ABSTRACT

SMITH, ADREINNE. Alteration of Nematode Gene Expression Results in Altered Infection Characteristics. (Under the direction of Eric Davis).

The southern root-knot nematode, Meloidogyne incognita, is an important obligate plant parasite with evolutionary adaptations such as a hollow, protrusible stylet and esophageal gland secretory cells that enable successful invasion and parasitism of roots of multiple crop species. Previous studies have identified more than fifty candidate genes that encode effector proteins synthesized in root-knot nematode esophageal gland cells and secreted from the stylet into plant cells during parasitism. Many of these candidate parasitism genes are without significant homology to any gene currently listed in public databases. Functional analysis of three of these M. incognita putative parasitism genes, designated Mi35F03, Mi4D01 and Mi5G05 was conducted by overexpression in plant tissue and RNA interference (RNAi) gene silencing assays. The effects of constitutive expression of each parasitism gene product with (+SP) and without (-SP) the secretion signal peptide were analyzed in Arabidopsis thaliana plants. Overexpression of Mi35F03-SP and Mi4D01-SP led to a decrease in root length and increase in root branching in transgenic Arabidopsis thaliana. There were no changes in the shoot phenotype of Arabidopsis lines that expressed any of the three parasitism gene products with or without the signal peptide. Interestingly, overexpression of Mi35F03 +/− SP and Mi4D01 +/− SP lead to a significant (p = 0.05) decrease in the number of galls/gram of root. Expression of double-stranded RNA complementary in sequence to each M. incognita parasitism gene transcript in transformed A. thaliana was utilized as a reciprocal way to analyze for potential RNAi effects on root-knot nematode parasitism of the host roots. A significant reduction in the number of galls/gram,
were observed in *Arabidopsis* lines expressing dsRNA of each gene. These findings indicate that Mi35F03, Mi4D01 and Mi5G05 play a role in root-knot nematode parasitism of host plants and support host-derived RNAi as a means to silence root-knot nematode parasitism genes and reduce nematode infection of plants.
Alteration of Nematode Gene Expression Results in Altered Infection Characteristics

by

Adreinne Dawn Smith

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Pathology

Raleigh, North Carolina

2014

APPROVED BY:

Dr. Eric Davis       Dr. Robert Franks
Committee Chair

Dr. David Shew       Dr. Paola Veronese
DEDICATION

I wish to dedicate this document to my daughter Annora Mackenzie Smith. You will always be mommy’s smart girl. I also wish to dedicate this document to my wonderful family and church family, Faith Christian Fellowship Center. I am eternally grateful for all of your love, support and most importantly your prayers through this difficult but rewarding experience. Last but not least, I would like to thank my grandmother, Bertha Weathers.
BIOGRAPHY

Adreinne Smith was born on March 28, 1979 in Sanford, North Carolina. She completed her Bachelor of Science degree in Zoology from North Carolina State University, Raleigh North Carolina in 2003. She received a Master of Science degree in Biology from Fayetteville State University in 2007 under the direction of Dr. Sherrice V. Allen. In the Fall of 2006, Adreinne accepted a graduate research program at North Carolina State University in the Department of Plant Pathology to pursue her Ph.D. Adreinne was a Ph.D. candidate of Dr. Eric Davis and conducted research on molecular plant-nematode interactions.
ACKNOWLEDGMENTS

I wish to express my sincere gratitude and appreciation to my Lord and Savior Jesus Christ. It is in Him that I live, move and have my being. I would like thank my major advisor Dr. Eric Davis for his guidance, support, correction, and encouragement throughout my graduate career at North Carolina State University. Thank you for believing in me when I didn’t believe in myself and for pushing me when I wanted to give up. I would like to thank my committee members for their guidance and support throughout my study and to the funding agencies and the NCSU Graduate School for providing financial support for the completion of my graduate career.

I would like to thank the past and current members of the Eric Davis Lab, Nrupali Patel, Laura Hudson, Nouredine Hamamouch, Bingye Xue, Kathryn Schweri, Torrey Gonzales, Megan Leach, Leah Vang and the many undergraduate members. Last but certainly not least, I would like to thank Chunying Li, who observed me during my most trying moments. I appreciate your sternness, your nurturing nature and for encouraging me to “just do it.”

I am grateful for my wonderful family who have always supported, encouraged and prayed me through countless situations. I am blessed to have the support, love and correction from Dr. Sherrice Allen who has always guided me through my personal and professional endeavors. A special thanks to Dr. Thomas Mitchell for connecting me to Department of Plant Pathology here at NC State. Finally, I would like to express gratitude and thanks to reverend Martin D. Valle for providing me with love, support and encouragement during the most challenging phase of my Ph.D. degree.
# TABLE OF CONTENTS

**LIST OF TABLES** ................................................................................................................................. vi

**LIST OF FIGURES** ................................................................................................................................. vii

**LITERATURE REVIEW** .......................................................................................................................... 1
  - General Introduction ................................................................................................................................. 1
  - The biology of *Meloidogyne* species ........................................................................................................ 3
  - Specific adaptations for plant parasitism .................................................................................................... 5
  - Plant parasitic nematode esophageal gland cell secretions ..................................................................... 7
  - Parasitism gene/effecter protein function in plant-nematode interactions ................................................. 10
  - Protein-protein interactions between nematode effectors and plant targets ........................................... 14
  - RNA-interference studies in nematodes .................................................................................................... 17
  - Engineering plants to express dsRNA and silence nematode gene function ............................................ 20

**MATERIALS AND METHODS** ................................................................................................................. 22
  - Plant material ........................................................................................................................................... 22
  - Nematode culture ..................................................................................................................................... 22
  - Parasitism genes 35F03, 4D01 and 5G05 that encode secreted nematode effectors .................................... 22
  - Expression of Mi35F03, Mi4D01 and Mi5G05 in transgenic Arabidopsis .................................................... 23
  - Relative reverse transcriptase polymerase chain reaction (RT-PCR) ......................................................... 26
  - Plant host-derived RNA-interference targeting Mi35F03, Mi4D01 and Mi5G05 ........................................... 27
  - Nematode infection assays using *Arabidopsis thaliana* as a host ............................................................ 29

**RESULTS** .................................................................................................................................................. 30
  - Expression of Mi35F03, Mi4D01 and Mi5G05 in transgenic *Arabidopsis thaliana* ................................. 30
  - Host-derived RNA-interference of Mi35F03, Mi4D01 and Mi5G05 ......................................................... 32

**DISCUSSION** .......................................................................................................................................... 33

**LITERATURE CITED** ............................................................................................................................... 38
LIST OF TABLES

LITERATURE REVIEW

Engineering plants to express dsRNA and silence nematode gene function

Table 1. Summary of three candidate parasitism genes encoding proteins for secretion and expressed exclusively within the esophageal gland cells of *Meloidogyne incognita* .................................................................53
LIST OF FIGURES

LITERATURE REVIEW

Figure 1. Life cycle of Meloidogyne incognita .................................................................49
Figure 2. Root-knot nematode feeding site (giant cell) ......................................................49
Figure 3. Illustration of the anterior end of the preparasitic second stage juvenile (J2) and parasitic adult female life stages of the root-knot nematode Meloidogyne incognita .................................................................50
Figure 4. Illustration of the yeast-two hybrid used to identify protein-protein interactions .................................................................51
Figure 5. Illustration of RNAi pathway .................................................................52

Expression of Mi35F03, Mi4D01 and Mi5G05

Figure 6. Illustration of the T-DNA region of the binary vector pBI121 containing the Meloidogyne incognita parasitism gene of interest .................................................................54
Figure 7. Illustration of pHANNIBAL construct to express double-stranded (dsRNA0 of the sense and antisense sequences of targeted nematode parasitism genes separated by the PDK intron .................................................................54

Expression of Mi35F03, Mi4D01 and Mi5G05 in transgenic Arabidopsis thaliana

Figure 8. Constitutive transcript expression of the Mi35F03 (AY142120) parasitism gene of Meloidogyne incognita without (-SP) and with (+SP) the predicted signal peptide for secretion in transgenic Arabidopsis thaliana .................................................................55
Figure 9. Constitutive transcript expression of the Mi4D01 (AF531162) parasitism gene of Meloidogyne incognita without (-SP) and with (+SP) the predicted signal peptide for secretion in transgenic Arabidopsis thaliana .................................................................56
Figure 10. Constitutive transcript expression of the Mi5G05 (AY135362) parasitism gene of Meloidogyne incognita without (-SP) and with (+SP) the predicted signal peptide for secretion in transgenic Arabidopsis thaliana .................................................................57
Figure 11. Root phenotype of Mi35F03-SP, Mi4D01-SP and Mi5G05-SP overexpression lines compared to wild-type (Col-0) controls at 12 days post-germination .................................................................58
Figure 12. Root length of transgenic *Arabidopsis* plants that constitutively express the *Mi35F03* parasitism gene +/- the predicted signal peptide for secretion ........................................58

Figure 13. Root length of transgenic *Arabidopsis* plants that constitutively express the *Mi4D01* parasitism gene +/- the predicted signal peptide for secretion ........................................59

Figure 14. Root length of transgenic *Arabidopsis* plants that constitutively express the *Mi5G05* parasitism gene +/- the predicted signal peptide for secretion ........................................59

Figure 15. Infection assay of overexpression transgenic *Arabidopsis thaliana* plant roots 6 weeks post-inoculation by *Meloidogyne incognita* (galls/gram of root) .................60

Figure 16. A repeat infection assay of over-expression transgenic *Arabidopsis thaliana* plant roots 6 weeks post-inoculation by *Meloidogyne incognita* (galls/gram of root) ............61

Host-derived RNA-interference of *Mi35F03, Mi4D01* and *Mi5G05*

Figure 17. Expression of hairpin double stranded (dsRNA) in transgenic *Arabidopsis* plants as measured by relative RT-PCR of the PDK intron sequence (291 bp) of the pHANNIBAL construct .........................................................62

Figure 18. Infection assay of RNAi transgenic *Arabidopsis thaliana* plant roots 6 weeks post-inoculation by *Meloidogyne incognita* .............................................................63

Figure 19. A repeat infection assay of RNAi transgenic *Arabidopsis thaliana* plant roots 6 weeks post-inoculation by *Meloidogyne incognita* .............................................................64
LITERATURE REVIEW

General Introduction

The microscopic roundworms called nematodes are members of the Kingdom Animalia (Agrios, 2005; Niblack et. al, 2006). They are the most abundant and ubiquitous animals in nature, inhabiting a variety of ecological niches ranging from arid deserts to frigid polar seas. Nematodes are often classified as either “free-living” or alternatively as parasites of animals and plants. Although “free-living” nematodes like Caenorhabditis elegans serve as premier biological models, animal and plant parasitic nematodes are prevalent in the scientific literature due to their effects on human and animal health as well as agricultural productivity (Maule and Curtis 2011). More specifically, plant parasitic nematodes are the causal agents of an estimated $100 billion in crop losses to the world-wide agro-economy annually (Sasser and Freckman, 1987; Chitwood, 2003; Maule and Curtis 2011; Li et. al, 2011), with cyst (Heterodera and Globedera species) and root-knot nematodes (Meloidogyne species) causing the most extensive damage (Li et. al, 2011; Lilley et. al 2007).

One key feature of the parasitic success of root-knot nematode (RKN) is its broad host range (over 2,000 host-plant species), which makes disease management a challenge (Barker 1998). Current integrated pest management strategies for control of root-knot nematode include crop rotation, nematicides, natural host-plant resistance and cultural practices (Li et. al, 2011; Mitchum et. al, 2007). Crop rotation is the oldest, most affordable and widely used form of pest management. The broad host range of RKN limits the effectiveness of crop rotation because the alternate crop could potentially serve as a host. In
addition, factors like the duration of rotation, the presence of other damaging nematodes and pest survival diminish the efficacy of this mechanism (Lilley et. al, 2007). Fumigant and non-fumigant pesticides have proven very effective in managing a variety of soil pathogens and pests including nematodes where crop value offsets the costs of chemical application (Li et al., 2011). However, many nematicides have been banned or phased-out due to their toxicity to the environment, human and animal health (Oka et. al, 2000, Lilley et. al, 2007). Alternatively, plant host resistance offers a more economical and efficient means of managing many species of plant parasitic nematodes, including RKN (Zasada et. al, 2010). Like other eukaryotic organisms, plants have evolved innate mechanisms to avoid or resist biotic stress caused by viruses, bacteria, fungi, and nematodes. One form of plant resistance to pathogens and pests is mediated by a single dominant resistance gene (R-gene) in the host that is effective if a corresponding dominant Avirulence (Avr) gene is present in the pathogen (Milligan et. al, 1998; Takken and Joosten et. al, 2000; Agrios 2005; and Atkinson et.al, 2013). This highly specific gene-for-gene interaction is commonly characterized by a hypersensitive response (HR), resulting in a rapid localized necrosis of the host tissue at the site of infection that inhibits the pathogen (Agrios 2005; Takken and Joosten et. al, 2000; Milligan et. al, 1998). The Mi gene in tomato, for example, is a single dominant R-gene that provides resistance against three major species of root-knot nematode, *M. incognita*, *M. arenaria*, and *M. javanica* (Jacquet et. al, 2005; Moens, 2011). Despite the successes of host resistance as a means of managing disease, resistance is not available in all crop plant species and is not always durable due to selection pressure on pathogen evolution that leads to resistance-breaking pathotypes (Mitchum et. al, 2007; Zasada et. al, 2010; Li et. al, 2011;
Atkinson et. al, 2013). The limitations of these current disease management strategies have intensified the need for alternative forms of control of plant parasitic nematodes.

The development of biotechnological techniques, where crops are engineered for pest resistance, has gained interest as an alternative approach to pest-management, including plant parasitic nematodes. For example, plant compounds like protease inhibitors that accumulate in response to wounding or herbivory have proven to be useful as anti-nematode agents in plants engineered to express them (Atkinson et.al, 2013). Constitutive transgenic expression of plant protease inhibitors (cystatins) in Arabidopsis thaliana suppressed the growth and fecundity of both sugar-beet cyst nematode (Heterodera schachtii) and root-knot nematode (Meloidogyne incognita) (Atkinson et. al, 2013). Current genomic and proteomic technologies now provide an expansive foundation for improving the knowledge base of host-pathogen interaction, where specific molecular aspects of parasitism can be targeted to develop alternative means of disease management (Lilley et. al, 2007).

The biology of Meloidogyne species

Root-knot nematodes (RKN), Meloidogyne spp., are the most damaging and economically important group of plant parasitic nematodes worldwide (Chitwood, 2003; Lilley et. al, 2007). The four major species of root knot nematode, Meloidogyne incognita, Meloidogyne arenaria, Meloidogyne hapla and Meloidogyne javanica are the most abundant world-wide (Sasser, 1980). All RKN species are obligate, biotrophic, sedentary endoparasites that spend the majority of their life-cycle within host-plant roots. The plant host-range of RKN is immense (Sasser, 1980). Oftentimes the damage associated with root-knot nematode goes undiagnosed because above ground symptoms such as, stunting, wilting
and chlorosis are indicative of a damaged root system and also characteristic of other plant pathogens as well as abiotic causes (Oka et. al, 2000; Lilley et. al, 2007). A key symptomatic feature of RKN infestation is galling or ‘knots’ at the sites of nematode infection along the root that inhibit the translocation of water and nutrients from the soil. Combined with the parasitic load of nematode feeding and potential secondary pathogen infections, the combined results are a decrease in crop yield and economic loss for the grower.

The lifecycle of RKN (Fig 1) is well established (Agrios, 2005; Davis et al., 2000; Lilley et. al, 2007; Baum et. al, 2007; Mitchum et. al, 2013). Mature adult females deposit eggs in masses in a gelatinous matrix along the surface of host roots. Following embryogenesis, a first stage juvenile (J1) develops and molts within the egg. Pre-parasitic second stage juveniles (J2) hatch from the egg and move through the soil in search of a suitable host root. The nematode stylet, a protrusible hollow oral spear, and secreted enzymes aid parasitic J2 in the penetration of the host behind the root tip and subsequent intercellular migration between the root cortical cells to the vascular cylinder (Vanholme et. al, 2004; Davis and Mitchum, 2005). Once in the vascular cylinder, the nematode selects 5 to 7 procambial cells to establish a permanent feeding site to enter and maintain its subsequent sedentary lifestyle (Vanholme et. al, 2004). Feeding site formation is coupled to the secretion of effector proteins that originate within the nematode esophageal gland cells (Baum et. al, 2007; Davis et al., 2000, 2008; Mitchum et. al, 2013). The selected plant cells from which the nematode feeds undergo tremendous cellular modifications that include highly vacuolated, multinucleate, hypertrophied cells with a dense granular cytoplasm and an increased number of cellular organelles (Fig 2) (Vanholme et. al, 2004; Davis and Mitchum
In essence, a normal plant cell is virtually turned into a giant metabolic ‘feeding factory’ or nutrient sink, called a ‘giant-cell’. The hypertrophy of the giant-cells and hyperplasia of the plant cells surrounding them causes the pronounced galling seen along the surface of the root (Vanholme et al., 2004; Davis and Mitchum 2005; Hassan et al., 2010; Jaouannet et al., 2013). Formation of giant-cells is required for RKN to initiate and maintain feeding for further growth and reproduction. The parasitic J2 then feeds, becomes swollen (pyriform) and all somatic musculature is lost, except for those muscles associated with feeding and reproduction (Dropkin, 1980). The nematode continues to feed and undergoes another molt into the J3 (Fig 1). After the third molt, the RKN male regains its vermiform shape, molts through fourth stage within the old swollen cuticle becoming a motile male that exits the root and stops feeding. The majority of infecting RKNs become adult females that remain swollen, molt quickly through the J3 and J4 stages to the adult stage, feeding within the host root for the duration of her life (Fig 1). Although M. hapla has demonstrated amphimixis, reproduction among the major species of Meloidogyne occurs via parthenogenesis, with the males having no sexual role (Dropkin, 1980). Each RKN female can lay up to 1,000 eggs per egg mass, with multiple generations occurring in one growing season. The entire life-cycle of M. incognita is approximately one month in optimal conditions (Dropkin, 1980).

Specific adaptations for plant parasitism

Specialized anatomical features including a hollow, protrusible stylet and the elaborate secretory gland cells within the nematode esophagus, promote the parasitic success of RKN and other species of phytoparasitic nematodes (Baum et al., 2007; Davis et al., 2000,
The stylet is a needle-like structure that is used to pierce plant cell walls for feeding and to release secreted effector proteins to aid in host penetration, migration, and feeding site formation (Baum et al., 2007). The origin of these secretory compounds is localized to two subventral esophageal gland cells and one dorsal esophageal gland cells within the nematode body (Fig 3). During the pre-parasitic stages (Pre-J2) and migration and penetration in roots (migratory parasitic stage), the subventral gland cells are enlarged, highly active and packed with membrane-bound secretory granules, while the dorsal gland remains small and relatively inactive (Hussey, 1989). The cellular extensions of the subventral gland cells open into ampullae connected to the esophageal lumen posterior to a muscular pump chamber in the esophageal median bulb (Baum et al., 2007; Davis et al., 2000, 2008; Hussey, 1989). During the sedentary parasitic (Par-J2) to the adult life stages, where feeding site initiation and formation commences, the dorsal gland becomes swollen, highly metabolically active and packed with membrane-bound secretory granules, while the subventral glands become small and relatively inactive (Baum et al., 2007; Davis et al., 2000, 2008; Hussey, 1989). The cellular extensions of the dorsal gland cells open into a single ampulla connected to the esophageal lumen at the base of the stylet (Hussey and Mims, 1990; Mitchum et al., 2013). Release of gland cell contents into the esophageal lumen occurs via the process of exocytosis (Hussey and Mims, 1990; Mitchum et al., 2013). Developmental changes in the activity of the esophageal gland cells indicate the significance of specific secretions to the vitality of the nematode during different stages of parasitism (Baum et al., 2007; Davis et al., 2000, 2008; Hussey, 1989; Mitchum et al., 2013). Although elaborate esophageal gland secretory cells are common in animal parasitic
nematodes, they are not present in ‘free-living’ nematodes like *C. elegans*, which further validates their role in parasitism (Baum et. al, 2007).

**Plant parasitic nematode esophageal gland cell secretions**

Like all pathogens, successful parasitism is accomplished by intricate molecular communication between the pathogen and its host (Agrios, 2005). The biology of plant parasitic nematodes (microscopic, obligate nature, requiring its host for survival) presents technical challenges for researchers. Previous research findings confirm that during pathogenesis, root-knot nematodes secrete compounds from their stylet and these secretions are essential in various developmental stages (egg hatching, root penetration, migration and feeding cell formation and establishment) and fecundity of the nematode (Hussey, 1989). Earlier studies sought to identify the nature of esophageal gland secretions and confirmed *via* histochemical and microspectrophotometric analyses that these compounds consisted of a mixture of proteins and not nucleic acid (Bird and Sauer, 1967; Hussey, 1989). The development and use of monoclonal antibodies (MAb) that bound to secretory granules formed in the esophageal gland cells was investigated for further identification and characterization of phytoparasitic nematode secretions involved in pathogenesis (Davis et al., 1994, 2000, 2008; Hussey et.al, 2002). Ultrastructural observation of various nematode life stages (Pre-J2, Par-J2, adult) of root knot and cyst nematodes, demonstrated MAb specific to the matrix of the secretory granules in either the sub-ventral gland (SvG), dorsal gland (DG) or both (Hussey and Mims 1990). Employing MAb’s as a means to purify and identify specific gland cell secretions required a sufficient amount of starting material and proved to be challenging due to the microscopic size and obligate-parasitic nature of plant-parasitic
nematodes. A study by Davis et al. (1992) used various immunization techniques to circumvent this issue and showed that intrasplenic immunization of mice produced a desirable amount of monoclonal antibodies that bound to esophageal gland antigens. Plant parasitic nematodes produce secretions through other orifices, like the amphids, which can limit the efficacy of purifying and identifying secretions from nematode esophagi (Davis et al., 1994; Goverse, 1994). In vitro studies showed that chemical 5-methoxy DMT oxalate and resorcinol could be used to isolate gland-cell specific proteins by inducing stylet thrusting and secretion from Pre-J2 of cyst nematode and RKN, respectively (Davis et al., 1994; Goverse et al., 1994, Jaubert et al., 2002). An immunopurification study using a MAb (MGR48) that binds to antigens specifically expressed within the subventral gland cells of cyst nematodes Globodera rostochiensis and Heterodera glycines yielded protein sequences subsequently used to identify the sequence of corresponding cDNA clones (Smant, 1998). Database analysis of these cDNA sequences resulted in the identification of the first $\beta$-1,4-endoglucanase (cellulase) genes of animal origin (Smant et al., 1998; Yan et al., 1998; Davis et al., 2000). Additional studies found the expression of $\beta$-1-4 endoglucanases in the egg, Pre-J2, male, and adult females of M. incognita (Rosso et al., 1999). Sequence similarities between endoglucanases of bacterial origin and the nematode endoglucanases suggest that nematode cellulases may have been acquired by horizontal gene transfer from prokaryotes (Smant et al., 1998; Davis et al., 2000, Danchin, 2010).

Given the obligate biotrophic nature, microscopic size and meager amounts of secretory compounds obtained from plant parasitic nematodes, more sensitive and powerful tools like the generation of cDNA libraries and expressed sequence tags (ESTs) became more
efficient for characterization of differentially expressed genes from whole nematodes at specific life stages (Dautova et. al, 2001; Vanholme et. al, 2004). The “parasitism genes” (Davis et. al, 2000) that encode secreted nematode effector proteins have remained a focus of molecular studies of endoparasitic nematodes to date (Davis and Mitchum 2005; Baum et. al, 2007, Mitchum et. al, 2013). Ribonucleic acid (RNA) fingerprinting was used to identify genes differentially expressed between Pre-J2 and Par-J2 in *M. incognita* and led to the discovery of a venom allergin-like gene (Ding et. al, 2000; Vanholme et. al, 2004). A cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technique was used on various life stages of the potato cyst nematode, *G. rostochiensis*, and led to the isolation and identification of genes expressed solely in the dorsal glands of Par-J2 (Qin et. al, 2000; Vanholme et. al, 2004). Differential expression of mRNA in the head and tail regions of *M. javanica* Par-J2 led to the identification of a nematode gene encoding a chorismate mutase (MjCM) that was expressed in the esophageal gland cells of *M. javanica* (Lambert et. al, 1999). This was the first chorismate mutase gene to be identified in an animal system, *M. javanica*, and complementation of a CM-deficient bacterial mutant with MjCM provided further support for the acquisition of prokaryotic genes within eukaryotes via horizontal gene transfer (Lambert et. al, 1999; Davis et. al, 2000, Vanholme et. al, 2004; Danchin et. al, 2010). An efficient and direct approach for differential study of effectors expressed throughout the nematode parasitic cycle involved the microaspiration of the cytoplasmic contents of nematode esophageal gland cells to enrich samples of mRNA needed to generate cDNA libraries of putative parasitism gene candidates (Gao et. al, 2001, 2003; Wang et. al, 2001; Huang et. al, 2003, 2004). Parasitism gene candidates were identified based on the
predicted presence of an N-terminal signal peptide sequence for secretion by Signal P algorithm (Bendsten et al., 2004) and in situ mRNA hybridization to determine differential expression and localization within the gland cells (Gao et al., 2001, 2003; Huang et al., 2003, 2004). Interestingly, Basic Local Alignment Search Tool (BLASTp www.blast.ncbi.nlm.nih.gov) analyses indicated that 27 of 37 putative M. incognita parasitism genes identified had no functional homology with any annotated genes (Huang et al., 2003). These ‘pioneer’ parasitism genes represent an attractive yet challenging suite of candidates in the characterization and functional analysis of M. incognita-host interactions (Huang et al., 2003).

**Parasitism gene/effector protein function in plant-nematode interactions**

Decades of research indicate that sedentary endoparasitic plant nematodes secrete proteinaceous compounds arising from esophageal gland secretory cells into host cells via the stylet (Baum et al., 2007; Davis et al., 2000, 2008; Hussey, 1989; Mitchum et al., 2013). These stylet secretions aid in host-invasion, cellular migration and feeding site formation and are essential to nematode parasitism of host plants. In addition, other pathogenicity factors, such as suppression of host defenses and immunity are being investigated (Hewezi and Baum, 2013; Mitchum et al., 2013). In the field of plant pathology, the term ‘effector’ has long been defined as a molecule produced by a pathogen pathogenicity/virulence gene that is exported into the plant for direct or indirect interactions with a plant R-gene protein (Agrios, 2005). More recently, the term ‘effector’ has been more broadly defined as all pathogen proteins that alter host-cell structure and functions regardless of whether these alterations facilitate infection or trigger defense responses (Hewezi and Baum, 2013).
The isolation of multiple differentially-expressed parasitism genes in plant parasitic nematodes has provided a platform for identifying nematode effector proteins (Hewezi and Baum, 2013; Mitchum et al., 2013). However, characterizing the functional role of these proteins in pathogenicity is necessary for understanding the complex and intricate relationship of plant-nematode interactions. In *M. incognita*, 30% of the isolated candidate parasitism genes (Huang et al., 2003) were expressed in the subventral gland cells, which are most active during early stages of parasitism (Pre-J2 to migratory Par-J2). Plants are equipped with structural barriers such as pectins, lignins, and rigid cell walls that can deter pathogen invasion. Two parasitism genes encoding pectate lyase enzymes were isolated from *M. incognita* and characterized (Huang et al., 2005). Pectate lyases are enzymes that break down the middle lamella, which is the primary wall component between plant cells and part of the cell wall matrix (Huang et al., 2005). This occurs via cleavage of α-1,4 linked galacturonosyl residues and β-elimation of glycosidic bonds (Huang et al., 2005). *In situ* hybridization revealed that these genes were expressed exclusively in the subventral gland cells and reverse transcription polymerase chain reaction (RT-PCR) revealed strong expression of transcripts in early parasitic stages but not in later stages (Huang et al., 2005). Xylan polymers make up hemicellulose, which is another structural component of plant cell walls. The first animal xylanase enzyme, endo-1,4 β-xylanase, was cloned from *M. incognita* (Dautova et al., 2006). The expression of cell wall degrading enzymes, in the subventral glands of migratory J2 suggests a role in penetration and migration within host roots (Mitchum et al., 2007, 2013; Davis et al., 2008; Hewezi and Baum, 2013).
During later parasitic stages (Par-J2 to adult), the dorsal gland cell swells and is highly metabolically active (Hussey, 1989). The increase in activity in the dorsal gland is coupled with an increase in the level of gene expression (Gao et al., 2001, 2003; Huang et al., 2003). Changes in the level of differentially-expressed genes in the dorsal gland, suggests that secretions from the dorsal gland are involved in the processes of feeding site formation and maintenance. Many of the effector proteins isolated from the dorsal gland in *M. incognita* have no sequence homology to other annotated genes in the database, with very few sharing sequence similarity with *C. elegans* (Huang et al., 2003). In the relative absence of known RKN effector identity our understanding of giant-cell phenotype (Jones, 1981) and gene expression (Escobar et al., 2011) can provide clues about the plant cellular processes affected during giant cell formation.

Nematode effector proteins encoding chorismate mutase (CM) have been identified in multiple species of both cyst and RKN (Lambert et al., 1999; Mitchum et al., 2007, 2013). Chorismate mutase is an enzyme of the shikimate pathway that is responsible for the production of cellular aromatic amino acids like tyrosine and tryptophan, plant defense compounds like salicylic acid, phytohoromones like auxin and other secondary metabolites (Mitchum et al., 2007; Lambert et al., 1999). Expression of CM from *M. javanica* in soybean hairy roots resulted in complete cessation of lateral root growth (Doyle and Lambert, 2003). This alteration of plant root development was overcome with the exogenous application of the auxin indole-3-acetic acid (IAA) (Doyle and Lambert, 2003). The calcium binding protein, calreticulin is involved in multiple cellular processes such as mRNA degradation,
cell adhesion and calcium homeostasis and has been identified as a secreted RKN effector (Jaubert et. al, 2005; Jaouannet et. al, 2013).

The sedentary nature of plant parasitic nematodes points to a network of complex and intricate signaling between nematode and its host. Secreted signaling peptides have been identified in plant parasitic nematodes. The Hg-SYV46 gene was identified as one of the most abundant genes exclusively expressed in the dorsal gland of Heterodera glycines (Wang et al., 2001). Protein domain analysis revealed that the C-terminus of HGSYV46 was similar to CLAVATA3/ESR-like (CLE) plant signaling peptide (Olsen and Skriver, 2003; Wang et al., 2005). Plant CLE genes have been well characterized in Arabidopsis thaliana (Leyser and Day, 2007). In plants, CLAVATA3 (CLV3) signaling is important for the growth and differentiation of meristematic tissues. CLV3 signaling results in the formation of an active membrane receptor kinase complex, CLAVTA1/CLAVATA2, which negatively regulates the expression of a protein called wuschel (WUS) (Leyser and Day, 2007). A balance between the levels of WUS and CLV3 are needed to maintain the proper growth of the apical meristem (Leyser and Day, 2007). Constitutive expression of SYV46 in A. thaliana resulted in arrested development of the shoot apical meristems, while expression of SYV46 in clv3 mutants restored wild-type development and differentiation of shoot apical meristem (Wang et. al, 2005). In M. incognita, an effector peptide, 16D10, expressed in the subventral glands (Huang et. al, 2003), was shown to have amino acid sequence similarity to a CLV3 motif (Huang et. al, 2006a). However, the 16D10 peptide could not restore functionality in clv3 A. thaliana mutants as was observed for CLE from cyst nematodes (Huang et. al, 2006a; Mitchum et al., 2012) Constitutive expression of 16D10 in A. thaliana did stimulate root
growth however, although no phenotypic changes were observed in shoot growth (Huang et al., 2006a). Contrastingly, constitutive expression of RKN parasitism gene Mi8D05 accelerated shoot growth and caused early flowering in Arabidopsis, but no changes in root growth were observed (Xue et al., 2013).

**Protein-protein interactions between nematode effectors and plant targets**

Functional analyses such as those involving Mi16D10 (Huang et al., 2006a) and Mi8D05 (Xue et al., 2013) prove that Arabidopsis thaliana is an efficient model system for understanding the RKN pathosystem. The obligate nature of plant endoparasitic nematodes, like RKN, requires an efficient and accessible model system for the study of plant-nematode interactions (Sijmons et al., 1991). Arabidopsis thaliana is a great model system being that its genome has been sequenced; it has a short life-cycle and is transformable for genetic and molecular studies (Sijmons et al., 1991). In addition, it is a compatible host for M. incognita (Sijmons et al., 1991; Huang et al., 2006a; Huang et al., 2006b; Xue et al., 2013). Previous studies have shown that nematode stylet secreted effector proteins are essential components of parasitism, from penetration and migration of and through host root tissues to feeding site induction (Baum et al., 2007; Davis et al., 2008; Mitchum et al., 2013). However, the various host-signaling pathways that are modulated by secreted nematode effector proteins are still being elucidated (Hewezi and Baum, 2013; Mitchum et al., 2013). Identifying host targets of nematode effector proteins could provide substantial evidence to the mechanisms that plant endoparasitic nematodes use to ‘hijack’ host cellular machinery and evade or suppress host defenses. Protein-protein interactions govern essentially every cellular process, from DNA replication and transcription to regulating the cell cycle (Coates and Hall, 2003; Ferro and
The yeast two-hybrid (Y2H) assay (Coates and Hall, 2003) can be employed to study the biologically complex protein-protein interactions that take place between nematodes and their hosts (Gheysen and Mitchum, 2011; Hewezi and Baum, 2013; Mitchum et al., 2013).

Yeast-two hybrid analysis is a powerful tool for studying protein-protein interactions under various cellular conditions (Coates and Hall, 2003). This method was initially described by Fields and Song (1989), who observed that the transcription factors (TF) of eukaryotes have a modular structure, a DNA binding domain (DBD) and a transcriptional activation domain (AD) (Coates and Hall, 2003; Ferro and Trabalzini, 2013). In the Y2H system (Coates and Hall, 2003; Ferro and Trabalzini, 2013), cDNAs encoding two proteins are co-expressed in yeast, where the protein of interest is fused to the DBD of a yeast TF and another protein is fused to the AD (Fig 4). The DBD is bound to an upstream activation sequence (UAS) or reporter gene (Ferro and Trabalzini, 2013; Coates and Hall, 2003). When a physical association between the two proteins occurs, bringing the domains DBD and AD into proximity, the reporter gene is transcriptionally activated (Ferro and Trabalzini, 2013; Coates and Hall, 2003). Although these two domains are physically separable and can function in proximity without covalent binding, neither domain can independently activate transcription (Ferro and Trabalzini, 2013). Yeast two-hybrid screens have been successful in identifying host targets of plant parasitic nematode effector proteins (Gheysen and Mitchum, 2011; Hewezi and Baum, 2013; Mitchum et al., 2013).

The first Y2H analysis of a plant parasitic nematode effector protein was conducted by Huang et al, (2006a) who demonstrated that Mi16D10 interacted with two *A. thaliana*
Scarecrow-like (SCL) transcription factors (TF) from the GRAS protein family. In *Arabidopsis*, SCL TF have role in plant root growth and development (Huang et. al, 2006a). The interaction Mi16D10 plant SCL TF provides evidence that some plant parasitic nematode effectors may function within the host nucleus to regulate host transcription to the benefit of the parasite (Huang et. al, 2006a). In a more recent study of a RKN effector, Y2H analysis of the *M. incognita* effector protein Mi8D05 revealed a specific interaction with a tonoplast intrinsic protein (TIP2), suggesting that Mi8D05 effector protein may potentially regulate water and solute transport in giant-cells (Xue et. al, 2013).

In cyst nematodes, the cellulose binding protein (CBP) from *Heterodera schachtii* interacted with pectin methylesterase 3 from *Arabidopsis thaliana* during early stages of syncytia formation (Hewezi et. al, 2008). Yeast two-hybrid analysis of the novel10A06 effector from *H. schachtii* (Hewezi et al., 2010) identified a positive interaction with *Arabidopsis thaliana* spermidine synthase 2 (SPDS2). SPDS2 is a key enzyme in the biosynthesis of polyamines that have multiple functions in plant cells (Hewezi et. al, 2010). Hewezi et. al, (2010) observed that interaction of Hs10A06 with SPDS2 resulted in an increase of spermidine content and polyamine oxidase cellular antioxidant activity in syncytia during the early stages of parasitism. Patel et. al, (2010) observed interaction of a *H. schachtii* annexin effector (Hs4F01) with a host oxidoreductase member of 2OG-F2 (II) oxygenase family in yeast co-transformation analyses. The 2OG-F2 (II) oxygenase plant enzyme is associated with plant defense and stress responses and interaction with Hs4F01 could potentially act to suppress host defenses (Patel et. al, 2010). In addition, the novel *H. schachtii* effector 30C02 was shown to interact with a host β-1,3 endoglucanase to potentially
alter host defenses (Hamamouch et. al, 2012). Novel *H. schachtii* effector protein 19C07 was shown to interact with an *Arabidopsis* auxin influx transporter LAX3 (Lee et. al, 2010). In *A. thaliana*, LAX3 is expressed in lateral roots and is essential for auxin signaling in the development of lateral root (Lee et. al, 2010). Interaction of Hs19C07 and host LAX3 suggests that nematode effector proteins modulate cellular auxin levels during parasitism (Lee et. al, 2010). In *Globodera rostochiensis*, an effector containing a SPRY domain (SPRYSEC19) was shown to directly interact with a host R-gene protein by binding to a coiled-coiled nucleotide-binding leucine-rich repeat motif (Rehman et. al, 2009; Hewezi and Baum, 2013).

These functional studies provide evidence that secreted nematode effector proteins can directly target host proteins to promote parasitism by modulating host transcription, suppressing host defenses, augmenting phytohormone balance, and regulating the movement of solutes and water for feeding cell formation.

**RNA-interference studies in nematodes**

Gene knockout strategies and gene silencing have been used to study and understand the function of genes involved in pathogenicity (Li et. al, 2011). The molecular basis of gene silencing activity as observed in earlier investigations was confirmed as RNA interference (RNAi) activity in the nematode *C. elegans* (Fire et. al, 1998). It was subsequently demonstrated that ingestion of double-stranded RNA (dsRNA) by *C. elegans* (Timmons et. al, 1998) and later by cyst nematodes (Urwin et. al, 2002), could induce post-transcriptional silencing of the complementary gene in the nematode. Gene silencing via RNAi provides the potential to better understand nematode effector function by mitigating parasitism gene
expression. The information gained from RNAi analysis of parasitism genes may also prove to be useful in the control of plant parasitic nematodes if genes essential to parasitism can be sufficiently silenced to disrupt the parasitic process.

RNA interference is a cellular process that leads to the degradation of homologous RNA molecules in a sequence specific manner, ultimately silencing expression of a complementary gene (Cogoni and Macino, 2000; Sijen and Kooter, 2000; Vaucheret et. al, 2001). Activation of RNA silencing (Fig 5) is initiated when a double-stranded RNA (dsRNA) molecule enters a cell (Cogoni and Macino, 2000; Vaucheret et. al, 2001; Boutla et. al, 2002; Bakhetia et. al, 2005). The exogenous dsRNA molecule is recognized by the host cell and cleaved by Dicer, an RNaseIII endonuclease, in an ATP-dependent manner (Vaucheret et. al, 2001; Boutla, et. al, 2002; Bakhetia et. al, 2005). Cleavage by the endonuclease Dicer, results in short double-stranded RNA molecules, known as small interfering RNA (siRNA) 21-25 nucleotides (nt) in length, with 2 nt long 3’ overhangs (Vaucheret et. al, 2001; Boutla et. al, 2002; Bakhetia et. al, 2005). A ribonucleoprotein complex or RNA silencing complex (RISC) is formed from these siRNAs (Boutla et. al, 2002; Bakhetia et. al, 2005). The RISC/siRNA duplex functions to unwind the double-stranded siRNA complex into single-stranded RNA molecules. Afterwards, these single-stranded siRNAs bind to a target messenger (mRNA) in a sequence-specific manner (Vaucheret et. al, 2001). Once the siRNA is bound to its target mRNA, the target mRNA undergoes cleavage by the protein called ‘slicer’ (Vaucheret et. al, 2001). The now cleaved RNA is recognized by the cell as aberrant or foreign and is destroyed, preventing the

Various methodologies have been utilized to elicit an RNAi response in *C. elegans*. These include direct micro-injection of dsRNA that target complementary mRNA sequences into germ cells, feeding the nematode bacteria expressing complimentary dsRNA or by soaking the nematode in complementary dsRNA solution (Fire et. al, 1998; Timmons and Fire, 1998; Tabara et. al, 1998; Lilley et. al, 2007). The obligate nature of plant parasitic nematodes renders them refractory to microinjection of dsRNA and they usually only ingest upon establishment of a feeding site in host plant tissues (Urwin et. al, 2002). Earlier studies showed that chemicals could be used to induce stylet secretions (Davis et al., 1994; Goverse et al., 1994). Conversely, a chemical stimulant, octopamine, was shown to induce pharyngeal pumping and ingestion of dsRNA by Pre-J2 of cyst nematodes in a soaking solution (Urwin et. al, 2002). Three cyst nematode genes were targeted for silencing in this RNAi-soaking study, an intestinal cysteine proteinase, a novel protein (hgctl), and major sperm protein (Uwrin et. al, 2002). The effects of RNAi on cysteine proteininases produced changes in sexual fate of cyst nematodes, with an increase in the number of males. Transcriptional analysis of major sperm protein demonstrated that silencing persisted for several days following RNAi treatment, suggesting that RNAi soaking of Pre-J2 could be used to study genes expressed at later parasitic stages (Uwrin et. al, 2002). In root-knot nematode, resorcinol was used to induce ingestion of dsRNA targeting calreticulin and polygalacturonidase (Rosso et. al, 2005). Targeted transcript levels were reduced following
RNAi treatment, but normal expression was restored by 72 hours after RNAi-soaking (Rosso et. al, 2005).

**Engineering plants to express dsRNA and silence nematode gene function**

Despite transient recovery of transcripts, methods for delivery of dsRNA *in vitro* have proved useful in functional analyses of nematode parasitism genes (Rosso et. al, 2009). However, plants that can be engineered to express dsRNA provide a more feasible gene silencing approach to investigate nematode effector function, especially for parasitism genes that are expressed only when nematodes are within plant tissues. Host-derived RNAi utilizes plants that are transformed with vectors that express hairpin dsRNA (Wesley et. al, 2001; Rosso et. al, 2009; Li et. al, 2011; Mitchum et. al, 2013). The coding region of the nematode gene of interest is cloned in the sense and antisense orientation and separated by a region of noncoding nucleotides (Rosso et. al, 2009; Mitchum et. al, 2013). The dsRNA complementary to the nematode gene of interest should be subsequently cleaved into siRNAs by host-plant DICER enzymes and of suitable size for ingestion by nematodes from within the plant tissues (Li et. al, 2011; Mitchum et. al, 2013; Rosso et. al, 2009; Wesley et. al, 2013). Ingestion of siRNA via the stylet can silence the complementary transcripts within the nematode and the plant can provide a continuous supply of dsRNA silencing triggers during the course of parasitism (Li et. al, 2011; Mitchum et. al, 2013; Rosso et. al, 2009; Wesley et. al, 2001). Successful silencing of nematode parasitism genes via host-derived RNAi leading to a decrease in susceptibility to nematode infection has been achieved in both cyst and root-knot nematodes (Mitchum et al., 2013; Rosso et al., 2009). *In planta*
expression of *Mi16D10* dsRNA and *Mi8D05* has led to a 90% reduction in transgenic Arabidopsis infection by root-knot nematodes (Huang et. al, 2006b; Xue et. al, 2013).

This dissertation is based upon the hypothesis that secretions from the esophageal gland cells of RKN are important for the establishment of parasitism by the nematode. *Arabidopsis thaliana* and *M. incognita* were chosen as a model pathosystem to determine the molecular processes involved in the functions of selected parasitism genes. As indicated above, the majority of parasitism genes identified in *M. incognita* (Huang et. al, 2003) encoded novel secreted effector proteins. In this study, constitutive expression and RNAi analysis of three novel nematode parasitism genes, *Mi35F03, Mi4D01* and *Mi5G05* (Table 1) were conducted in Arabidopsis to investigate potential changes in host plant phenotype and susceptibility to *M. incognita*. 


MATERIALS AND METHODS

Plant material

*Arabidopsis thaliana* (ecotype Columbia-0) was used as wild-type to generate all transgenic plant lines for plant phenotype analyses and infection assays with *Meloidogyne incognita*. Seeds were surface sterilized with 10% sodium hyperchlorite and 0.01% Tween 20 solution for 3 minutes followed by treatment with 70% ethanol for 3 minutes. Sterilized seeds were washed 5 times with sterile distilled water to remove excess sterilization solution. Seeds were plated on Murashige and Skoog (MS) medium, sealed with parafilm and kept at 4˚C for 48 hours. After 48 hours, plates were kept in a growth chamber at 24˚C with 16 hour light/8 hour dark cycle for 5 days.

Nematode culture

*Meloidogyne incognita*, race 4, was propagated on the roots of greenhouse grown tomato plants (*Solanum esculentum* cv. Rutgers) (Hussey and Barker, 1973). RKN egg masses were extracted from the roots of host tomato plants using a 0.5% sodium hypochlorite solution and collected on a 25µm sieve, as previously described (Hussey and Barker1973). Eggs were hatched over water at 28˚C for 48 hours. Pre-parasitic second-stage juveniles (pre-J2) were collected on a Baermann pan and used in subsequent root infection assays.

Parasitism genes *35F03, 4D01 and 5G05* that encode secreted nematode effectors

The selected parasitism genes *Mi35F03, Mi4D01*, and *Mi5G05* of *Meloidogyne incognita* (Table 1) were isolated and characterized by Huang et al. (2003). The longest ORF of *Mi35F03* (accession number AY142120) cDNA (398-bp) encoded a deduced protein of 47
amino acids (aa) that included an N-terminal signal peptide as predicted by Signal P (Bensted et al., 2004). BLASTp analysis of the 398-bp sequence of Mi35F03 against multiple genomes identified no database sequence similarity or predicted peptide sequence, suggesting that Mi35F03 is novel.

The longest ORF of Mi4D01 (accession number AF531162) cDNA (785-bp) encoded a deduced protein of 174 aa that included an N-terminal signal peptide as predicted by Signal P. BLASTp analysis of the 785-bp sequence of Mi4D01 against multiple genomes identified no significant sequence similarity, but showed similarity to a putative protein domain in C. elegans, the ground-like superfamily. In C. elegans, this domain helps to bind and modulate the activity of patch-like membrane proteins, similar to the activity of neuropeptides (Marchler-Bauer et al., 2011).

The longest ORF of Mi5G05 (accession number AY135362) cDNA (977-bp) encoded a deduced protein of 271 aa that included an N-terminal signal peptide as predicted by Signal P. BLASTp analysis of the 977-bp sequence of Mi5G05 against multiple genomes identified no significant sequence similarity, but showed predicted protein similarity to that of Ascaris suum. Pfam analysis identified a protein domain similar to a leukocyte cell-derived chemotaxin (Marchler-Bauer et al., 2013).

**Expression of Mi35F03, Mi4D01, and Mi5G05 in transgenic Arabidopsis**

The Mi35F03, Mi4D01, and Mi5G05 parasitism genes were constitutively expressed with and without the signal peptide in transgenic Arabidopsis plants. Constitutive expression without the signal peptide should target the protein for localization within the cytosol of the transformed plant cells. If the nematode signal peptide is active in the plant cell, inclusion of
the signal peptide should target protein localization for the secretory pathway and apoplast of the host cell.

To conduct expression assays in transgenic Arabidopsis, the coding regions of Mi35F03, Mi4D01 and Mi5G05 with or without the signal peptide sequence, were amplified from full length cDNA clone with gene specific primers 35F03-For 5’

ATGTATCCTTGGACAATTTT 3’ and 35F03-Rev 5’ CTAGATGAATGCCCAACAGA 3’; 4D01-For 5’ ATGCAAAATTAATTTTATT 3’ and 4D01-Rev 5’

TCATTGAATTTTGAAGACTG 3’ 5G05-For 5’ ATGGTTTTATTTATTTTTATTTT 3’ and 5G05-Rev 5’ CTATAGGTGAATAAATTTTG 3’.

The coding region products replaced the β-glucoronidase (GUS) gene at the SacI and XbaI restriction sites, in binary vector pBI121 (Chen et. al, 2003), under the control of the CaMV35S promoter (Fig 6) to generate 35S::35F03, 35S::4D01, 35S::5G05. The sequence and orientation of each expression cassette was confirmed by polymerase chain reaction (PCR) of plant genomic DNA and by DNA sequencing (Eton Bioscience, Durham, NC).

Each expression cassette, 35S::35F03, 35S::4D01, 35S::5G05 was introduced into Agrobacterium tumefaciens C58C1 by electroporation (Shen and Forde, 1989) and transformed into Arabidopsis thaliana Col-0 by floral dip-mediated transformation (Clough and Bent, 1998). Seeds from transformed Arabidopsis plants were collected and sterilized as previously described and plated on MS media supplemented with 50µg/mL of kanamycin. Segregation of transgenic seedlings was based upon their ability to grow in the presence of kanamycin. The presence of each transgene in transformed Arabidopsis lines was confirmed by PCR analysis using DNA extracted from the transgenic plant lines as a template and gene-
specific primers. The following gene specific primers were used to confirm the presence of each transgene, with the inclusion of the signal peptide. 35F03-For 5’ ATGTATCCTTGGACAATTTT 3’ and 35F03-Rev 5’ CTAGATGAATGCCCAACAGA 3’
4D01-For 5’ ATGCAAAATTAATTTTTAT 3’ and 4D01-Rev 5’
TCATTGAATTTTGAGACTG 3’ 5G05-For 5’ ATGTTTTATTTATTTATTTTATTT 3’ and 5G05-Rev 5’ CTATAGGTGAATTAATTTTG 3’. The following gene specific primers excluding the signal peptide sequence were used to confirm the presence of each transgene. 35F03-SP-For 5’ TGAAATAATTGAGGAAAG 3’ and 35F03-Rev 5’
CTAGATGAATGCCCAACAGA 3’, 4D01-SP-For 5’ TGGTTTTTGAGGATGTG 3’ and 4D01-Rev 5’ TCATTGAATTTTGAGACTG 3’, 5G05-SP-For 5’ TTGTATGAGACTATTTTAT 3’ and 5G05-Rev 5’ CTATAGGTGAATTAATTTTG 3’. The PCR parameters were as follows 95°C 5 minutes, 95°C 50 seconds, 50°C 45 seconds, 72°C 1 minute, 72°C 10 minutes for 30 cycles. Transformed Arabidopsis lines that were PCR positive for each transgene of interest were cultured on MS media for 5 days at 24°C with 16 hours light/8 hours’ dark. After five days some seedlings were transferred to soil for T2 collection and ten homozygous seedlings per gene (Mi35F03, Mi4D01, Mi5G05, where n=30) were aseptically transferred to square petri plates containing MS media and grown at 24°C with 16 hours light/8 hours dark and placed vertically for assessment of root length every other day for 11 days. Shoot phenotypes were assessed on these same ten plant lines, where leaf number and leaf morphology was observed every other day for 15 days.
Relative reverse transcription polymerase chain reaction (RT-PCR)

The RNeasy Plant Mini Kit was used to extract and purify total RNA from ground plant tissues, according to the manufacturer’s instructions (Qiagen, Valencia, CA). All mRNA samples were DNase-treated (Turbo DNA-free Ambion, Life Technologies). RT-PCR was conducted to determine transgene expression (with or without encoding signal peptide sequence) in T3 lines. Four micrograms (4µg) of DNase-treated RNA was used to synthesize first-strand cDNA by PCR amplification using Superscript II Reverse Transcriptase kit, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). RT-PCR amplification was carried using primers 35F03-For 5’
GAATGTATCCTTGAGACAATTTT 3’ or 35F03-SP-For 5’
TGAAATAATTGGAGAGAAAAA 3’ and 35F03-Rev 5’ CTAGATGATGCCCCACACAGA
3’ 4D01-For 5’ ATGCAAAATTTTATTATTATT 3’ 4D01-SP-For 5’
TGGGTGTTGGTGGAGGATGTG 3’ and 4D01-Rev 5’ TCATTGAATTGGGACTG 3’
5G05-For 5’ ATGTTTATTATTATTTATTATTATTATTATTT 3’ or 5G05-SP-For 5’
TTGTATGAAAGACTATTGTGT 3’ and 5G05-Rev 5’ CTATAGGTGAATAAATTTTG 3’.

An amplified fragment of uniformly expressed *Arabidopsis* actin 8 (ACT8) (accession number NM_103814) was used as a control. Actin-For 5’ GCCCCGAGGCAGCAGTGAAGAT 3’ and Actin-Rev 5’ GCTGGAAAGTGCTGAGGGAAGC 3’. Twelve microliters (12µl) of each RT-PCR reaction was separated on 1% agarose electrophoresis gel. Transgenic *Arabidopsis* lines confirmed to express *Mi35F03, Mi4D01* and *Mi5G05* were used for subsequent infection assays with *Meloidogyne incognita* to assess potential effects of transgene expression on plant development.
Plant host-derived RNA-interference targeting *Mi35F03, Mi4D01 and Mi5G05*

Double-stranded RNA (dsRNA) complementary to transcript sequence of *Mi4D01, Mi35F03* and *Mi5G05* was expressed in transgenic Arabidopsis to assay for potential effects of RNAi-silencing of target nematode genes on successful parasitism. The sense and antisense arms of the full ORFs of *Mi4D01, Mi35F03* and *Mi5G05* (Fig 7) were cloned in the sense (*XhoI-EcoRI* and *XhoI-KpnI* restriction sites respectively) and antisense (*BamHI-XbaI* restriction sites) orientation of the pHANNIBAL vector (Wesley et. al, 2001). Constitutive expression of each transgene in plants was under the control of the CaMV35S promoter. cDNA complementary to green fluorescent protein (GFP) was constructed and utilized as a negative RNAi control since this gene does not exist in nematodes. GFPRNAi-For 5’ GATCCCAACGAAAAGAGAGACCACAT 3’ and GFPRNAi-Rev 5’ CAACAAAGAATTGGGACAACGACCACAT 3’. All pHANNIBAL clones containing the sense and antisense of each gene of interest were subcloned as *NotI* fragments (Fig 7) into the binary vector pART 27 (Gleave, 1992). Introduction of RNAi-GFP, RNAi-35F05, RNAi-4D01 and RNAi-5G05 into wild-type *Arabidopsis* was accomplished using *Agrobacterium mediated* floral transformation (Clough and Bent, 1998). Seeds from transformed *Arabidopsis* plants were collected and sterilized as previously described and plated on MS media supplemented with 50µg/mL of kanamycin. Segregation analysis of transformed *Arabidopsis* lines (T1) containing the sense and the antisense of *Mi35F03, Mi4D01* and *Mi5G05*, was based on antibiotic resistance to kanamycin. The presence of each transgene in transformed *Arabidopsis* lines was confirmed by PCR analysis using template DNA extracted from transgenic lines and gene-specific *Mi35F03, Mi4D01*, and *Mi5G05*. The
following primer pairs were for PCR confirmation 35F03-ForSense 5’ TTGGAGGAAAAGGTCGAA 3’ and 35F03-RevSense 5’ TCAGCTGCGCAGTTTCTA 3’; 4D01-ForSense 5’ TTGCTTGTATTATATTGCC 3’ and 4D01-RevSense 5’ CCTGATGTCCATCCACAC 3’; 5G05-ForSense 5’ CCGATTCTCGACACCCCTG 3’ and 5G05-RevSense 5’ TTGGAGTTCCCCATTCTT 3’. The PCR cycling parameters were set at 95˚C 5 minutes, 95˚C 50 seconds, 45˚C 50 seconds, 72˚C 1 minute, 72˚C 10 minutes, for 35 cycles. Positively transformed transgenic plant lines were cultured on MS media for 5 days at 24˚C with 16 hours light/8 hours’ dark. After five days some seedlings were transferred to soil for T2 collection. Ten homozygous seedlings (Mi35F03, Mi4D01, Mi5G05, where n=30) were aseptically transferred to square petri plates containing MS media and grown at 24˚C with 16 hours light/8 hours dark and placed vertically for root length assays. Shoot phenotypes were assessed on these same ten plant lines, where leaf number and leaf morphology was observed every other day for 15 days.

Root samples from independent homozygous Arabidopsis lines of RNAi-GFP, RNAi-35F05, RNAi-4D01 and RNAi-5G05 were used to analyze transgene expression of the PDK intron of each hairpin dsRNA (Wesley et al., 2001) using RT-PCR. Total RNA from plant material was extracted using the RNeasy Plant Mini Kit according to the manufacturer’s instructions. RT-PCR analyses for PDK intron expression was conducted using the First-Strand cDNA synthesis kit using Superscript II RT. Gene specific primers’ were used for amplification of a 500bp product using primers PDKFor 5’- GATCATGTCATTTGTATCATTGATC-3’ and PDKRev 5’- GCTAGTATACATCTTACATGTTC-3’. The products of the RT-PCR reaction were
separated on 1% agarose electrophoresis gel. Transgenic *Arabidopsis* lines RNAi-GFP, RNAi-35F05, RNAi-4D01 and RNAi-5G05 confirmed to express dsRNA were used for subsequent infection assays with *Meloidogyne incognita* to assess potential effects of RNAi transgene expression on the parasitic interaction.

**Nematode infection assays using *Arabidopsis thaliana* as a host**

The procedure of Xue et al. (2013) was used for all infection assays of Arabidopsis with *M. incognita*. *Arabidopsis thaliana* ecotype (Col-0) was used as wild-type to generate all transgenic plant lines for infection assays with *M. incognita*. For nematode infection assays, sterile seeds were plated on MS media supplemented with kanamycin 50µg/mL and kept at 24˚ C and 16 hour light/ 8 hour dark for five days. Five days post-germination the *Arabidopsis* seedlings were transplanted into 3-inch plastic pots of soil (93.3% sand, 3.3% silt, and 3.4% clay), one plant per pot, under greenhouse conditions. One-week after transplanting, 1,000 freshly hatched pre-parasitic second-stage juveniles (Pre-J2) of *M. incognita* were added to the soil at the base of each *Arabidopsis* test plant. At six weeks post-inoculation, root systems were gently washed in water and blotted dry to assess root fresh weight. The number of galls induced by *M. incognita* infection on each root system was counted under a dissecting microscope. The mean and standard error of 10 replicates per treatment were calculated and statistical differences in mean (n=10) were determined using ANOVA statistical analysis (SAS Institute, Cary, NC). Each infection assay was repeated to assess reproducibility.
RESULTS

Expression of Mi35F03, Mi4D01 and Mi5G05 in transgenic Arabidopsis thaliana

To assess potential effects of individual nematode effector proteins on plant cells and resultant plant phenotype the Mi35F03, Mi4D01 and Mi5G05 parasitism genes were each constitutively expressed in Arabidopsis, with and without the signal peptide. The exclusion of the signal peptide targeted the nematode protein to the cytoplasm of transformed plant cells while the presence of the signal peptide targeted the nematode protein to the host secretory pathway for export from the plant cell. There were three independent transgenic lines generated and analyzed from each gene construct. Expression of each nematode parasitism gene in transgenic Arabidopsis lines was confirmed by RT-PCR (Figs 8-10). Relative gene expression levels were variable between Mi35F03, Mi4D01 and Mi5G05 transgenic lines. There were similarities in transgene expression levels in Mi35F03 lines 3, 8 and 9 (with the signal peptide) and Mi35F03 lines 5, 6 and 10 (without the signal peptide), compared to actin controls (Fig 8). Varying levels of transgene expression was observed among Mi4D01 transgenic lines 5, 11 and 12 (with the signal peptide). Mi4D01 lines 5 and 12 show relatively low levels of expression compared to Mi4D01 line 11 and actin controls. Similar levels of transgene expression are seen in Mi4D01 lines 8, 9 and 18 (without the signal peptide), compared to controls (Fig 9). Mi5G05 transgene expression levels are relatively high in lines with (lines 5, 7 and 8) and without the signal peptide (lines 7, 8 and 14), compared to controls (Fig 10).
The potential effects of expression of each nematode parasitism gene expression on transgenic Arabidopsis plant phenotype were observed visually. Root length assays were conducted on agar plates to observe potential phenotypic differences between transformed *Arabidopsis* versus wild-type control. Visual Differences in root length and root architecture were observed (Fig 11) in Mi35F03 transgenic lines 5, 6 and 10 and Mi4D01 transgenic lines 8, 9 and 18 without the signal peptide lines compared to wild-type controls. Two independent tests revealed significant differences (paired t-test, *p*≤0.05) in the root growth in transformed *Arabidopsis* lines that expressed Mi35F03 and Mi4D01 without the signal peptide compared to wild-type controls (Figs 12 & 13). There were no significant differences in root growth observed in Mi35F03+SP, Mi4D01+SP and Mi5G05 +/- SP compared to wild-type controls (Figs 12-14). No significant differences in leaf morphology, leaf number or growth rate were observed in shoots of transgenic *Arabidopsis* that expressed Mi35F03, Mi4D01 and Mi5G05.

Infection assays with *M. incognita* were conducted on *Arabidopsis* lines that were confirmed to express the Mi35F03, Mi4D01 and Mi5G05 parasitism genes to assess potential affects of constitutive effector expression on root-knot nematode infectivity in two separate tests (Figs 15 & 16). At six weeks post inoculation, significant differences (paired t-test *p*≤0.05) compared to controls were observed in the number of galls/gram of root for plant lines that overexpressed Mi35F03 and Mi4D01 with and without the signal peptide, in both tests (Figs 15 & 16). A 56-80% and a 57-76% reduction in the galls/gram of root was observed in nematode infected Mi35F03+SP transgenic *Arabidopsis* lines, in the first and second assay respectively. Nematode infected Mi35F03-SP transgenic *Arabidopsis* lines resulted in an 86-94% and a 70-82% reduction, in the first and second assay respectively. In
both assays, *Mi4D01* lines with and without the signal peptide resulted in an 80% reduction in the galls/gram of root, compared to controls. The galls/gram of root for nematode infected *Mi5G05* transgenic *Arabidopsis* showed no significant differences compared to controls for both assays.

**Host-derived RNA-interference of *Mi35F03, Mi4D01 and Mi5G05***

Plant-host derived RNAi was used as a means to silence the expression of the target *M. incognita* parasitism genes within the nematode and to assess potential effects on nematode parasitism. Transformed homozygous *Arabidopsis* lines that expressed dsRNA of *Mi35F03, Mi4D01* and *Mi5G05* were confirmed by RT-PCR of the hairpin intron (Fig 17) and used for subsequent assays. No visual changes in plant shoot or root phenotypes were observed among the wild-type, GFP control lines, and transgenic *35F03*RNAi, *4D01*RNAi, and *5G05*RNAi *Arabidopsis* lines (data not shown). Six weeks post inoculation with *M. incognita*, significant (paired t-test p≤0.05) reductions were observed in the number of galls per gram of root for plant lines that expressed dsRNA of *Mi35F03* and *Mi5G05*, compared to GFP-RNAi and wild-type plant controls (Figs 18 and 19). Nematode infected plant lines expressing *Mi35F03* dsRNA showed an 85-100% and a 70-90% reduction in the galls/gram of roots in the first and second tests respectively. Nematode infected *5G05* transgenic RNAi *Arabidopsis* plants showed a 60-80% reduction in galls/gram of root for both analyses. There was a 10-20% increase observed in the galls/gram of roots for nematode infected RNAi *4D01* plant lines in test one (Fig 18) compared to controls while a 55-94% reduction in the galls/gram of root was observed in the repeated test of 4D01-RNAi (Fig 19).
DISCUSSION

Secreted effector proteins encoded by parasitism genes expressed in the esophageal gland cells of root-knot nematodes are essential to form complex feeding sites (giant-cells) localized in host root tissues (Baum et. al, 2007; Davis et al., 2008; Mitchum et. al, 2013). Our work here suggests that the root-knot nematode parasitism genes \textit{Mi35F03}, \textit{Mi4D01} and \textit{Mi5G05} may potentially play a role in the parasitic process and could prove to be useful novel targets for anti-nematode agents. The expression of nematode parasitism genes within a host such as \textit{A. thaliana} to assess potential affects in plant phenotype and nematode susceptibility gives initial insight into the function of the nematode parasitism proteins that are secreted during the infection process (Davis et al., 2008; Huang et. al, 2006; Wang et. al, 2005). Expression analyses of \textit{Mi35F03} and \textit{Mi4D01} without the signal peptide in \textit{Arabidopsis} produced an observable root growth phenotype, resulting in primary roots that were shorter and more branched than WT controls. Previous studies of ectopic expression of nematode parasitism genes implicate that altered host susceptibility by plant parasitic nematodes is independent of host root system length and mass (Hewezi et al., 2008, 2010; Patel et al., 2008, 2010). No observable morphological phenotype was seen in the shoots of transformed \textit{Arabidopsis} plants expressing \textit{Mi35F03}, \textit{Mi4D01} and \textit{Mi5G05} with or without the signal peptide. This does not exclude the possibility of bio-molecular changes in the host that do not produce a visual phenotype upon transgene expression, as observed with nematode annexin effect proteins (Patel et al., 2010). In our study, plants that expressed \textit{Mi35F03} and \textit{Mi4D01} were significantly less susceptible to infection by \textit{M. incognita}. 

33
Previous studies conducted on other nematode parasitism genes (Wang et al., 2005, Haung et al., 2006; Xue et al., 2013), revealed that plants expressing nematode parasitism genes were more susceptible to infection by plant parasitic nematodes. The decrease in host susceptibility observed in our study could potentially be due to the disruption of cellular pathways that are important for nematode feeding site formation and parasitism. Huang et al. (2003) have confirmed that Mi35F03 and Mi4D01 are expressed in the early stages of root-knot nematode parasitism in plants, during the time of feeding site formation. It is possible that overexpression of Mi35F03 and Mi4D01 with and without the signal peptide may modulate phytohormone signaling (Gheysen and Mitchum, 2011), thus altering nematode development and feeding site formation. Constitutive ectopic expression of both Mi35F03 and Mi4D01 could produce amplification signals similar to that of environmental stress, signaling that the root system is saturated with nematode population, potentially resulting in a switch in reproductive development, where more males are produced (Patel et al., 2008).

Future studies where selected developing M. incognita females are excised from the roots of transgenic overexpression lines could be used to test this hypothesis. The levels of transgene expression for Mi35F03, Mi4D01 and Mi5G05 without the signal peptide were shown to be highly-expressed (Figs 8-10). It is also possible that overexpression of Mi35F03 and Mi4D01 may potentially be toxic or act as avr genes within host plant cells, where their expression levels could trigger activation of a host defense pathway, or regulation of a host genetic element, resulting in deleterious effects on RKN development. A comparative analysis measuring the level of expression of host defense genes in infected versus uninfected tissues (Hamamouch et al., 2010, 2012) combined with protein-protein analyses could provide more
clues about Mi35F03 and Mi4D01 functionality during parasitism. Balanced gene expression or dosage is important for normal gene function (Lejeune et. al, 1959; Prelich, 2012). For example overexpression of the exoenzyme ExoS, a toxin important for *Pseudomonas* infectivity in humans, in Chinese hamster ovary cells showed inhibition of the ExoS toxin (Arnolodo et. al, 2008; Prelich, 2012). Constitutive expression of Mi35F03, Mi4D01 and Mi5G05 at high levels in inappropriate tissues could potentially cause deleterious effects on the nematode development (Kondou et. al, 2010). Furthermore an increase in the amount of target protein does not mean that the activity of that protein has increased (Prelich, 2012). The biological basis for the observed decrease in host susceptibility remains unclear. Further genomic and proteomic approaches such as, transcriptome analysis from infected host and yeast two-hybrid analysis could provide insight as to what was observed. Host localization studies of Mi35F03, Mi4D01 and Mi5G05 could also provide more insight on their role in nematode parasitism.

Plant host-derived RNAi was conducted to further investigate the potential roles of nematode parasitism genes Mi35F03, Mi4D01 and Mi5G05 in host response and susceptibility to nematode infection. Post-transcriptional gene silencing is a molecular process that is common to both plants and nematodes. Thus, plants expressing dsRNA complementary to the nematode transcripts can cleave dsRNA into small interfering RNAs (siRNAs) via the endoribonuclease, Dicer (Baulcombe, 2004). Nematodes feeding inside host-plant roots can potentially ingest the siRNAs through the feeding tube, potentially silencing the expression of endogenous nematode parasitism genes (Atkinson et. al, 2013; Fairbain et. al, 2007; Hamamouch et al., 2012; Patel et al., 2008, 2010; Sindhu et al., 2009;
Xue et al. 2013). The constitutive expression of dsRNA for, *Mi35F03, Mi4D01,* and *Mi5G05* via the CaMV35S promoter did not result in any significant morphological changes in root or shoot tissues. However, constitutive expression of dsRNA *Mi35F03* and *Mi5G05* led to significant suppression of RKN parasitism as indicated by significant differences observed in the number of galls/gram of root during infection assays, resulting in strong disruption of root-knot nematode parasitism. This suggests that Mi35F03 and Mi5G05 have essential functions in nematode parasitic interactions. Host-derived RNA of *Mi16D10* and *Mi8D05* have also resulted in significant disruption of nematode parasitism of host roots in three major species of root-knot nematode (Huang et. al, 2006; Xue et. al, 2013). The significant decreases observed in nematode infected plants expressing dsRNA to *Mi35F03* and *Mi5G05* could be due to lack of functional redundancy in expression of these parasitism genes during the various stages of nematode development and feeding site formation. Excision of nematodes from infected plants could be used to determine the level of gene silencing at various developmental stages via quantitative RT-PCR (Patel et al., 2008, 2010; Sindu et al., 2009; Xue et al., 2013). It is unclear why *Mi4D01* RNAi had little effect on *M. incognita* parasitism in the first test and provided a strong reduction in gall numbers in the second test. The relative expression level of *Mi4D01* dsRNA was low compared to *Mi35F03* and *Mi5G05* (Fig 17) and may have contributed to the variability observed between experiments. The Mi4D01 target transcript is considerably longer than the other two parasitism genes assayed and may contribute to overall efficacy in silencing. Use of high-throughput RNA sequencing to determine *Mi4D01* siRNA expression levels and species (Hamamouch et al., 2012) in transgenic Arabidopsis between experiments may also provide
insight into this variability in RNAi effect between experiments. In total, the significant
reductions in galls/gram of root in the RNAi lines and effects on parasitism in overexpression
lines suggests that secretion of Mi35F03, Mi4D01 and Mi5G05 play a role in promoting
nematode parasitic success.


Hewezi T., Howe P., Maier T.R., Hussey R. S., Mitchum M.G., Davis E. L, and Baum T.J. 2008. Cellulose binding protein from the parasitic nematode Heterodera schactii interacts
with Arabidopsis pectin methylesterase: cooperative cell wall modification during parasitism. The Plant Cell. 20: 3080-3093.


Figure 1. Life cycle of *Meloidogyne incognita*. A developed first-stage juvenile (J1) forms in the egg. The J1 molts within the egg, becoming a second-stage juvenile (J2) that hatches from the egg. The J2 penetrates the host root, forms specialized feeding cells (giant-cells), feeds and swells and develops rapidly through third and fourth juvenile stages (J3 and J4 respectively) to reproductive adults. A few vermiform adult males are formed that leave the root, but the majority develop into swollen adult females within the root and produce eggs in an egg mass. These egg masses protrude along the surface of the root. Reprinted with permission from APS Press.

Figure 2. Root-knot nematode feeding site (giant cell). Cross section of the vascular cells of a plant root modified into a multinucleate giant cell by a root knot nematode (Davis et. al, 2004). Reprinted with permission from Elsevier.
Figure 3. Illustration of the anterior end of the preparasitic second-stage juvenile (J2) and parasitic adult female life stages of the root-knot nematode *Meloidogyne incognita* (Davis et al., 2004). Note the differences among the life stages in the morphology and the activity of the subventral and dorsal esophageal gland secretory cells that connect to the oral stylet for the secretion of protein effectors. Reprinted with permission from Elsevier.
Figure 4. Illustration of the yeast two-hybrid used to identify protein-protein interactions. Association between two proteins, bait and prey, brings the transcriptional activation domain and DNA binding domain into proximity and transcription of the reporter gene occurs (Coates and Hall 2003).
Figure 5. Illustration of RNAi Pathway. Activation of RNAi is initiated by a dsRNA molecule. The dsRNA is recognized and cleaved by endoribonuclease III Dicer into siRNAs of 21-25nt in length with 3’ overhangs. The siRNAs associate with the RNA induced silencing complex (RISC). The siRNAs are unwound, activating RISC. Activated RISC complex associated with the unwound ssRNA binds to the target mRNA. The assembly of RISC and the target RNA leads to degradation of the target mRNA in a sequence specific manner. (Vaucheret et. al, 2001).
Table 1. Summary of three candidate parasitism genes encoding proteins for secretion and expressed exclusively within the esophageal gland cells of *Meloidogyne incognita*.

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Accession No.</th>
<th>CDS Sequence Size (bp)</th>
<th>Predicted Protein Accession No.</th>
<th>Predicted Protein Size</th>
<th>BlastP/Conserved Domain</th>
<th>Gland Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>35F03/msp30</td>
<td>AY142120</td>
<td>398/141</td>
<td>AAN52094.1</td>
<td>47 aa</td>
<td>Novel</td>
<td>...</td>
</tr>
<tr>
<td>4D01/msp3</td>
<td>AF531162</td>
<td>785/522</td>
<td>AAQ10017.1</td>
<td>174 aa</td>
<td>Ground-like domain</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pfam 0.4155</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38% identity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Haemonchus contortus</em></td>
<td></td>
</tr>
<tr>
<td>5G05/msp26</td>
<td>AY135362</td>
<td>977/813</td>
<td>AAN15806.1</td>
<td>271 aa</td>
<td>Peptidase M23;</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>leukocyte cell-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>derived chemotaxin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pfam 01551</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44% identity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Ascaris suum</em></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 6.** Illustration of the T-DNA region of the binary vector pB1121 containing the *Meloidogyne incognita* parasitism gene of interest. The β-galacturonase (GUS) gene was originally in the vector and was replaced with each *M. incognita* parasitism gene of interest. Expression of each cassette was confirmed in *Arabidopsis thaliana* transgenic homozygous lines in Figures 8-10. LB = left border; RB = right border; RKN PG = root knot nematode parasitism gene.

**Figure 7.** Illustration of pHANNIBAL construct (Wesley et al, 2001) to express hairpin double-stranded RNA (dsRNA) of the sense and antisense sequences of targeted nematode parasitism genes separated by the PDK intron. The T-DNA region of the binary vector pART27 containing the insert is under constitutive control of the CaMV 35S promoter. LB = left border; RB = right border; nptII = neomycin phosphotransferase gene (kanamycin resistance); Nos-terminator sequence.
Figure 9. Constitutive transcript expression of the *Mi4D01* (Accession No. AF531162) parasitism gene of *Meloidogyne incognita* without (-SP) and with (+SP) the predicted signal peptide for secretion in transgenic *Arabidopsis thaliana*. Relative RT-PCR confirming the expression of 4D01 -/+SP in three independent *Arabidopsis* homozygous lines including –RT as a negative control and expression of *Arabidopsis* actin 8 (Accession No. NM_103814) transcripts as a positive control. **Top:** Lane 1. 1 Kb ladder. Lane 3. Line 8. Lane 5. Line 8 –RT. Lane 7. Line 9. Line 9–RT. Lane 11. Line 18. Lane 13. Line 18 –RT. **Middle:** Lane 1. 1 Kb ladder. Lane 3. Line 5. Line 5 –RT. Lane 7. Line 11. Line 11 –RT. Lane 11. Line 12. Line 13. Line 12 –RT. **Bottom:** *Arabidopsis* actin expression. **Product size:** 4D01 –SP ~ 451 bp. 4D01 +SP ~ 525 bp. *Arabidopsis* actin control ~ 390 bp.
Figure 10. Constitutive transcript expression of the Mi5G05 (Accession No. AY135362) parasitism gene of Meloidogyne incognita without (-SP) and with (+SP) the predicted signal peptide for secretion in transgenic Arabidopsis thaliana. Relative RT-PCR confirming the expression of 5G05 -/+SP in three independent Arabidopsis homozygous lines including –RT as a negative control and expression of Arabidopsis actin 8 transcripts (Accession No. NM-103814) as a positive control.

Figure 11. Root phenotypes of *Mi35F03*-SP, *Mi4D01*-SP and *Mi5G05*-SP Arabidopsis overexpression lines compared to wild-type (Col-0) controls (right side of each plate) at 12 days post germination. Significant differences in root length for *Mi35F03*-sp and *Mi4D01*-sp were observed for these lines with p-value < 0.05.

Figure 12. Root length of transgenic Arabidopsis plants that constitutively express the Mi35F03 parasitism gene +/- the predicted signal peptide for secretion. The average root lengths of three independent lines and standard error are compared to wild-type (WT) plants at 5, 7, 9, and 11 days post germination. Significant differences (*) in root length compared to WT (n=10, P < 0.05).
Figure 13. Root length of transgenic Arabidopsis plants that constitutively express the Mi4D01 parasitism gene +/- the predicted signal peptide for secretion. The average root lengths of three independent lines and standard error are compared to WT plants at 5, 7, 9, and 11 days post germination. Significant differences (*) in root length compared to WT (n=10, P < 0.05).

Figure 14. Root length of transgenic Arabidopsis plants that constitutively express Mi5G05 parasitism gene +/- the predicted signal peptide for secretion. The average root lengths of three independent lines and standard error are compared to WT plants at 5, 7, 9, and 11 days post germination. There were no significant differences in the root length of Mi5G05 +/- sp lines compared to WT (n=10, P < 0.05).
Figure 15. Infection assay of overexpression transgenic Arabidopsis thaliana plant roots 6 weeks post-inoculation by Meloidogyne incognita (galls/gram of root). There were significant differences in the galls/gram on transgenic Arabidopsis homozygous lines 6 weeks post-inoculation. Significant difference in galls/gram of root on transgenic lines expressing Mi35F03+/−SP and Mi4D01+/−SP compared to wildtype and GUS controls (P ≤ 0.05). Asterisks (*) indicate significant differences; n=10. (t-test, P ≤ 0.05 versus control)
Figure 16. A repeat infection assay of overexpression transgenic Arabidopsis thaliana plant roots 6 weeks post-inoculation by Meloidogyne incognita (galls/gram of root). There were significant differences in the galls/gram root on transgenic Arabidopsis homozygous lines 6 weeks days post-inoculation. There was a significant difference in galls/gram of root for Mi35F03+/−SP and 4D01+/−SP compared to wildtype and GUS controls (P ≤ 0.05). Asterisks (*) indicate significant differences; n=10. (t-test, P ≤ 0.05 versus control)
Figure 17. Expression of hairpin double-stranded RNA (dsRNA) in transgenic Arabidopsis plants as measured by relative RT-PCR of the PDK intron sequence (291 bp) of the pHANNIBAL construct (Wesley et al., 2001) designed to induce RNAi-mediated silencing of target transcripts. Expression of dsRNA complementary to transcripts of the Mi35F03, Mi4D01 Mi5G05 parasitism genes of Meloidogyne incognita are demonstrated in three independent transgenic lines of Arabidopsis thaliana. Controls include transgenic Arabidopsis that express dsRNA (RNAi) complementary to green fluorescent protein (GFP) used as a positive control and reverse transcriptase (-RT) treatment of extracted template RNA.
Figure 18. Infection assay of RNAi transgenic *Arabidopsis thaliana* plant roots 6 weeks post-inoculation by *Meloidogyne incognita*. There were significant differences in the number of galls/gram of on transgenic *Arabidopsis* homozygous 6 weeks post-inoculation. There was a decrease in the rate of infection for Mi35F03 lines 1, 3 and 7 and Mi5F05 lines 2, 3 and 4 expressing dsRNA, compared to wild-type and GFP controls (P ≤ 0.05). There was a significant increase observed in the rate of infection for Mi4D01 lines 3, 4 and 6 expressing dsRNA (P ≤ 0.05) compared to wildtype. n=10. (t-test, P ≤ 0.05 versus control)
Figure 19. A repeat infection assay of RNAi transgenic Arabidopsis thaliana plant roots 6 weeks post-inoculation by Meloidogyne incognita. There were significant differences in the galls/gram root on transgenic Arabidopsis homozygous lines 6 weeks post-inoculation. There was a decrease in the galls/gram root in Mi35F03, Mi4D01 and Mi5G05 lines expressing dsRNA (P ≤ 0.05) compared with wildtype and GFP-RNAi controls. n=10. (t-test, P ≤ 0.05 versus control)