples of parasitism proteins are those produced in the esophageal glands and released as secretory proteins. These proteins are synthesized as preproteins with N-terminal signal peptides that target the nascent protein chain during translation of the parasitism gene mRNA to the endoplasmic reticulum. There, the signal peptide is cleaved off and the mature protein passes along the secretory pathway. However, there are a few examples of parasitism protein candidates that presumably use a different mode of secretion not requiring a signal peptide. Nonetheless, even when considering such exceptions, the majority of currently known parasitism genes are expressed exclusively in the esophageal-glands and code for secretory parasitism proteins requiring a signal peptide.

POTENTIAL ROLES OF PARASITISM PROTEINS

When considering parasitism genes in the narrow sense described above, i.e., esophageal-gland-expressed genes coding for secretory proteins released through the nematode stylet, an array of possible involvements of parasitism proteins in the nematode life cycles can be postulated. First of all, nematodes need to penetrate the roots of their host plants and migrate through root tissues. Considering the moderate size of root-knot nematodes and cyst nematode infective J2s, cell walls pose formidable obstacles, and, as will be discussed later on, both nematodes use a mixture of cell-wall-digesting enzymes to break structural integrity of plant cell-walls. In addition to these important functions, the most impressive achievement appears to be the nematode-directed formation of the elaborate feeding cells by root-knot nematodes (giant-cells) and cyst nematodes (syncytia). As mentioned above, the nematodes need to communicate with mostly differentiated root cells and induce the development of the parasitized cells into the different feeding cell types. Furthermore, the nematodes need to maintain these cells, which probably include suppressing plant defenses and/or cell death programs that may be activated during parasitism. Finally, video footage of a feeding cyst nematode (12, 15) and micrographs of other nematode feeding sites including root-knot nematodes (17-19), clearly show following the release of secretions through the dorsal-gland-valve the formation of a tubular structure (feeding tube) at the stylet orifice inside the cytoplasm of the feeding cell (Figure 4). Hence, feeding tube formation along with feeding cell maintenance during food uptake, more than likely, are roles of parasitism proteins. The size exclusion of molecules ingested by root-knot nematodes and cyst nematodes has been documented to be between 28 and 40 kD (20, 21), suggesting that the feeding tube acts as a molecular sieve.

PARASITISM GENE IDENTIFICATION

The identities of parasitism proteins have intrigued scientists, and an array of approaches to identify parasitism genes and proteins have been devised and tried. Most of these approaches targeted the esophageal-glands because of their obvious involvement in parasitism. Antibodies specific to esophageal-gland antigens were generated using *in vitro* purified nematode stylet secretions or fractions of nematode homogenates and used to screen cDNA expression libraries (22) or to affinity purify the nematode antigens (23). Furthermore, efforts were expended to directly identify purified stylet-secreted proteins (24-29). Also, the mining of ever-growing databases containing the nucleotide sequences of expressed genes (Expressed Sequence Tags, ESTs), revealed parasitism gene candidates because of their similarity to already identified parasitism genes from other nematode species or to proteins with obvious functions in parasitism (30-35). Finally, gene expression at the RNA level at different time points or in different nematode tissues was assessed in hopes of identifying parasitism genes because of their developmental expression patterns or their localized expression in the esophageal-glands (36-42). However, the most exhaustive and direct approach to identify parasitism genes targeted the esophageal-glands directly *via* microaspiration of gland-cell cytoplasm followed by the construction and mining

of gland-specific cDNA libraries (37, 38, 43-49). All these approaches have been detailed and compared in recent reviews and will not be repeated here (4-6, 8). One of the greatest conceptual advances in nematology over the last decade has been the discovery that sedentary plant-parasitic nematodes produce in their esophageal-glands a large array of secretory proteins with putative functions in parasitism (5). Determining the identity of parasitism genes, however, is only the first step toward unraveling the mechanisms of plant parasitism by nematodes. Understanding the functions of the parasitism proteins, individually or in concert, currently represents the biggest obstacle in this research.

KNOWN PARASITISM GENES

A current list of root-knot and cyst nematode genes with known putative functions in parasitism, mostly based on similarities to characterized proteins in other organisms, is presented in a recent review (8) and shall not be repeated here. In addition to the parasitism proteins with similarity to characterized proteins, there is an even larger number of parasitism genes from root-knot and cyst nematodes for which no similarities to characterized proteins in other organisms exist (44, 47).

It is an interesting observation that when parasitism proteins are similar to known proteins, this similarity usually is not with proteins from *C. elegans*, a non-parasitic nematode whose genome is fully sequenced. Rather, if similarities to nematode proteins are found, these similarities are frequently only with proteins from other parasitic nematodes. Most frequently, however, similarities are with proteins from bacteria, fungi, or plants for which there are no functions in nematodes. For example, plant-parasitic nematodes produce cellulases and pectinases, yet there are no substrates for these enzymes found within the nematode. Similarly, these nematodes do not have a shikimate pathway, yet they produce a key enzyme of this pathway. Also, nematode parasitism proteins sometimes represent secretory versions of known cellular effector proteins. These curiosities all point in one direction, namely that these nematode proteins do not have a function within the nematode but function as instruments of parasitism when secreted within the parasitized plant.

CURRENT HYPOTHESES OF PARASITISM PROTEIN FUNCTIONS

Despite the fact that the majority of parasitism protein candidates currently known are without similarity to characterized proteins, interesting conclusions can be drawn, nonetheless, from a relatively small group of parasitism proteins. In this group, similarities of parasitism proteins with functionally characterized proteins from other organisms and the functional characterization of parasitism proteins that already has been accomplished allow the formulation of credible working hypotheses about mechanisms of parasitism used by root-knot nematodes and/or cyst nematodes.

Cell-Wall-Digesting Enzymes

As already mentioned above, it has been established that 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. To date, there have been cellulase and pectinase genes described for root-knot nematode (38, 47, 48, 50) and cyst nematode species (23, 44, 46, 51-53). Discovery of cellulase genes in the soybean and potato cyst nematodes represented the first major breakthrough in parasitism gene discovery and was of particular interest because at that time, no cellulase genes had been reported from animals (23). In addition, nematode cellulases were highly similar to bacterial proteins, which raised the interesting hypothesis that a certain subset of parasitism genes was actually acquired by horizontal gene transfer (23, 52). Similarly, pectinases had not been reported from animals as well, and the nematode pectinase proteins were of the pectate lyase type found in fungi and bacteria, cyst and root-knot nematodes; (35, 47, 48, 51, 54) or the polygalacturonase type of bacteria (root-knot nematode; 55). An involvement of these enzymes in penetration and migration is backed by the fact that cell-wall-digesting enzymes are produced and secreted during nematode penetration and migration and to a much smaller extent, or not at all, during the later sedentary stages (48, 50, 56-58; A. Elling and T. J. Baum, unpublished data). Interestingly, males of cyst nematodes, who regain mobility and leave host roots, reinitiate cellulase production during this life stage (56, 58). Very convincing support is also gained from experiments in which genes for cell-wall-digesting enzymes are inactivated by gene-silencing techniques (see below) and J2 infectivity is reduced (59). While it is clear that these enzymes are used for the purpose of cell-wall softening, it is not clear why the nematodes have large gene families for some of these proteins and what exactly are the functions of the individual gene family members (57). Similarly, the function of cellulose-binding proteins discovered in root-knot and cyst nematodes remains elusive (36, 44, 45, 47): do these proteins function in concert with cellulase enzymes that lack a cellulose-binding domain or do these proteins have functions in their own right? The latter is suggested by the finding that in planta overexpression of a bacterial cellulose-binding domain led to accelerated cell growth (60). Research outside the realm of sedentary nematodes also reported beta-1,4-endoglucanase genes from the lesion nematode *Pratylenchus penetrans* (61), which is a migratory parasite that obviously also requires successful means to breach plant cell-walls. Very interestingly, a cellulase of the beta-1,3-endoglucanase type recently was reported from the pinewood nematode *Bursaphelenchus xenophilus* (a fungus-feeding, insect-vectored nematode living in pine trees) where it is hypothesized of being involved in nematode feeding from fungal mycelium (62).

Expansins

In addition to the ability to break down covalent bonds found in plant cell-walls (i.e., through cellulases and pectinases) there is evidence that the potato cyst nematode also secretes a protein having the ability to break non-covalent bonds. This activity is accomplished by an expansin-like protein discovered in the potato cyst nematode (41), which represented the first confirmed report of such a protein outside the plant kingdom. Expansins soften cell-walls by breaking non-covalent bonds between cell-wall-fibrils, thereby allowing a sliding of fibrils

past each other. The resultant plant cell-wall softening could be demonstrated for the potato cyst nematode expansin parasitism protein (41). No such genes have been found in root-knot nematodes or other cyst nematodes to date.

Metabolic Enzymes

Discoveries in both root-knot (39, 49) and cyst nematodes (32, 44) identified parasitism genes coding for proteins similar to chorismate mutases. 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. Expression of a root-knot nematode chorismate mutase gene in soybean hairy roots produced an auxin-deficient phenotype, which gave rise to this model of chorismate mutase function (63). A lack of salicylic acid production in response to nematode chorismate mutase injection could result in a downregulation of plant defenses. In line with a putative function in defense deactivation, it was observed that chorismate mutases represent a polymorphic gene family in soybean cyst nematodes and that presence and expression of certain gene family members correlates with the nematodes' ability to infect certain soybean genotypes harboring soybean cyst nematode resistance genes (64, 65).

Ubiquitination/Proteasome Functions

Targeted and timed protein degradation is a final and powerful means to regulate gene expression. Cyst nematodes apparently are using 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. This hypothesis is founded in the discovery that cyst nematodes produce secretory isotypes of otherwise purely 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 E3 ubiquitin protein ligase complex (42, 44). An additional level of complexity exists in the fact that the nematode-produced ubiquitin molecules also contain a short C-terminal extension with unknown function. Unlike known non-nematode ubiquitin extension proteins (66), the nematode extension apparently is not a ribosomal protein and, therefore, its function remains unknown.

Small Bioactive Peptides

Recent scientific progress has begun to establish significant roles for small peptides in plant development (67). For example, the small extracellular ligand CLAVATA3 in Arabidopsis has been established as a key factor determining shoot meristem differentiation (68). It was particularly intriguing when it was discovered that the soybean cyst nematode produces a small parasitism peptide with a conserved C-terminal motif found in CLAVATA3-like ligand peptides (46, 69). Expressing the cDNA of this soybean cyst nematode CLAVATA3-like peptide in the *clavata3* (*clv3*) Arabidopsis mutant restored the wild-type phenotype, thereby confirming a first case of ligand mimicry in phytonematology (70). In other words, the soybean cyst nematode has evolved a secreted ligand for an endogenous plant receptor in order to parasitize the host plant successfully. Functionality also has been shown for a small 13 amino acid root-knot nematode parasitism peptide that previously had been discovered (47). This root-knot nematode peptide, when produced in planta, increased the rate of cell division in root meristems and was shown to bind to a plant transcription factor of the SCARE-CROW family (G. Huang and R. S. Hussey, unpublished data). This finding represents a first discovery of a direct regulatory interaction between nematode and plant proteins and, therefore, represents a powerful starting point for further exploration of this pathosystem. Considering the established importance of small peptides in signaling roles in plant development as well as plant–parasite interactions, it also will be interesting to determine if the small C-terminal extension of the cyst nematode ubiquitin extension proteins mentioned above (42, 44) will have regulatory functions in the recipient plant cell. Additional support for a role of small peptides in nematode-plant interactions is presented by an unknown peptide fraction smaller than 3 kDa isolated from potato cyst nematode secretions. This protein fraction was shown to have biological activity by stimulating proliferation of tobacco leaf protoplasts and human peripheral blood mononuclear cells (71).

Nuclear Localized Parasitism Proteins

Analyses of parasitism proteins using computational approaches to predict protein localization and fate identified a significant subset of putative parasitism proteins with predicted nuclear localization signals (NLS), i.e., protein domains that mediate active uptake into the nucleus (44, 47). However, these proteins also contained N-terminal signal peptides directing them into the endoplasmic reticulum. This conflict can be resolved by postulating that NLS-containing nematode parasitism proteins first are targeted to the nematode gland-cell endoplasmic reticulum and the secretory pathway and only after secretion into a plant cell are they taken up into the plant nucleus. In testing this hypothesis, the active uptake of nematode parasitism proteins into plant nuclei has been shown for a small group of cyst nematode parasitism proteins (42; A. Elling and T. J. Baum, unpublished data). It will be of utmost interest now to decipher protein functions within the plant nucleus.

RanBPM

In a project comparing gene expression patterns among discrete developmental stages of the potato cyst nematode, a group of parasitism gene candidates was identified (40). Further analyses of these genes revealed the presence of a small family of genes coding for secretory proteins with high similarity to proteins binding to the small G-protein Ran, so-called RanBPMs (Ran-Binding Protein in the Microtubule organizing center). Several of these genes were expressed in the dorsal-gland (72). Exact functions of RanBPMs remain elusive and appear to be complex and diverse including the regulation of the cell cycle. Therefore, it is a tempting hypothesis that potato cyst nematode proteins with similarity to RanBPM may have a function in regulating the cell cycle activities observed in developing syncytia (72). As a first step it remains to be seen if Ran-binding activity or an effect on plant cell phenotype can be demonstrated for these nematode peptides.

Venom-Allergen Proteins

The parasitism proteins listed above are similar to functionally characterized proteins from other organisms, which allowed the formulation of clearly-defined hypotheses about protein function during parasitism. On the other hand, there are those parasitism protein candidates that 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 (73) and cyst nematodes (37, 44) that are collectively called venom-allergen proteins (vaps). Gene sequences for these venom proteins were first described from hymenopteran insects (74), and vaps were also identified as secreted proteins (ASP) in the animal-parasitic nematode *Ancylostoma caninum* (75). Genes encoding vaps have since been found in other nematodes, including parasites as well as the free-living *C. elegans*. While several of these proteins were found to be secreted, or in the case of soybean cyst nematodes to be expressed in the subventral-glands (37), their function remains elusive.

Calcirecticulin

In a similar development, a calcirecticulin-like protein preceded by a signal peptide was identified as being produced in the subventral-glands of a root-knot nematode (27). Calcirecticulin-like proteins are secreted from other parasitic nematodes and, therefore, are good candidates for being involved in parasite-host interactions (76, 77). However, the puzzling array of putative or demonstrated calcirecticulin functions reported (76) make it difficult to postulate a function in plant parasitism by root-knot nematodes.

Annexin

Similarly, 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 (44).

Annexin genes represent a large family coding for calcium-dependent phospholipid-binding proteins with a wide range of reported functions. Therefore, no clear postulation about annexin functions in cyst nematode parasitism can be made at this time. 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 (78).

Chitinase

Also, there is an example of a parasitism protein with clearly defined function but no obvious role for this function at the time of the protein production. This putative parasitism protein is a chitinase identified in the subventral glands of the soybean cyst nematode (43). The only report of chitin in a nematode has been in the egg shell (79) and chitinases have been discussed as having a role in nematode hatch. However, *in situ* expression analyses (43) as well as microarray expression analyses (A. Elling and T. J. Baum, unpublished data) clearly demonstrate that this chitinase gene is not expressed in the eggs but that it shows a strong expression peak during the early phases of parasitism after penetration. As with many other parasitism proteins, further research has to explore a role for chitinase production during this stage of parasitism.

PARASITISM GENES IN A WIDER SENSE

In addition to the aforementioned parasitism proteins that satisfy the requirements of being produced exclusively in the esophageal-glands and harboring an N-terminal signal peptide, a small number of potentially interesting candidate genes that differ in at least one of these criteria have been identified.

Peroxidase

It appears likely that nematodes deploy means to cope with reactive oxygen species (ROS) produced by the host plant as a defense means in response to nematode attack (80). Such ROS-detoxifying enzymes have been reported in the form of peroxidases from the potato cyst nematode (33, 81). Peroxidase genes are expressed in the potato cyst nematode hypodermis and the peroxidase proteins accumulate on the nematode body surface presumably to detoxify ROS.

FAR

Another example of secreted nematode proteins with potential roles in negating plant defenses is a surface associated retinol- and fatty acid-binding (FAR) protein found in the potato cyst nematode *G. pallida*. This protein was found to bind to lipids that are precursors of the jasmonic acid signaling pathway as well as plant defense compounds (82). The reported accumulation of this

protein at the nematode surface makes it a strong candidate for a protein that could interfere with plant defense mechanisms.

SXP/RAL-2

Another hypodermis-expressed gene coding for a secretory protein as well as a related gene expressed in glands associated with the anterior chemosensory organs (amphids) were identified from the potato cyst nematode *G. rostochiensis*. Both genes code for proteins of the nematode SXP/RAL-2 family, for which no functions could be ascertained to date (31).

Avr Protein

The amphids of a root-knot nematode were found to express a secreted protein that, while of unknown primary function, appears to represent a nematode avirulence protein, i.e., a protein whose presence leads to the initiation of effective plant resistance mechanisms triggered by the tomato *Mi* resistance gene (83). It will be of utmost interest to decipher the primary role of this protein and the mode by which it appears to trigger a resistance response.

14-3-3

A final protein with the potential of being involved in nematode parasitism has been discovered in the root-knot nematode *M. incognita*. This dorsal-gland-expressed gene codes for a protein of the 14-3-3 family that appears to be secreted despite lacking an N-terminal signal peptide (28). 14-3-3 proteins are well conserved in eukaryotes with a diverse spectrum of putative functions, and a role in nematode parasitism, if any, remains obscure.

DIFFERENCES BETWEEN ROOT-KNOT NEMATODES AND CYST NEMATODES

As mentioned above, root-knot and cyst nematodes use similar strategies to enable their sedentary parasitic life styles. However, it appears that these nematodes use very different tools of fulfilling their strategies because root-knot nematodes usually have wide host ranges and cyst nematodes narrow ones and the ontogeny of their feeding sites (giant-cells *versus* syncytia) is very different in certain aspects. Fully characterizing the root-knot nematode and cyst nematode parasitism genes should provide more definite answers. When assessing the currently identified panels of parasitism genes found in root-knot nematodes and cyst nematodes, one can find support for the hypothesis that root-knot nematodes and cyst nematodes use different molecular tools for their otherwise similar life habits—at least during the sedentary phase of parasitism. During the migratory phase, both nematode groups (root-knot nematodes and cyst nematodes) use cellulase and pectinase enzymes produced in their subventral glands in order to penetrate into and migrate through plant roots. Also, during the early phases of parasitism both nematode groups produce cellulose-binding proteins

and venom-allergen proteins for unknown reasons and both root-knot nematodes and cyst nematodes produce chorismate mutase enzymes potentially to inactivate plant host defenses. However, as a first significant difference, a cyst nematode was shown to use an expansin parasitism protein to soften host cell walls, which is a group of proteins so far not identified in root-knot nematodes. Even more profound differences exist beyond these early stages of parasitism. While the soybean cyst nematode uses a small ligand with similarity to CLAVATA3-like proteins and appears to employ an ubiquitination pathway, no such proteins were discovered in root-knot nematodes. Instead, a large percentage of parasitism protein candidates without any database similarities (including cyst nematode genes) were found in root-knot nematodes. Also, while both root-knot nematodes and cyst nematodes produce a high proportion of unknown parasitism protein candidates in their dorsal-gland, root-knot nematodes appear to produce a relatively large proportion of unknown parasitism proteins also in their subventral glands. Of course, these assessments can only rely on the current state of knowledge and can only be completely validated when all parasitism proteins of several species of both nematode groups are identified. In summary, parasitism protein identities so far confirm that root-knot nematodes and cyst nematodes share certain aspects of their parasitic strategies but that key components of their arsenals of molecular tools likely are very different.

WHICH GLAND HAS WHICH FUNCTION?

Over the years, theories about the functions of the subventral glands *versus* the dorsal-gland have changed considerably. Early observations led to the conclusion that only the dorsal-gland is involved in direct parasitism functions because the subventral-glands emptied into the esophagus behind to the pump chamber, suggesting a transport of subventral gland-produced proteins only posteriorly into the intestine. However, the first parasitism gene to be identified was a subventral-gland-expressed gene coding for a cyst nematode cellulase which was definitively secreted through the nematode stylet, thereby, refuting the earlier hypotheses about subventral-gland proteins (84). When more and more cell wall-digesting or cell wall-modifying enzymes that were produced in the subventral-glands and secreted through the stylet were identified, it was plausible to speculate that the subventral-glands function during migration whereas the dorsal-gland proteins would be involved in mechanisms needed for feeding site formation and feeding. This was even more intriguing when considering that many of the subventral-gland-produced parasitism proteins were candidates for horizontal gene transfer acquisition by plant-parasitic nematodes because these proteins were most similar to prokaryotic or fungal proteins or had not even been reported from animals. In other words, it seemed intriguing to think of the subventral-gland as expressing a group of parasitism genes with a narrow function during nematode migration and obtained from other organisms. However, soon exceptions were reported that showed subventral-gland-produced proteins without known function during migration and that were produced even after the nematode had become sedentary. Currently, it appears most likely that subventral-

gland-produced proteins have a pronounced but not exclusive role during nematode migration. Apparently, subventral-gland and dorsal-gland function in concert during the induction phases of feeding sites. Only the later stages, when feeding site maintenance and feeding appear to be the main functions, seem to be the dorsal-gland's exclusive domain.

FUNCTIONAL CHARACTERIZATION OF PARASITISM PROTEINS

The above-mentioned hypotheses about parasitism protein function have been formulated because of similarities of parasitism proteins with known, already characterized proteins. Additionally a variety of approaches have been employed to advance parasitism protein functional characterization. Such approaches are particularly important when considering that the majority (>70%) of currently identified parasitism proteins have no similarity to known proteins, particularly, those parasitism proteins produced in the dorsal-gland. A panel of molecular approaches is currently being used that will be instrumental in advancing knowledge of parasitism protein function. The following paragraphs provide short summaries of some of the most powerful approaches currently used.

Parasitism Gene Expression Profiling

Determining the exact locale of gene expression is of utmost interest for any gene-of-interest and of particular importance for parasitism genes. Expression in the 'wrong' cell can eliminate a gene from consideration while the opposite can provide the needed confirmation. Case in point, specific expression in one or more esophageal-glands has been a key criterion for parasitism gene discovery. Techniques for assessing gene expression at the mRNA as well as the protein level have been well established in the form of *in situ* mRNA hybridization (85) as well as *in situ* immunofluorescence analyses (86) (Figure 5). Similarly, not only the location but also the timing of expression is extremely valuable since it can provide insight into gene function. Characterization of cellulase parasitism genes, for example, was advanced when the developmental expression of cellulase gene family members was assessed, which determined a likely involvement of cellulases in the migratory phases of nematode parasitism – an observation complementing the fact that cellulases most likely aid in digesting cell walls during penetration and migration (56, 58). Analysis of gene expression over time can be accomplished using the *in situ* methods mentioned above, although processing high numbers of gene candidates proved to be challenging (44, 47, 56, 58). An alternative for the temporal assessment at the mRNA level is presented by microarray analyses. This approach has been employed with glass slides containing a small set of soybean cyst nematode cDNA sequences (87) as well as with Affymetrix GeneChips® containing oligonucleotide probe sets for more than 7,000 soybean cyst nematode genes. This latter approach identified the temporal expression of all currently known parasitism gene candidates along with all currently known soybean cyst nematode genes from eggs to adult female (A. Elling and T.J. Baum, unpublished data).
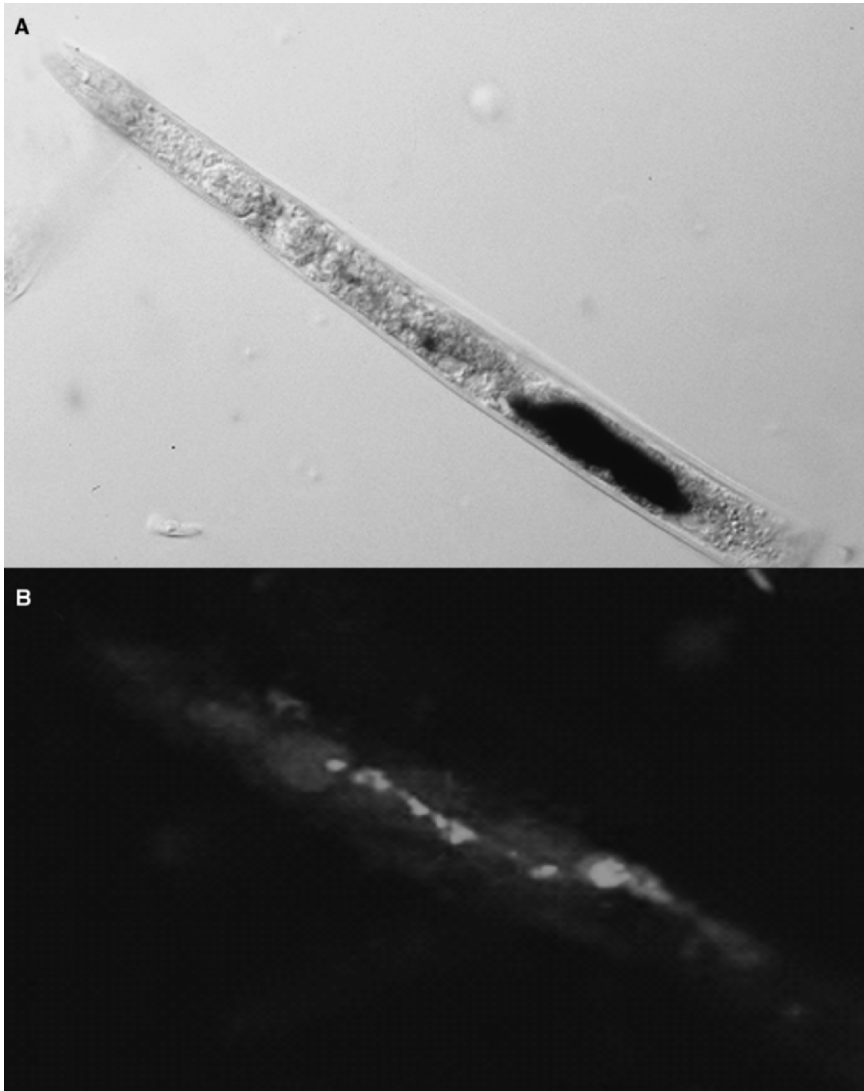
Figure 5. Assessing parasitism gene expression in the nematode. Determining the locale of gene expression in the nematode can be accomplished on the mRNA as well as the protein level. *In situ* hybridization (A) reveals the mRNA accumulation of this parasitism gene in the subventral esophageal-glands (dark stain). This result is confirmed by immunolocalization (B) of the corresponding parasitism protein in the same nematode glands (green fluorescence) (Pictures by G. Huang).

In Planta Localization of Parasitism Proteins

An equally crucial area of research is the documentation of secretion of nematode parasitism proteins inside the plant tissue. This not only again provides meaningful insight into protein function but it represents the ultimate proof that

a protein-of-interest in fact can serve a direct function in nematode–plant inter-actions. Unfortunately, documenting a secreted protein is challenging at best, as several major hurdles pose obstacles to achieving success. A most elegant approach would be the production of a protein-of-interest as a reporter protein fusion in the nematode itself to follow the protein's fate when secreted into plant tissue/cells. Unfortunately, to date, no reliable protocols for the transformation of plant-parasitic nematodes have been published. The only other alternative is immuno-detection in planta, which requires high quality antibodies. But even with a specific antibody or serum, detection of a nematode protein in planta is difficult and frequently inconclusive. So far, documentation of in planta accu-mulation of parasitism proteins (Figure 6) has been very limited (54, 63, 84). Problems arise from the small amount of protein secreted from nematodes and the fact that once deposited into a plant cell, nematode proteins most likely form complexes with plant proteins or are processed, both of which can seriously impede antibody binding. Additionally, these obstacles don't even take into account the low probability of fixing a plant specimen and preparing an appro-priate tissue section of the exact place and time when a given parasitism protein is secreted. A large number of sera to soybean cyst nematode and root-knot nem-atode parasitism proteins has been produced recently (E.L. Davis, R.S. Hussey and T.J. Baum, unpublished data) and these challenging assays are under way.
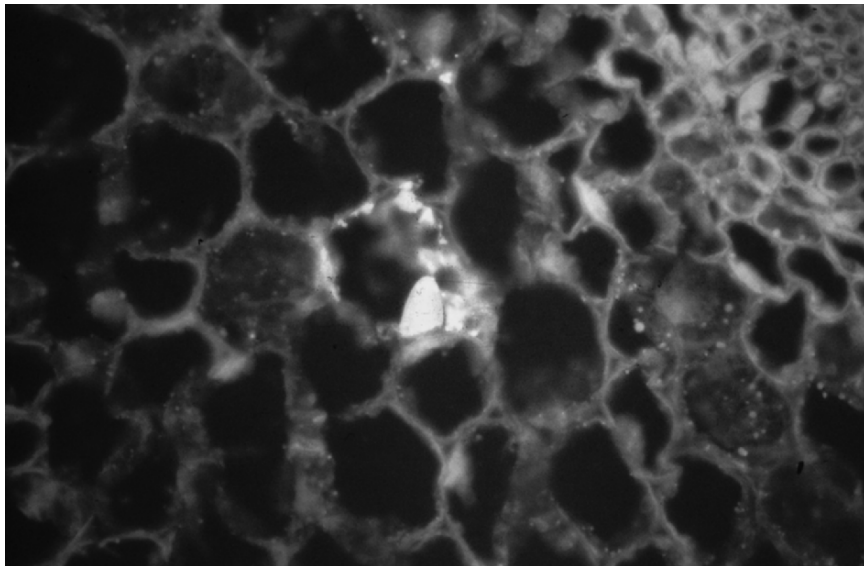

Figure 6. In planta accumulation of parasitism proteins. This section shows the head of a cyst nema-tode second-stage juvenile that was migrating through a soybean root. Immunolocalization of a cellu-lase parasitism protein clearly shows the accumulation of this parasitism protein (green fluorescence) along the migration path and on the outside of the nematode cuticle, thereby confirming in planta secretion of this protein (Picture by X. Wang).

Intracellular Localization of Parasitism Proteins

Another useful approach is the assessment of the subcellular localization of a parasitism protein once delivered to plant cells. Although not a substitute for the in planta localization, it represents a good tool for further characterization. For this purpose, nematode parasitism proteins are produced in planta as fusion proteins with reporter proteins like GUS or gfp. A significant number of cyst nematode parasitism proteins has been shown to be transported into the plant nucleus using this approach (42; A. Elling and T.J. Baum, unpublished data) (Figure 7).

Plant Expression of Parasitism Genes

Expression of parasitism genes in planta can be used to establish the fate of the encoded protein as well as to assess phenotypic changes of the plant or
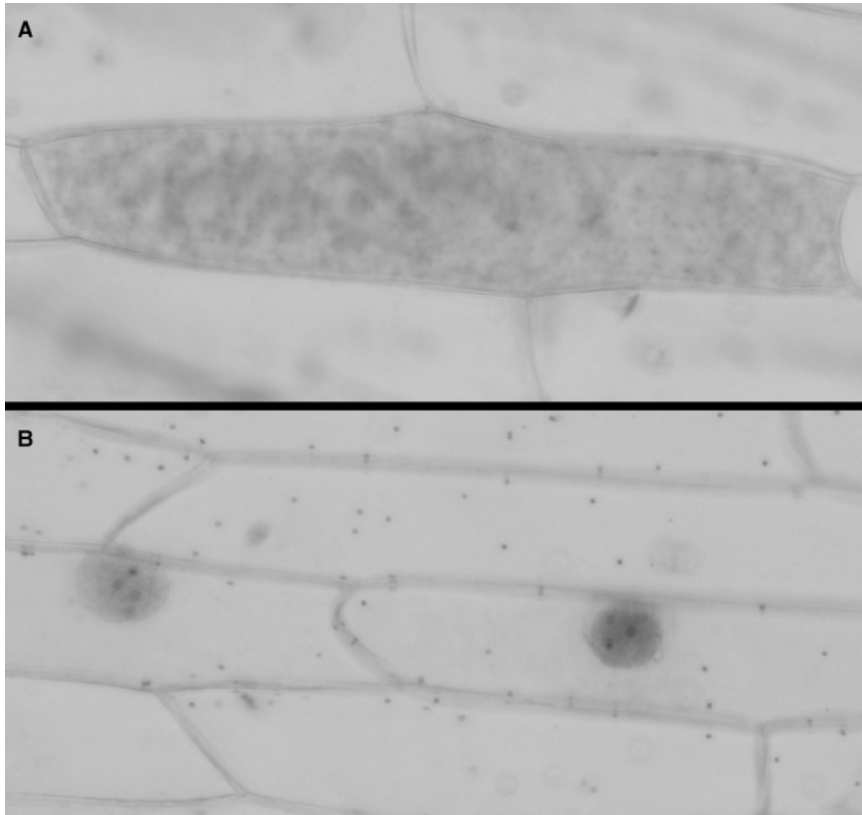

Figure 7. Intracellular localization of parasitism proteins. Translational fusion of parasitism proteins with the GUS reporter gene allows the visualization of protein localization. (A) A protein without specific targeting domains accumulates in the cytoplasm of onion epidermal cells. (B) A parasitism protein containing a nuclear localization signal is efficiently transported into the onion cell nucleus and accumulates there exclusively (Pictures by A. Elling).

parts thereof resulting from its overexpression. Because root-knot nematode and cyst nematode parasitism is accompanied by dramatic plant changes, it can be speculated that individual parasitism proteins will contribute to these changes by changing a certain aspect of the normal plant phenotype. Of particular interest here is the decision whether to include the parasitism protein signal peptide, i.e., whether one suspects the parasitism protein to function in the plant cell cytoplasm or in the apoplast. Furthermore, the choice of promoter is crucial and can influence the results and the conclusions to be drawn from a particular experiment, as this choice determines in which tissues, when, and to what strength a given parasitism gene is transcriptionally turned on. Of particular interest here are inducible promoters that can be used to customize parasitism gene expression. Expression of a few parasitism genes so far resulted in detectable phenotype changes in wild-type plants (G. Huang and R.S. Hussey, unpublished data). Particularly interesting could also be the expression of a parasitism gene suspected to code for an avirulence protein in a resistant host background because correct parasitism gene expression should trigger a visible resistance response.

Mutant Complementation

Another very powerful application of parasitism gene expression in heterologous organisms is the use of mutants as recipient organisms with the goal to restore the wild-type phenotype, thereby proving parasitism protein function. This approach was used to determine chorismate mutase function by complementing a bacterial chorismate mutase mutant (39, 49). As already mentioned above, producing the soybean cyst nematode parasitism protein containing a CLAVATA3-like conserved domain in the Arabidopsis *clv3* mutant restored the wild-type phenotype. Unfortunately, it is rather the exception that suitable protein similarities exist and well-defined mutants are available. Nonetheless, when successful, such complementation data provide strong support for a protein function.

Gene Silencing

Reverse genetics have been powerful in many biological systems because understanding gene functions can be achieved by inactivating a gene-of-interest. With the recent increased understanding of double-stranded (ds) RNA-induced gene silencing pathways, so-called RNA interference (RNAi), reverse genetics also became available to plant-parasitic nematodes despite our inability to stably transform these organisms. The obstacle remains how to expose plant-parasitic nematodes to the RNA species required to induce the RNAi mechanism. The observation that RNAi can be initiated in *C. elegans* by ingestion of dsRNA molecules (88) provided an important breakthrough for plant-parasitic nematodes. Incubating plant-parasitic nematodes in solutions containing dsRNA complementary to regions of a gene-of-interest led to a decrease of that gene's mRNA abundance (59, 89-91). In some cases phenotypes could be associated with this mRNA decrease, thereby revealing valuable insights into putative gene functions. For example, inactivating cellulase genes in the potato cyst nematode *G. rostochiensis* by soaking in dsRNA resulted in a decrease in nematode

parasitism (59). Undoubtedly, further use of this technique will be a crucial advancement in determining contributions to and roles in parasitism of individual parasitism genes. A variation to this approach is currently explored in which dsRNA is produced in planta (92) within the nematode-induced feeding sites with the goal to allow a direct uptake of siRNA (<28 kD) by the feeding nematode. In addition to revealing parasitism protein function using this approach, the identification of which parasitism genes are essential for plant parasitism could lead to the development of novel and durable resistant transgenic plants using the RNAi technology.

Search for Interacting Proteins

It is likely that many parasitism proteins once delivered into the host plant will engage in interactions with plant proteins. Knowing the identity of such plant proteins has the potential to advance the understanding of parasitism protein function plus it will open additional avenues for further research. For example, parasitism proteins translocated into the plant nucleus will have to interact with plant cytoplasmic proteins to enable nuclear uptake where they in turn may interact with other proteins in order to exert their main function. Promising approaches to identify plant proteins that interact with nematode proteins are yeast-two-hybrid analyses and direct identification of such proteins through affinity purification. However, such approaches are not straight-forward and prone to many artifacts. To make matters worse, the confirmation of a suspected protein-protein interaction is equally tricky. Conceptual problems exist for example when considering that nematodes appear to secrete multiple protein and that it is conceivable that more than one nematode parasitism protein needs to be present to accomplish correct binding to plant proteins. Also, nematode parasitism proteins pass through the nematode gland-cell secretory pathway and there could be subject to modifications like glycosylation and/or cross-linking, which could alter protein-protein interactions. None of these protein modifications is easily reproduced in standard assays targeting the identification of interacting proteins. Nonetheless, first successes have been reported. As already mentioned above, SCARECROW transcription factor-like proteins were found to interact with a small parasitism peptide from a root-knot nematode, which could be confirmed through co-immunoprecipitation (G. Huang and R.S. Hussey, unpublished data).

PRESENT AND FUTURE

When assessing the putative identities of the parasitism genes described above, one can find four groups of parasitism gene similarities. In the first group, nematode parasitism gene candidates are found that have similarity to non-nematode, non-animal, or even non-eukaryotic genes. Classic examples are the nematode cellulase genes that code for proteins very similar to bacterial cellulases. Such genes are strong candidates for genes acquired by horizontal gene transfer. In a second group, one can find nematode parasitism genes that are similar to genes found in other, non-parasitic nematodes. For example, the annexin or venom-allergen genes mentioned above. These genes are found throughout the

Nematoda and other animals and may have evolved in root-knot nematodes and cyst nematodes to allow their protein products to assume functions during parasitism. In a third class, there are genes without similarity among animal genes but whose protein products exhibit weak similarities with plant proteins or domains thereof, and which apparently can function in the context of plant regulatory mechanisms. Good examples are the small soybean cyst nematode protein with similarity to the plant CLAVATA3 ligand or the root-knot nematode peptide that binds to a plant SCARECROW transcription factor. The final group of parasitism genes is the largest and contains genes coding for secretory proteins with unknown identity. These parasitism genes present the most difficult candidates to investigate for function. A combination of increased genomic data, bioinformatics and *in vivo* functional analyses discussed above, particularly RNAi, will be critical to unravel potential roles of these "pioneer" proteins in parasitism.

This review is a snapshot of our current understanding and thinking regarding the molecular basis of nematode parasitism of plants. The next decade holds tremendous promise in advancing our knowledge of parasitism genes and proteins and it will be interesting to compare our knowledge now with the level attained then. It will be particularly interesting to learn more about the reports that plant-parasitic nematodes release cytokinine plant hormones (93) or that root-knot nematodes potentially use a NOD factor-like signaling compound (94). These discoveries open the door to an additional realm of complexity and difficulty, namely the fact that nematodes may release compounds other than proteins in order to determine the outcome of their interactions with plants. It will be particularly rewarding to determine the origins of such compounds, which genes are involved in their synthesis, and their potential functions in parasitism.

As the genome sequencing efforts of the first species of phytonematodes are just beginning, the existing cache of expressed parasitic nematode genes underscores the urgency for robust analyses of gene function in the post-genomic era. The obligate nature of nematodes as parasites makes application of "routine" *C. elegans* technologies challenging, yet recent success with applications of RNAi to parasitic nematodes are encouraging. These emerging technologies not only provide critical analyses of gene function, but they offer the exciting potential to identify novel targets to interfere in parasite biology to protect human, animal, and crop health.

Finally, it is for the most part completely unclear how the nematodes manage secretion of their parasitism proteins. That is, are parasitism proteins secreted as mixes within individual secretory granules in the esophageal-glands or are they separately packaged? Is the secretion of individual proteins a process under the regulation other than gene expression, i.e., can the nematode deliberately secrete one protein and not another while both are present within the same gland? These are just a few of the truly interesting biological questions that need to be answered to obtain a more complete picture of plant-parasitic nematode parasitism. However, already now, available knowledge opens the way to several avenues to create novel means for nematode control, which is the main charge that warrants research on plant-parasitic nematodes in the first place, and maybe the most interesting developments of the near future will be the realization of new mechanisms to render plants resistant to plant-parasitic nematode attack.

REFERENCES

1   Poinar G. (1983) A Natural History of Nematodes. Prentice-Hall, Englewood Cliffs, NJ.
2   Ainscough, R., Bardill, S., Barlow, K. et al. *C. elegans* Sequencing Consortium (1998) Science 282, 2012-2018.
3   Blaxter, M., De Ley, P., Garey, J., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J., Mackey, L., Dorris, M., Frisse, L., Vida, J. and Thomas, W. (1998) Nature 392, 71-75.
4   Davis, E., Hussey, R., Baum, T., Bakker, J., Schots, A., Rosso, M.-N. and Abad, P. (2000) Annu. Rev. Phytopathol. 38, 365-396.
5   Davis, E., Hussey, R. and Baum, T. (2004) Trends Parasitol. 20, 134-141.
6   Hussey, R., Davis, E. and Baum, T. (2002) Braz. J. Plant Physiol. 14, 183-194.
7   Jasmer, D., Goverse, A. and Smant, G. (2003) Annu. Rev. Phytopathol. 41, 245-270.
8   Vanholme, B., de Meutter, J., Tytgat, T., van Montagu, M., Coomans, A. and Gheysen, G. (2004) Gene 332, 13-27.
9   Sijmons, P., Atkinson, H. and Wyss, U. (1994) Annu. Rev. Phytopathol. 32, 235-259.
10 Gheysen, G. and Fenoll, C. (2002) Annu. Rev. Phytopathol. 40, 191-219.
11 Hussey, R. (1989) Annu. Rev. Phytopathol. 27, 123-141.
12 Wyss U. (1992) Fund. Appl. Nematol. 15, 75-89.
13 Wyss U., Grundler, F.M.W. and Muench, A. (1992) Nematologica 38, 98-111.
14 Wyss, U. and Zunke, U. (1985) Rev. Nematol. 8, 89-92.
15 Wyss, U. and Zunke, U. (1986) Rev. Nematol. 9:153-65.
16 Sundermann, C. and Hussey, R. (1988) J. Nematol. 20, 141-49.
17 Hussey, R. and Mims, C. (1991) Protoplasma 162, 99-107.
18 Cohn, E. and Mordechai, M. (1977) Phytoparasitica 5, 83-93.
19 Endo, B. (1990) in Electron Microscopy of Plant Pathogens (K. Mendgen and D.E. Lesemann, eds.), pp. 291-305. Springer Verlag, Berlin.
20 Böckenhoff, A. and Grundler, F. (1994) Parasitology 109, 249-54.
21 Urwin, P., Moller, S., Lilley, C., McPherson, M. and Atkinson, H. (1997) Mol. Plant-Microbe Interact. 10, 394-400.
22 Ray, C., Abbott, A. and Hussey, R. (1994) Mol. Biochem. Parasitol. 68, 93-101.
23 Smant, G., Stokkermans, J., Yan, Y., de Boer, J., Baum, T., Wang, X., Hussey, R., Gommers, F., Henrissat, B., Davis, E., Helder, J., Schots, A. and Bakker, J. (1998) Proc. Nat. Acad. Sci. USA 95, 4906-4911.
24 De Meutter, J., Vanholme, B., Bauw, G., Tytgat, T., Gheysen, G. and Gheysen, G. (2001) Mol. Plant Pathol. 2, 297-301.
25 Hussey, R., Paguio, O. and Seabury, F. (1990) Phytopathology 80, 709-714.
26 Vanholme, B., De Meutter, J., Tytgat, T., Gheysen, G.D.C., Vanhoutte, I. and Gheysen, G. (2001) Med. Fac. Landbouww. 66, 93-95.

27   Jaubert, S., Ledger, T.N., Laffaire, J., Piotte, C., Abad, P. and Rosso, M.-N.
        (2002) Mol. Biochem. Parasitol. 121, 205-211.
28   Jaubert, S., Laffaire, J., Ledger, T., Escoubas, P., Amri, E., Abad, P. and
        Rosso, M.-N. (2004) Int. J. Parasitol. 34, 873-880.
29   Robertson, L., Robertson, W. and Jones, J. (1999) Parasitology 119,
        167-176.
30   Dautova, M., Rosso, M., Abad, P., Gommers, F., Bakker, J. and Smant,
        G. (2001) Nematology 3, 129-139.
31   Jones, J., Smant, G. and Blok, V. (2000) Nematology 2, 887-893.
32   Jones, J., Furlanetto, C., Bakker, E., Banks, B., Blok, V., Chen, Q.,
        Phillips, M. and Prior, A. (2003) Mol. Plant Pathol. 4, 43-50.
33   Jones, J., Reavy, B., Smant, G. and Prior, A. (2004) Gene 324, 47-54.
34   Popeijus, H., Blok, V., Cardle, L., Bakker, E., Phillips, M., Helder, J.,
        Smant, G. and Jones, J. (2000) Nematology 2, 567-574.
35   Popeijus, H., Overmars, H., Jones, J., Blok, V., Goverse, A., Helder, J.,
        Schots, A., Bakker, J., and Smant, G. (2000) Nature 406, 36-37.
36   Ding, X., Shields, J., Allen, R. and Hussey, R. (1998) Mol. Plant-
        Microbe Interact. 11, 952-959.
37   Gao, B., Allen, R., Maier, T., Davis, E., Baum, T. and Hussey, R. (2001)
        Mol. Plant-Microbe Interact. 14, 1247-1254.
38   Huang, G., Dong, R., Maier, T., Allen, T., Davis, E., Baum, T. and
        Hussey, R. (2004) Mol. Plant Pathol. 5, 1-6.
39   Lambert, K., Allen, K. and Sussex, I. (1999) Mol. Plant-Microbe
        Interact. 21, 328-336.
40   Qin, L., Overmars, H., Helder, J., Popeijus, H., van der Voort, J.R.,
        Groenink, W., van Koert, P., Schots, A., Bakker, J. and Smant, G. (2000)
        Mol. Plant-Microbe Interact. 13, 830-836.
41   Qin, L., Kudla, U., Roze, E., Goverse, A., Popeijus, H., Nieuwland, A.,
        Overmars, H., Jones, J., Schots, A., Smant, G., Bakker, J. and Helder,
        J. (2004) Nature. 427, 30.
42   Tytgat, T., Vanholme, B., De Meutter, J., Claeys, M., Couvreur, M.,
        Vanhoutte, I., Gheysen, G., Van Criekinge, W., Borgonie, G., Coomans, A.
        and Gheysen, G. (2004) Mol. Plant-Microbe Interact. 17, 846-852.
43   Gao, B., Allen, R., Maier, T., McDermott, J., Davis, E., Baum, T. and
        Hussey, R. (2002) Int. J. Parasitol. 32, 1293-1300.
44   Gao, B., Allen, R., Maier, T., Davis, E., Baum, T. and Hussey, R. (2003)
        Mol. Plant-Microbe Interact. 16, 720-726.
45   Gao, B., Allen, R., Davis, E., Baum, T. and Hussey, R. (2004) Int.
        J. Parasitol. 34, 1377-1383.
46   Wang, X., Allen, R., Ding, X., Goellner, M., Maier, T., de Boer, J.,
        Baum, T. Hussey, R. and Davis, E. (2001) Mol. Plant-Microbe Interact.
        14, 536-544.
47   Huang, G., Gao, B., Maier, T., Davis, E., Baum, T. and Hussey, R.
        (2003) Mol. Plant-Microbe Interact. 16, 376-381.
48   Huang, G., Dong, R., Allen, R., Davis, E., Baum, T. and Hussey, R.
        (2005) Int. J. Parasitol. 35, 685-692.

49   Huang, G., Dong, R., Allen, R., Davis, E., Baum, T. and Hussey, R. (2005) Mol. Plant Pathol. 6, 23-30.

50   Rosso, M.-N., Favery, B., Poitte, C., Arthaud, L., de Boer, J., Hussey, R., Bakker, J., Baum, T. and Abad, P. (1999) Mol. Plant-Microbe Interact. 12, 585-591.

51   de Boer, J., McDermott, J., Davis, E., Hussey, R., Popeijus, H., Smant, G. and Baum, T. (2002) J. Nematol. 34, 9-11.

52   Yan, Y., Smant, G., Stokkermans, J., Qin, L., Helder, J., Baum, T., Schots, A. and Davis, E. (1998) Gene 220, 61-70.

53   Yan, Y., Smant, G. and Davis, E. (2001) Mol. Plant-Microbe Interact. 14, 63-71.

54   Doyle, E. and Lambert, K. (2002) Mol. Plant-Microbe Interact. 15, 549-556.

55   Jaubert, S., Laffaire, J., Abad, P. and Rosso, M.-N. (2002) FEBS Lett. 522, 109-112.

56   De Boer, J., Yan, Y., Wang, X., Smant, G., Hussey, R., Davis, E. and Baum, T. (1999) Mol. Plant-Microbe Interact. 12, 663-669.

57   Gao, B., Allen, R., Davis, E., Baum, T. and Hussey, R. (2004) Mol. Plant Pathol. 5, 93-104.

58   Goellner, M., Smant, G., De Boer, J., Baum, T. and Davis, E. (2000) J. Nematol. 32, 154-165.

59   Chen, Q., Rehman, S., Smant, G. and Jones, J. (2005) Mol. Plant-Micobe Interact. 18, 621-625.

60   Shpigel E., Roiz, L., Goren, R. and Shoseyov O. (1998) Plant Physiol. 117, 1185-1194.

61   Uehara, T., Kushida, A. and Momota Y. (2001) Nematology 3, 335-341.

62   Kikuchi, T., Shibuya, H. and Jones, J. (2005) Biochem. J. 389, 117-125.

63   Doyle, E. and Lambert, K. (2003) Mol. Plant-Microbe Interact. 16, 123-131.

64   Bekal, S., Niblack, T. and Lambert, K. (2003) Mol. Plant-Microbe Interact. 16, 439-446.

65   Lambert, K., Bekal, S., Domier, L., Niblack, T., Noel, G. and Smyth, C. (2005) Mol. Plant-Microbe Interact. 18, 593-601.

66   Callis, J., Raasch, J. and Vierstra, R. (1990) J. Biol. Chem. 265, 12486-12493.

67   Lindsey, K., Casson, S. and Chilley, P. (2002) Trends Plant Sci. 7, 78-83.

68   Fletcher, J., Brand, U., Running, M., Simon, R. and Meyerowitz, E. (1999) Science 283, 1911-1914.

69   Olsen, A. and Skriver, K. (2003) Trends Plant Sci. 8, 55-57.

70   Wang, X., Mitchum, M., Gao, B., Li, C., Diab, H., Baum, T., Hussey, R. and Davis, E. (2005) Mol. Plant Pathol. 6, 187-191.

71   Goverse, A., van der Voort, J., Rouppe van der Voort, C., Kavelaars, A., Smant, G., Schots, A., Bakker, J. and Helder, J., (1999) Mol. Plant-Microbe Interact. 12, 872-881.

72   Qin, L. (2001) Molecular Genetic Analysis of the Pathogenicity of the Potato Cyst Nematode *Globodera rostochiensis*, Thesis Wageningen, The Netherlands.

73	Ding, X., Shields, J., Allen, R. and Hussey, R. (2000) Int. J. Parasitol. 30, 77-81.
74	Fang, K., Vitale, M., Fehlner, P. and King, T. (1988) Proc. Nat. Acad. Sci. U.S.A. 85, 895-899.
75	Zhan, B., Liu, Y., Badamchian, M., Williamson, A., Feng, J., Loukas, A., Hawdon, J.M. and Hotez, P.J. (2003) Int. J. Parasitol. 33, 897-907.
76	Nakhasi, H., Pogue, G., Duncan, R., Joshi, M., Atreya, C., Lee, N. and Dwyer, D. (1998) Parasitol. Today 14, 157-160.
77	Pritchard, D., Brown, A., Kasper, G., Mcelroy, P., Loukas, A., Hewitt, C., Berry, C., Füllkrug, R. and Beck, E. (1999) Parasite Immunol. 21, 439-450.
78	Fioretti, L., Warry, A., Porter, A., Haydock, P. and Curtis, R. (2001) Nematology 3, 45-54.
79	Bird, A. and Bird, J. (1991) The structure of nematodes, Academic Press, San Diego, CA.
80	Waetzig, G., Sobczak, M. and Grundler, F. (1999) Nematology 1, 681-686.
81	Robertson, L., Robertson, W., Sobczak, M., Helder, J., Tetaud, E., Ariyanayagam, M., Ferguson, M., Fairlamp, A. and Jones, J. (2000) Mol. Biochem. Parasitol. 111, 41-49.
82	Prior, A., Jones, J., Blok, V., Beauchamp, J., McDermott, L., Cooper, A. and Kennedy, M. (2001) Biochem. J. 356, 387-394.
83	Semblat, J., Rosso, M., Hussey, R., Abad, P. and Castagnone-Sereno, P. (2001) Mol. Plant-Microbe Interact. 14, 72-79.
84	Wang, X., Meyers, D., Yan, Y., Baum, T., Smant, G., Hussey, R. and Davis, E. (1999) Mol. Plant-Microbe Interact. 12, 64-67.
85	De Boer, J., Yan, Y., Smant, G., Davis, E., and Baum, T. (1998) J. Nematol. 30, 309-312.
86	Goverse, A., Davis, E., and Hussey, R. (1994) J. Nematol. 26, 251-259.
87	De Boer, J., McDermott, J., Wang, X., Maier, T., Qui, F., Hussey, R., Davis, E. and Baum, T. (2002) Mol. Plant Pathol. 3, 261-270.
88	Timmons, L and Fire, A. (1998) Nature 395, 854.
89	Rosso, M.-N., Dubrana, M., Cimbolini, N., Jaubert, S. and Abad, P. (2005) Mol. Plant-Microbe Interact. 18, 615-620.
90	Fanelli, E., Di Vito, M., Jones, J. and De Giorgi, C. (2005) Gene 349, 87-95.
91	Urwin, P., Lilley, C. and Atkinson, H. (2002) Mol. Plant-Microbe Interact. 15, 747-752.
92	Wesley, S., Helliwell, C., Smith, N., Wang, M., Rouse, D., Liu, Q., Gooding, P., Singh, S., Abbott, D., Stoutjesdijk, P., Robinson, S., Gleave, A., Green, A. and Waterhouse, P. (2001) Plant J. 27, 581-590.
93	De Meutter, J., Tytgat, T., Witters, E., Gheysen, G., Van Onckelen, H. and Gheysen, G. (2003) Mol. Plant Pathol. 4, 271-277.
94	Weerasinghe, R., Mck. Bird, D. and Allen, N. (2005) Proc. Nat. Acad. Sci. U.S.A. 102, 3147-3152.