Identification and characterization of effectors secreted from sedentary endoparasitic phytonematodes

by

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TABLE OF CONTENTS

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ABSTRACT

Sedentary endoparasitic phytonematodes are a group of taxa comprised of cyst nematodes (*Heterodera* and *Globodera spp*.) and root-knot nematodes (*Meloidogyne spp*.), which are some of the most economically important crop pathogens on earth. These pests infect plant roots by creating elaborate feeding sites around the vasculature, which diverts nutrients away from the plant to feed the nematode and causes yield reduction in the plant. The nematodes create their feeding sites by delivering effector proteins into plant tissues. Effectors interact with plant components to modify development, metabolism, and defense pathways within plant cells, ultimately forming and maintaining the feeding site within the host root.

In order to find ways to mitigate the damages caused by these nematode pathogens, it is vital to identify nematode effectors and understand how effector proteins are able to manipulate the plant host. This dissertation first summarizes what is currently known about nematode effector proteins and then contributes to that body of knowledge. Our data show that two effectors from *Heterodera schachtii* (*Hs*4E02 and *Hs*25A01) are likely to function in the plant-nematode interaction by binding to plant proteins. Through the preparation and mining of gland transcripts we have also identified 18 additional putative effectors that are expressed specifically within the esophageal gland cells of *Meloidogyne incognita* during infective life stages. Two of these putative effectors are part of the major avirulence protein (MAP) family of effectors. Interestingly, we were able to identify conserved amino acid motifs within this effector family that resemble plant CLAVATA3/ESR (CLE) signaling peptides found in plants and cyst nematodes. These data indicate that the MAP effector

family from root-knot nematodes may manipulate plant developmental signaling in a manner analogous to the CLE effectors secreted from cyst nematodes.

CHAPTER 1. AN INTRODUCTION TO SEDENTARY ENDOPARASITIC PHYTONEMATODES

The phylum *Nematoda* contains over 25,000 described species. This makes it one of the most diverse phylums of metazoans, second only to *Arthropoda*. A large proportion of nematode species are parasites of either animals or plants. The annual yield loss attributed to plant-parasitic nematodes has been estimated to be in excess of 100 Billion U.S. dollars (Koenning, Overstreet et al. 1999), which makes them one of the most devastating groups of agricultural pests.

The etiology of plant-parasitic nematodes is quite diverse. Though most species feed on the roots of the plant, there are several species that parasitize the aerial portions of plants as well. The root parasites are classified by their mode of infection. Species are either ectoparasites (feed from outside the host root) or they are endoparasites (boring into and feeding from inside the host root). At the same time, the parasites are classified as either mobile (migrating while feeding) or sedentary (stationary while feeding). By far the most economically important group of plant parasitic nematodes are the sedentary endoparasites (cyst nematodes and root-knot nematodes) (Sasser and Freckman 1986; Koenning, Overstreet et al. 1999; Chitwood 2003), and consequently a majority of the research on the nematode-plant interaction has also focused on this group. This literature review specifically concentrates on the interaction between the sedentary endoparasitic nematodes and their host plants.

As a group, sedentary endoparasitic nematodes are defined as plant-parasitic nematodes that bore into a host plant root and establish an elaborate feeding site. This feeding site sustains these nematodes throughout their sedentary lifecycle. This group of

parasites is composed of two taxonomic groups, commonly referred to as the cyst nematodes (*Heterodera* and *Globodera* spp.) and root-knot nematodes (*Meloidogyne* spp.). Though root-knot and cyst nematodes share similar modes of infection, it is thought that the sedentary endoparasitic lifestyle evolved separately in the two taxonomic groups (Holterman, Karssen et al. 2009). Because of their unique evolutionary paths, it isn't surprising that the two groups exhibit very distinct morphology and infection etiology.

Root-Knot Nematodes (*Meloidogyne spp*.)

The genus *Meloidogyne* is composed of 97 described species as of June 2009 (Koenning, Overstreet et al. 1999; Trudgill, Bala et al. 2000; Trudgill and Blok 2001; Perry, Moens et al. 2009). These species are distributed worldwide and are considered to be some of the most devastating crop pathogens on earth (Abad and Williamson 2010). One of the reasons *Meloidogyne spp*. are so successful and prolific is that they have an enormous range of potential host plants. Indeed, it is thought that one of the prolific species, *Meloidogyne incognita*, has a host range that exceeds 3,000 plant species (Ehwaeti, Fargette et al. 1999). Taken as a group, the *Meloidogyne* genus is thought to infect nearly every flowering plant (Trudgill and Blok 2001). This expansive host range allows *Meloidogyne* species to overcome the classical crop rotation strategies used to combat many other soil borne pathogens. In the past, fumigation was used to try and control these pests. However, many of the nematicides successfully used in the past have been removed from the market in recent years due to environmental concerns. As a result, controls strategies have increasingly relied on a limited number of resistance genes such as Mi-1. Unfortunately, *Meloidogyne* species have shown the ability to overcome these resistance genes, particularly when they are

overused (Castagnone-Sereno 2002; Meher, Gajbhiye et al. 2009). The destructive and obdurate nature of *Meloidogyne* infestation makes it important that researchers understand how these pests infect their plant hosts.

Infection and life cycle

All *Meloidogyne* species begin their life cycle when they hatch from eggs in the soil surrounding a plant root. The parasites emerge as second-stage pre-parasitic juveniles (pre-J2), having already completed one molting within the egg. The newly hatched vermiform juveniles use plant exudates as chemotactic signals to migrate to the root tip of a potential host plant. Once at the root tip, a pre-J2 will penetrate the host near the zone of elongation. Unlike cyst nematode species, the J2 will migrate intercellularly slipping between the cell walls of adjacent cells. Because of this unique mode of migration, little root damage can be observed as the nematode travels through the root (Gravato Nobre, Von Mende et al. 1995; Mende 1997). Once within the root of the host plant the nematodes first migrate to the region near the root tip, where they proceed to make a 180 degree U-turn into the vascular cylinder of the root (Wyss, Grundler et al. 1992; Mende 1997). It has been speculated that this circuitous migration pattern allows the nematode to avoid the casparian strip, which may pose a significant barrier to the nematode's migration.

Once inside the vascular cylinder of a host root, the J2 migrates back up the root until it reaches a group of cells that it will use to form a feeding site. The signals and mechanisms used by the nematode to choose these specific plant cells are still not well understood. However, it is well documented that the juvenile nematode chooses a group of procambial cells near or directly adjacent to the sieve elements of the xylem (Hoth, Stadler et al. 2008).

To begin the process of forming the feeding site, the J2 nematode uses a hollow protrusible mouth spear (a stylet) to pierce the outer plant cell wall and invaginate the cell membrane. The stylet is a direct extension of the nematode's esophagus and functions similarly to a hypodermic needle, conducting a flow of effector proteins from the esophagus into the host plant's tissues (Hussey 1989). Using its stylet, the parasite injects 5-10 procambial cells with a cocktail of proteins. The cells that receive these injections soon begin to enlarge and take on unique morphological characteristics. Ultimately, they form the giant-cells (GC), which make up the feeding sites of all *Meloidogyne* species (see Giant-Cell Morphology and Physiology). In addition to the drastic morphological changes that take place within the GC, *Meloidogyne* species also cause systemic hypertrophic growth in the surrounding root tissue. This increased growth causes a gall to form around the growing nematode. This characteristic "root-knotting" can cause extensive damage in root crops; deforming the roots to a point where market value is reduced (Coyne, Tchabi et al. 2006). The growing nematode spends the remainder of its sedentary life cycle within the gall feeding from the GC.

In order to feed from a GC, the nematode will reinsert its stylet and will produce a long feeding tube that protrudes into the cytoplasm of the GC. Electron micrographic images indicate that the feeding tube extends from the end of the stylet opening and into the GC cytoplasm. It appears to be a crystalline structure that may polymerize from stylet secretions, which are as of yet unidentified (Sobczak, Golinowski et al. 1999; Mitchum, Hussey et al. 2013). Though the exact constituents of the feeding tube are not yet known, it has been shown experimentally that the feeding tube creates a size barrier for molecules ingested by the nematode. Allowing only molecules under 40kDa to pass into the nematode's esophagus (Hussey and Mims 1991; Mitchum, Hussey et al. 2013). In *Meloidogyne* species the multiple

discarded feeding tubes can be seen within each giant-cell, indicating that the parasite creates a new feeding tube each time it probes a cell and discards it after feeding. Researchers have postulated that the feeding tube functions to prevent clogging of the stylet while the nematode feeds from the cytoplasm of the GC.

Sedentary J2 nematodes continue to feed off of the GCs for many days, and subsequently begin to enlarge and soon will proceed to go through three consecutive molts. Within a matter of hours it will progress through the J3 and J4 stages to become a reproductive adult. During the relatively short J3 and J4 stages, sexual dimorphisms become apparent. Females will form extensive ovaries, a vagina, and rectal glands, taking on a rotund pear-like shape. Males recapitulate a vermiform shape, albeit with a mass several times that of the J2 worms. The vermiform males will leave the root tissues, and in sexually reproducing species such as *M. hapla,* they will seek out and mate with the adult females. However, many species of *Meloidogyne* are asexual. In the case of these obligately parthenogenic species, such as *M. incognita*, males exit the host root but don't successfully mate with females (Rohini, Ekanayake et al. 1986; Trudgill and Blok 2001).

Once females have reached sexual maturity, they begin to secrete a gelatinous matrix from glands in their rectum (Maggenti and Allen 1960). The components of the this matrix have cellular lysis activity, which induces the formation of a pore through the surrounding gall tissue (Orion 1987; Orion and Franck 1990). Adult females deposit their eggs within the gelatinous matrix to create an egg mass, which flows out of the pore and into the rhizosphere. In addition to forming the pore, components of the gelatinous matrix have been shown to have anti-microbial activity, and will protect the egg mass in the soil environment until the new J2 nematodes hatch.

The length of the life cycle for *Meloidogyne* spp. varies depending on the species, as well as the environmental conditions. In the case of *M. incognita*, perfect conditions can produce reproductive cycles as short as 20 days from egg to egg. This short life-cycle allows multiple generations of nematodes to re-infect host plants multiple times and can result in increasing infection loads over the course of a growing season (Rohini, Ekanayake et al. 1986).

Giant cell morphology and physiology

The GCs induced by *Meloidogyne* spp. are drastically different, both morphologically and physiologically, from the procambial cells they develop from. After the nematode secretes its effector cocktail, the first and most apparent morphological change within these cells is the observation of successive nuclear divisions without cytokinesis, resulting in multiple nuclei within each cell (Huang 1985). Soon the cells begin to hypertrophy, eventually increasing to many times their original volume and containing as many as 100 lobed nuclei. Other overt morphological changes are apparent: the central vacuole dissolves into many smaller vesicles and the cell wall thickens and produces extensive ingrowths, which are particularly prolific on the side adjacent to the xylem tissue (Huang 1985). These ingrowths have been likened to the folding structures present in transfer cells (Bird 1961; Jones and Northcot.Dh 1972; Hoth, Stadler et al. 2008), and are thought to increase the surface area available for nutrient transfer from the vasculature. At the same time, root-knot nematode infections also have systemic effects on the surrounding root tissue. Hypertrophic cell division in the tissue around the GCs produces a gall. Detailed anatomical analysis of this gall tissue has revealed that it contains nucleated sieve elements that adjoin the GCs

(Hoth, Stadler et al. 2008). Since the GCs are symplastically isolated, these sieve elements are presumed to provide nutrients that can be absorbed through the apoplast of the GCs.

The morphological changes in the feeding site are accompanied by physiological changes as well. Early histochemical studies revealed that the GCs display marked amylase and invertase activity along with a distinct lack of starch compared to uninfected tissues(Orion and Bronner 1973). More recent transcriptomic analysis of GCs has revealed that their gene expression patterns are drastically different from uninfected cells. Barcala et. al. showed that the transcriptome of GCs induced in Arabidopsis showed similarities to differentiating xylem cells, as well as the crown galls induced by *Agrobacterium tumefaciens* (Barcala, Garcia et al. 2010). Another recent study has found that a number of specific metabolic pathways are differentially regulated during GC formation (Ibrahim, Hosseini et al. 2011). These pathways included cell cycle regulators, cell wall remodeling enzymes, glycolysis and gluconeogenesis, as well as plant defense response genes. Furthermore, it has been found that 26 different membrane transporter genes are significantly up-regulated in GCs (Hammes, Schachtman et al. 2005), which is consistent with the idea that GCs are symplastically isolated and must import nutrients from the apoplast.

Together, this body of evidence paints an elaborate picture of the feeding site produced by *Meloidogyne* spp, one that depicts the GCs and surrounding gall tissue as a specialized organ that diverts nutrients away from the plant to feed the growing nematode.

Cyst Nematodes (*Heterodera* and *Globodera* spp.)

Cyst nematodes are exceptionally devastating pathogens of crop plants worldwide (Koenning, Overstreet et al. 1999; Chitwood 2003). Species from both *Heterodera* and

Globodera infect a wide range of host plants. However, individual species display a relatively narrow host specificity, particularly when compared to root-knot nematodes (Abad and Williamson 2010). The relatively narrow host range of cyst nematodes seems to be compensated for by their extreme survivability in the soil; eggs can remain dormant for many years waiting for a suitable host plant. This degree of persistence makes control of these pests difficult when using crop rotation strategies (Fleming and Powers 1998). The most effective means of control has been through the use of resistant varieties of crop plants. However, integrating resistance genes into multiple crops has proven to be a challenge, and specific field populations have been known to overcome resistance genes (Dale and Phillips 1982; Lilley, Atkinson et al. 2005). Although the life cycle of cyst nematodes is similar to root-knot nematodes species, there are distinct differences in how they infect as well as in the feeding site they create.

Infection and life cycle

As in root-knot nematode species, all cyst nematodes emerge from the egg as J2-stage nematodes, after being stimulated to hatch by host root exudates. The J2 nematodes migrate to the host root using the plant exudates as chemotactic factors (Masamune, Anetai et al. 1982). Once they reach the root, the nematodes tend to preferentially penetrate the root at the zone of elongation, boring through the walls of host cells. Unlike root-knot nematodes, cyst nematodes collapse the cytoplasm of each plant cell as they migrate to the vascular cylinder, causing significant damage to the host root (Wyss 1986; Golinowski, Sobczak et al. 1997). Once in the vascular cylinder, the nematode selects a procambial cell adjacent to the xylem to be the initial feeding site cell. The nematode then uses its stylet like a hypodermic needle; it

carefully pierces the cell wall, invaginates the underlying membrane, and secretes a cocktail of effector proteins (Hussey 1989). The secretion of this effector cocktail into plant tissues initiates the development of the nematode's syncytial feeding site (see syncytium morphology and physiology). Upon initiation of the syncytium, the nematode produces a feeding tube very similar to that produced by root-knot nematodes. This structure extends into the syncytium and acts as a molecular sieve through which the nematode ingests the symplastic contents (Bockenhoff and Grundler 1994; Sobczak, Golinowski et al. 1999). The nematode feeds from the growing syncytium; undergoing three successive molts to become a sexually mature adult. Unlike many root-knot nematode species, cyst nematode species reproduce sexually. Female cyst nematodes feed from the syncytium all the way through adulthood. They grow drastically taking on a rotund body shape that eventually forces its way out of the root, exposing the posterior of the female to the surrounding rhizosphere. By contrast, male cyst nematodes only feed through the J3 life-stage, after which they undergo two successive molts to become a much larger sexually mature vermiform. The mature male exits the plant root to mate with exposed females. The uterus of the adult female fills with fertilized eggs, and she eventually dies. The tough outer cuticle persists, forming the 'cyst' that typifies the group. The cyst forms a protective sheath around the eggs inside. Many of the eggs will hatch within a few days, allowing the nematode to have multiple infection cycles over the course of a growing season. However, some cyst eggs will remain dormant to wait for future growing seasons. These eggs can remain viable within the soil for up to twenty years waiting for the presence of a viable host to repeat the infection cycle (Lilley, Atkinson et al. 2005).

Syncytium morphology and physiology

The syncytium produced by cyst nematodes has some distinct differences compared to the GCs produced by root-knot nematodes. After the nematode injects its effector proteins, the initial syncytial cell begins to exhibit hypertrophic growth. The cell walls of adjacent cells begin to break down, and their cytoplasms fuse with the initial cell. This process continues until several hundred cells become fused into one continuous symplast directly adjacent to the vasculature tissue (Jones and Northcote 1972; Jones 1981). The cytoplasm of the mature syncytium becomes dense and highly metabolically active, with proliferation of mitochondria, free ribosomes, and smooth endoplasmic reticulum. Other subcellular changes also become apparent. The Vacuole is broken down into smaller vesicular bodies (Golinowski, Grundler et al. 1996; Golinowski, Sobczak et al. 1997). Nuclear morphology also changes dramatically; multiple rounds of DNA endoreduplication in the absence of karyokinesis results in increased nuclear and nucleolar size (Niebel, de Almeida Engler et al. 1996; Goverse, de Engler et al. 2000). Eventually, the cell wall around the syncytium thickens, and in-growths similar to those seen in GCs begin to appear (Jones and Northcote 1972). In contrast to GCs, it is clear that the syncytium is symplastically connected to the vasculature tissue (Hoth, Schneidereit et al. 2005; Hoth, Stadler et al. 2008), indicating that cyst and *Meloidogyne* species have very different strategies for extracting nutrients from their host plant into the feeding site. In female cyst nematodes, the syncytium will continue to expand as the nematode progresses through the J3 stage of development, incorporating several hundred root cells surrounding the vasculature. After the J3 stage, the feeding site will maintain a stable volume through the remainder of the female nematode's lifecycle.

Males produce smaller syncytia that only last through the J3 life stage, when the male exits the root.

As in GCs, the morphological changes are accompanied by drastic changes in physiology. This has been highlighted in recent gene expression studies that have isolated and the syncytial cytoplasm. Numerous differentially expressed genes have been identified within the syncytium when compared to uninfected root cells (Ithal, Recknor et al. 2007; Klink, Overall et al. 2007; Szakasits, Heinen et al. 2009). Gene ontology analysis has revealed that a significant number of these differentially expressed genes are associated with metabolic activities and defense responses. Interestingly, it was also found that there are genes expressed within the syncytium that are not normally expressed in root tissues, such as those involved in pollen and seed development (Szakasits, Heinen et al. 2009). This data suggests that the nematode causes drastic shifts in the normal physiology of root cells during the formation of the syncytium.

Effector Proteins Secreted By Cyst and Root-Knot Nematodes

Hogenhout defines effectors as any molecule secreted by a parasite that "alter host-cell structure and function" (Hogenhout, Van der Hoorn et al. 2009). In the past decade, there has been a huge increase in the number of secreted proteins identified from plant-parasitic nematodes. The vast majority of these putative effectors have been isolated from the three esophageal gland cells, which secrete proteins directly into the esophagus and out the stylet (Gao, Allen et al. 2001; Gao, Allen et al. 2003; Huang, Gao et al. 2003). Only a few of these secreted proteins have confirmed or even putative effector functions *in planta*. Despite the

limited scope of our understanding, a picture of how these proteins function to alter host plants is beginning to emerge.

Despite differences in the etiology and evolutionary history of cyst and root-knot nematodes, the effector repertoires secreted by these two groups do show some similarities. In fact, there are some effector families that are found in both cyst and root-knot species. At the same time, there are also certain effectors that are only found in either root-knot or cyst nematodes, and still others that seem to be confined to only certain species in either group (summarized in Table 1). Despite these differences, the nematode effectors characterized to date can generally be divided into groups based on their function *in planta*: cell wall modifying effectors, defense suppressing effectors, and development altering effectors.

Cell wall modifying effectors

The cell wall modifying effectors are currently the largest class of defined nematode effectors. This is largely because they contain detectable enzymatic domains that are not normally found in metazoans, making them relatively easy to identify. Interestingly, bioinformatics analysis has shown that most of these cell wall associated effectors have arisen from multiple horizontal gene transfers from prokaryotes (Bird, Scholl et al. 2003; Danchin, Rosso et al. 2010; Paganini, Campan-Fournier et al. 2012). All of these enzymes are used by the nematode to mechanically disrupt the plant cell wall during nematode migration, or to allow for the morphological changes that take place within the feeding site.

Multiple classes of cell wall modifying enzymes are known to be secreted from the esophageal gland cells, or are present within the nematode's genome. Endoglucanases (Smant, Stokkermans et al. 1998; Bera-Maillet, Arthaud et al. 2000), Expansins (Qin, Kudla

et al. 2004; Danchin, Haegeman et al. 2011), and Pectate Lyases (Kudla, Milac et al. 2007; Vanholme, W et al. 2007; Danchin, Rosso et al. 2010) have been identified in both cyst and root-knot nematodes. Additionally, investigations of the genomes of root-knot nematodes have facilitated the identification of polygalacturonases and xylanases that as of yet have not been found in cyst nematodes (Abad, Gouzy et al. 2008; Opperman, Bird et al. 2008; Danchin, Haegeman et al. 2011).

In addition to enzymatic cell wall modifiers, other proteins that contain cellulose binding domains have been identified from both cyst and root-knot nematodes (Ding, Shields et al. 1998; Adam Mohamed A. M. 2008; Hewezi, Howe et al. 2008), and have been shown to have functions *in planta*. One such protein, secreted from *Heterodera schachtii,* was shown to bind to a pectin methylesterase. When expressed *in planta*, this effector caused increased susceptibility to nematode infection and increase root growth (Hewezi, Howe et al. 2008). These data indicate that this cellulose-binding effector likely functions by facilitating the modification of plant cell walls through its interaction with a plant pectin methylesterase. The presence of this large and conserved class of cell wall modifying effectors indicates that these types of modifications are important in facilitating the nematode's ability to infect its host plant.

Defense suppressing effectors

Plants are continuously assailed by pathogens from their surrounding environments, and have evolved multiple layers of defense to sense and resist being infected by these pests. At the same time, successful biotrophic pathogens have evolved effectors that suppress the

host plant defenses (Hogenhout, Van der Hoorn et al. 2009). Unsurprisingly, multiple effectors from both cyst and root-knot nematodes have been shown to subvert plant defenses.

One such effector (10A06) is secreted from *Heterodera schachtii*, and has been shown to interact with spermidine synthase *in planta* (Hewezi, Howe et al. 2010). This interaction is thought to alter polyamine synthesis, thereby reduce the host plant's ability to produce defense associated compounds such as salicylic acid. Another effector from *H. schachtii* (30C02) binds and inhibits the pathogenesis related protein β-1,3-endoglucanase and increases host susceptibility to nematode infection (Noureddine Hamamouch 2012). Another cyst nematode, *Globodera rostochiensis*, secretes a venom allergen-like protein (Gr-VAP1) which interacts with the papain-like cysteine protease $Rcr3^{pim}$, a protein known to be involved in plant defenses (Lozano-Torres, Wilbers et al. 2012). Yet another effector secreted by *Globodera spp.*, SprySec19, interacts directly with a plant resistance protein, yet doesn't elicit a defense response (Rehman, Postma et al. 2009). This raises the intriguing possibility that SprySec19 inhibits the R-protein's function. These nematode effectors are the only nematode effector proteins to date that are known to directly target defense machinery.

However, many other secreted proteins have been identified that are likely to play a role in suppressing host plant defenses. Superoxide dismutases have been identified in the secretions from both cyst and root-knot nematodes (Robertson, Robertson et al. 1999; Bellafiore, Shen et al. 2008). It is hypothesized that these proteins may blunt the plant defense response by acting as antioxidants to counteract the reactive oxygen species produced by the plant. Similarly, root-knot nematode species secrete glutathione-s-transferases, which are thought to detoxify anti-microbial compounds produced by the plant (Dubreuil, Magliano et al. 2007). Nearly all plant-parasitic nematodes also secrete chorismate

mutase, a key enzyme in the plant shikimate pathway (Bekal S 2003; Doyle and Lambert 2003; Huang, Dong et al. 2005; Vanholme, Kast et al. 2009). These effectors are thought to alter the shikimate metabolic pathway, thereby disrupting salicylic acid production and defense signaling. Another effector (4F01) secreted from *Heterodera spp.* shows sequence similarity to plant annexins and has even been shown to complement endogenous plant annexins *in planta* (Patel, Hamamouch et al. 2010). Since annexins are known to function in plant stress responses, it is thought that this effector directly modulates these same stress response pathways during nematode infection. Still other secreted effectors from cyst nematodes are homologous to ubiquitin-associated proteins (Gao, Allen et al. 2003; Tytgat, Vanholme et al. 2004; Jones, Kumar et al. 2009). Though these proteins have not yet been shown to function *in planta*, recent studies have identified effectors with similar domains in both bacterial and oomycete pathogens that target plant resistance machinery proteins for degradation (Gohre, Spallek et al. 2008; Birch, Armstrong et al. 2009). This raises the intriguing possibility that nematodes may have evolved similar effectors to targets analogous defense machinery.

Though the list of nematode effectors with confirmed roles in defense suppression is still short, the prevalence of these types of effectors in other plant pathogens, and the large number of uncharacterized effectors in nematodes, indicates that there are likely to be more effectors added to this list in the future.

Development altering effectors

Nematodes profoundly alter the morphology and physiology of the root cells they infect (see giant-cell and syncytium sections), it is therefore not so surprising to find that

certain nematode effectors have been shown to alter plant developmental pathways. One such effector (19A07) is secreted from *Heterodera schachtii*, interacts with the auxin transporter LAX3 *in planta*, and is thought to alter auxin influx into the syncytium (Lee, Chronis et al. 2011). Since auxin signaling has been implicated in syncytium formation (Grunewald, Cannoot et al. 2009), it is reasonable to think that 19A07 could play a role in transporting auxin into the developing feeding site.

Evidence is accumulating that plant-parasitic nematodes also manipulate other plant developmental pathways. It has recently become apparent that cyst nematodes secrete effector proteins that mimic the 12- and 13-amino acid CLAVATA3/ESR (CLE) signaling peptides from plants (Olsen and Skriver 2003). These CLE mimics have been shown to interact with receptor-like kinases within plants, and subsequently initiate developmental changes (Wang, Mitchum et al. 2005; Lu, Chen et al. 2009; Replogle, Wang et al. 2011; Wang, Replogle et al. 2011). More recently, other small secreted proteins have been identified in root-knot nematodes which may mimic plant IDA signaling peptides (Tucker and Yang 2013). It is increasingly apparent that multiple groups of small signaling peptides play important roles in plant development (Stahl and Simon 2012). Because of their importance in plant development and their small size, it is possible that nematodes still contain many other small plant peptide mimics that have yet to be discovered.

Another small effector (16D10) from root-knot nematodes has been shown to play a very different role in altering plant development. It has been shown that 16D10 interacts *in planta* with a SCARECROW-like transcription factor, and when expressed in plants it drastically increases root growth (Huang, Dong et al. 2006). This evidence indicates that 16D10 likely functions to promote the development of the GCs.

Some other effectors that disrupt defense pathways (Doyle and Lambert 2003; Hewezi, Howe et al. 2010) are also known to alter root growth when expressed *in planta.* This highlights that there is signaling cross that occurs between developmental and defense pathways in plants, and raises the possibility that some effectors could have dual roles in altering both defense and development during nematode infection.

Effectors with unknown functions

The vast majority of proteins secreted from plant-parasitic nematodes still have no known or putative function within the host. This lack of information is largely due to the unique nature of these proteins, which tend to have few or no homologs in other species (Gao, Allen et al. 2001; Gao, Allen et al. 2003; Huang, Gao et al. 2003). These 'pioneers' require *ab initio* scientific approaches, making it difficult to determine how they function in the plant-nematode interaction. Still other effectors are known to have a function in plant parasitism, yet their exact mechanism of functionality is still a mystery. Two of these effectors are MAP-1 and the putative Cg-1 from root-knot nematodes. Both of which have been isolated as potential avirulence factors against the Mi-1 resistance gene in tomato (Semblat, Rosso et al. 2001; Gleason, Liu et al. 2008). Both of these genes have been confirmed to have effects on nematode parasitism, and the *MAP-1* gene has even been shown to be part of a family of secreted proteins within root-knot nematodes (Castagnone-Sereno, Semblat et al. 2009; Tomalova, Iachia et al. 2012). Yet neither of these proteins, or their family members, have been attributed a function. Studies in this dissertation hypothesize a function for the MAP-1 effector family, but future studies will be needed to confirm the function of these proteins and others like them.

It is also worth noting that there are other factors that likely contribute to virulence of these nematodes *in planta.* To date, the majority of known effectors are expressed within the esophageal gland cells of plant parasitic nematodes. However, this doesn't mean that there aren't other proteins from other organs that alter the host plant and facilitate nematode parasitism. Indeed, a protein isolated from the outer cuticle surface of *Globodera pallida* was shown to bind the precursors of plant defense compounds (Prior, Jones et al. 2001). It is not unreasonable to think that other proteins that come into contact with plant tissues, such as those secreted from the rectal glands or phasmids of the nematode, may function to alter the plant host and increase nematode virulence.

After appreciating the wealth of knowledge that has been accumulated on nematode effector proteins over the past decade, it is clear that these are very complex plant-pathogen interactions. Much more research will be needed in order to understand how all of these effectors work in concert to facilitate nematode parasitism.

Dissertation Organization

This dissertation is composed of four independent journal papers (chapters 2 through 5); each contains its own introduction, results, and discussion sections, with accompanying figures and references at the end of each chapter. The author of the dissertation was responsible for the majority of the research as well as the writing of each paper. Chapter 1 is a general introduction to sedentary endoparasitic nematodes and the previous research preformed on their effector proteins. Chapter 6 presents general conclusion, and relates the data from this dissertation to the general knowledge of plant-nematode interactions, and states how it could be useful in the future.

Table 1. A compiled list of effector proteins from both root-knot (*Meloidogyne* spp.) and cyst nematodes (*Globodera and Heterodera* spp.) that have confirmed or putative functions in the plant-nematode interaction.

Effector	Species	Functions	Research
Major Avirulence Proteins (MAP-1)	*Meloidogyne spp.*	Early interactions between J2 and host plant. May trigger *Mi*-1 mediated resistance in tomato	(Semblat, Rosso et al. 2001; Castagnone-Sereno, Semblat et al. 2009; Tomalova, Iachia et al. 2012; Rutter et al. unpublished)
Endoglucanases	*Heterodera*, *Globodera* and *Meloidogyne spp*.	Cellulose degradation	(Smant, Stokkermans et al. 1998; Bera-Maillet, Arthaud et al. 2000; Danchin, Rosso et al. 2010)
Pectate Lyase	*Heterodera*, *Globodera* and *Meloidogyne spp*.	Pectate degradation	(Kudla, Milac et al. 2007; Vanholme et al. 2007; Danchin, Rosso et al. 2010)
Chorismate mutases	*Heterodera*, *Globodera* and *Meloidogyne spp.*	Disruption of shikimate pathway and salicylic acid signaling	(Bekal S 2003; Doyle and Lambert 2003; Huang, Dong et al. 2005; Vanholme, Kast et al. 2009)
Polygalacturonases	*Meloidogyne spp.*	Galacturonan degradation	(Abad, Gouzy et al. 2008; Opperman, Bird et al. 2008; Danchin, Haegeman et al. 2011)
Xylanases	*Meloidogyne spp.*	Xylan degradation	(Abad, Gouzy et al. 2008; Danchin, Haegeman et al. 2011)
Cellulose binding proteins	*Heterodera*, *Globodera* and *Meloidogyne spp.*	Cell wall modifications	(Ding, Shields et al. 1998; Adam Mohamed A. M. 2008; Hewezi, Howe et al. 2008)
Expansins	*Heterodera*, *Globodera* and *Meloidogyne spp.*	Loosening of cell wall components	(Qin, Kudla et al. 2004; Danchin, Haegeman et al. 2011)
Proteases	*Heterodera*, *Globodera* and *Meloidogyne spp.*	Degradation of host proteins (Unconfirmed)	(Vanholme, Mitreva et al. 2006; Jones, Kumar et al. 2009; Vieira, Danchin et al. 2011)

Table 1 (continued)

Effector	Species	Functions	Research
Glutathione-S-Transferases	*Meloidogyne spp.*	Detoxification of plant defense compounds (Unconfirmed)	(Dubreuil, Magliano et al. 2007)
superoxide dismutase	*Globodera* and *Meloidogyne spp.*	Antioxidation (Unconfirmed)	(Robertson, Robertson et al. 1999; Bellafiore, Shen et al. 2008)
Gp-RBP-1 (aka SPRYSEC)	*Globodera spp.*	Triggers plant resistance through the Gpa2 protein.	(Sacco, Koropacka et al. 2009)
10A06	*Heterodera spp.*	Stimulates polyamine biosynthesis and suppresses host defenses	(Hewezi, Howe et al. 2010)
Ubiquitin associated proteins	*Heterodera* and *Globodera spp.*	Alter Ubiquitination *in planta* (Unconfirmed)	(Gao, Allen et al. 2003; Tytgat, Vanholme et al. 2004; Jones, Kumar et al. 2009)
19C07	*Heterodera spp.*	Binds Auxin importer *in planta* Alters Auxin transport into the feeding site	(Lee, Chronis et al. 2011)
Plant signaling peptide mimics	*Heterodera*, *Globodera* and *Meloidogyne spp.*	Manipulate developmental signaling pathways in the host plant	(Wang, Mitchum et al. 2005; Lu, Chen et al. 2009; Tucker and Yang 2013)
16D10	*Meloidogyne spp.*	Interacts with a host transcription factor to alter plant development	(Huang, Dong et al. 2006)
Annexin mimics (4F01)	*Heterodera spp.*	Can complement endogenous plant annexins and alter host defenses	(Patel, Hamamouch et al. 2010)
Venom allergen-like proteins	*Globodera spp.*	Bind to papain-like cysteine proteases *in planta* and interfere with host defenses	(Lozano-Torres, Wilbers et al. 2012)
30C02	*Heterodera spp.*	Binds and inhibits a pathogenesis related gene in the host plant	(Noureddine Hamamouch 2012)

Table 1 (continued)

Effector	Species	Functions	Research
30C02	*Heterodera spp.*	Binds and inhibits a pathogenesis related gene in the host plant	(Noureddine Hamamouch 2012)
Cg1	*Meloidogyne spp.*	Silencing results in virulence against Mi-1 medeated resistance	(Gleason, Liu et al. 2008)

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CHAPTER 2. THE PUTATIVE EFFECTOR PROTEIN HS4E02 INTERACTS WITH THE PAPAIN-LIKE CYSTEINE PROTEASE RD21A FROM *ARABIDOPSIS THALIANA* IN YEAST

Modified from a manuscript to be submitted for publication to the *Journal of Nematology*

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Abstract

4E02 is a putative effector protein secreted from the esophageal gland cells of both *Heterodera glycines* and *Heterodera schachtii.* Previous studies have shown that *Hs*4E02 localizes to the nucleus *in planta.* However, the functional role of this effector has not yet been assigned and attempts to show that this protein has an effector function have been unsuccessful. In the current study we have used a yeast two-hybrid system to show that *Hs*4E02 interacts with the active site of the papain-like cysteine protease RD21A from Arabidopsis. Since RD21A and its homologs have been shown to play integral roles in other pathosystems, it is likely that this interaction has functional relevance in the plant-nematode interaction.

Introduction

The plant-parasitic soybean cyst nematode (*Heterodera glycines*) engages in complex interactions with its host plant. During infection, this parasite bores into the roots of its host

[1]Primary researcher and writer
[2]Intellectual and editorial contributions
[3]Intellectual and research contributions
[4]Intellectual contributions
[5]Intellectual, editorial, and financial contributions, primary corresponding author

plant and secretes a cocktail of effector proteins into plant tissues. In the past decade, a large body of evidence has shown that these effector proteins are important factors that facilitate the parasite's ability to infect its host (Mitchum, Hussey et al. 2013). Once within the root tissue, the nematode's effector proteins interact with and manipulate host factors, causing drastic morphological and physiological changes within the host. These changes ultimately result in the formation of a syncytium composed of several hundred fused root cells (Golinowski, Sobczak et al. 1997). This syncytium acts as the nematode's sole source of nourishment throughout its entire life cycle. Interestingly, the formation of the syncytium is also accompanied by drastic morphological changes within the nuclei, which increase in size due to DNA endoreduplication in the absence of nuclear division (Endo 1971; Goverse, de Engler et al. 2000). This increase in genomic DNA along with a concomitant increase in transcriptional activity indicates that the nematode's effector proteins alter plant nuclear biology to create the syncytium. Understanding how the secreted effector proteins are able to induce the formation of the feeding site is a necessary first step in the development of new control strategies to mitigate the damage this pathogen causes to crop plants.

To date, over 60 putative effector proteins have been isolated from *H. glycines* (Gao, Allen et al. 2001; Wang, Allen et al. 2001; Gao, Allen et al. 2003). The process of attributing a function to these putative effectors is hampered by the unique nature of their amino acid sequence. Indeed, the majority of these secreted proteins have no homologs within the National Center for Biotechnology Information (NCBI) non-redundant protein database and few contain detectable protein domains. The cryptic nature of these proteins, and the limited resources available to investigate their function, has prompted researchers to prioritize these proteins based on other criteria.

One putative effector that has sparked considerable interest is *Hg*4E02. This protein was first identified in an effector screen as a transcript encoding an N-terminal signal peptide that was specifically expressed within the subventral esophageal gland cells of *H. glycines* (Gao, Allen et al. 2003), which are known to be a major source of secreted effector proteins (Hussey 1989). Like other putative effector proteins, *Hg*4E02 has no homologs within the NCBI non-redundant protein database and contains no known protein domains with the exception of a SV40-type nuclear localization signal (NLS) (PSORT II). The functionality of this NLS was confirmed experimentally by localizing Hg4E02 to the nuclei of onion epidermal cells and Arabidopsis protoplasts (Elling, Davis et al. 2007). The localization of *Hg*4E02 to the plant cell nucleus highlights this protein as one that may elicit changes within the host nucleus.

In an attempt to test whether *Hg*4E02 performs a virulence function for cyst nematodes, Patel et. al. (2008) cloned a homolog of *Hg*4E02 from the closely related sugar beet cyst nematode (*Heterodera schachtii*), which was found to be 99% identical to the *H. glycines* sequence (Patel, Hamamouch et al. 2008). Unlike *H. glycines, H. schachtii* is able to infect the model plant *Arabidopsis thaliana*, making it a much more tractable pathosystem for studying the nematode-plant interaction (Sijmons, Grundler et al. 1991). In fact, the *H. schachtii*-Arabidopsis pathosystem has been used to confirm the virulence functions of many cyst nematode effectors (Hewezi, Howe et al. 2010; Lee, Chronis et al. 2011; Noureddine Hamamouch 2012; Mitchum, Hussey et al. 2013). In an unexpected twist, Arabidopsis lines expressing an *Hs*4E02 transgene, with or without a signal peptide, showed no change in their susceptibility to *H. schachtii* infection (Patel, Hamamouch et al. 2008). At the same time, Arabidopsis lines expressing hairpin RNAs against the *Hs*4E02 transcript were tested for

their susceptibility to *H. schachtii* infection. In spite of the fact that these lines caused a significant reduction of *Hs*4E02 transcript abundance within the nematode, no statistically significant difference in nematode susceptibility could be detected (Patel, Hamamouch et al. 2008). Though these data do not support a strong virulence function for *Hs*4E02, it does not exclude the possibility that it is an important effector protein.

In this study, we have gone a step further in the characterization of the *Hs*4E02 protein and have identified plant proteins with which this effector could potentially interact to carry out a virulence function.

Materials and Methods

Ectopic expression in Arabidopsis

The *Hs*4E02 coding sequence was amplified without the signal peptide sequence using specific primers containing the restriction sites *Bam*HI and *Sac*I on the forward and reverse primers respectively (Forward primer: 5'-TAT AGG ATC CAT GGA AGA GGG AGG GCG AGT GAA GCG C-3' and reverse primer: 5'-TAT AGA GCT CTT AAT GTT TGG GCT TCT TCC CGC AAC ATG-3' (restriction sites underlined). The attached restrictions sites were used to directionally clone the amplified *Hs*4E02 fragment into the multiple cloning site of the pBI121 binary vector. This created a T-DNA cassette containing the *Hs*4E02 coding sequence under the control of the *CaMV* 35S promoter. The construct was confirmed using Sanger sequencing, and transformed into *Agrobacterium tumefaciens* strain C58 using the freeze thaw method (An 1988). *A. tumefaciens* transformed with the *Hs*4E02 expression construct were used to transform *Arabidopsis thaliana* ecotype Columbia-0 using the previously described floral dip method (Clough and Bent 1998). T1

seeds were screened on MS media containing 50mg/L kanamycin) to select for the presence of the *NPTII* selection marker. Transgenic lines were self-fertilized and selected to the T3 generation, and segregation was used to determine which lines were stable carriers of the transgene.

Quantitative Real-Time RT-PCR

Total RNA was isolated from three separate biological replicates of each *Hs*4E02 expressing line and wild type (ecotype columbia-0), using the perfect pure RNA fibrous tissue extraction kit (5 Prime) according to the manufacturer's instructions. DNase treatment was performed with the DNase I provided with the RNA extraction kit according to the manufacturer's instructions. Gene-specific primers for the Hs4E02 coding sequence and Arabidopsis actin (AT1G49240) were designed (*Hs*4E02 forward primer: *5'*-CCT TTT CCG TCC TCA ATC GCT GC-3' *Hs*4E02 reverse primer: 5'-GTT TGG GCT TCT TCC CGC AAC ATG-3'). One ng of DNase treated RNA and the appropriate primer pairs [10mM] were used in a 15uL reaction for cDNA synthesis and PCR reaction with component mixtures from a One-step RT-PCR kit (Quanta) according to the manufacturer's instructions. The PCR reactions were run in an I-Cycler (Bio-Rad) using the following program: 50°C for 10 min, 95°C for 5 min, and 40 cycles of 95°C for 30 sec and 60°C for 30 sec. Following PCR amplification, the reactions were subjected to a temperature ramp to create the dissociation curve, determined by changes in fluorescence measurements as a function of temperature, by which the nonspecific products can be detected. The dissociation program was 95°C for 1 min, 55°C for 10 sec, followed by a slow ramp from 55°C to 95°C. Four technical replicates of each reaction were performed, averaged, and then normalized to the

internal control (actin). Expression levels of the transgene were calculated a using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The baseline was calculated using the non-specific background detected in the reactions using the *Hs*4E02 primer set with the wild type RNA.

Nematode assays

Seeds from all five *Hs*4E02 expressing Arabidopsis lines and wild type (Columbia-0) were surface sterilized using 50% bleach. Individual sterilized seeds were planted in individual wells of 12-well culture plates (Falcon) containing modified Knop's medium in a randomly blocked experiment using 60 seeds from each line. Plates were grown at 23°C under 16-h-light/8-h-dark conditions. Two week old seedlings were inoculated with ~250 pre-parasitic J2 *Heterodera schachtii* per seedling that were surface sterilized using a previously described protocol (Baum, Wubben et al. 2000). Inoculated plants were grown under the same conditions for an additional 3 weeks. After which, each plant was individually scored for the number of adult J4 female nematodes. P-values for the difference in the average number of adult females between *Hs*4E02 expressing lines and wild type were calculated using a least squares mean model (nematode=plate genotype) (SAS).

Yeast two-hybrid assay

A yeast-two-hybrid screening was performed as described in the BD Matchmaker Library Construction and Screening Kits (Clonetech). The *Hs*4E02 coding sequence was amplified without the signal peptide using specific primers containing the restriction sites *Eco*RI and *Pst*I on the forward and reverse primers respectively (Forward primer: 5'-TAT AGA ATT CGA AGA GGG AGG GCG AGT GAA GCG C-3' and reverse primer 5'-ATG

ATC TGC AGG TCA ATG TTT GGG CTT CTT CCC GCA AC-3' restriction sites underlined). The attached restrictions sites were used to directionally clone the amplified *Hs*4E02 fragment into the multiple cloning site of the pGBKT7 bait vector to produce a fusion construct of *Hs*4E02 bound to the GAL4 DNA binding domain (BD). This construct was verified by sequencing, and transformed into *Saccharomyces cerevisiae* strain Y187 to create the bait strain. The previously generated prey libraries using cDNA made from the roots of Arabidopsis infected with *H. schachtii* at 3, 7, and 14 days after infection (Hewezi, Howe et al. 2008). The bait strain was mated separately with the three prey libraries in the compatible *S. cerevisiae* strain AH109. Screening and subsequent co-transformation analysis of interacting prey vectors was carried out according to Clonetech protocols, using the empty bait vector as well as human lamin C containing bait vector as negative controls.

Results

Arabidopsis lines expressing *Hs*4E02 display an inconsistent susceptibility trend

To detect a potential virulence function that *Hs*4E02 may have during the interaction between *H. schachtii* and Arabidopsis, the *Hs*4E02 coding sequence, without the signal peptide was constitutively expressed within Arabidopsis. Five independently transformed lines were developed, which stably express the *Hs*4E02 transgene. The level of transgene expression was assessed for each line using quantitative real-time RT-PCR (qPCR). Each line was shown to express the *Hs*4E02 transgene mRNA, but variability was observed in the level of transgene expression in each line (Fig. 1).

To assess the relative susceptibility of each *Hs*4E02-expressing line to *H. schachtii* infection, all five lines were infected with *H. schachtii* alongside untransformed controls, and

the number of successfully developing adult female nematodes was counted 21 days after inoculation (Fig. 2). Two of the lines (line 1-3 and line 5-1) showed significant increases in the number of J4 female nematodes compared to control plants, although this susceptibility phenotype was not consistently observed in all five lines.

Identification of *Hs*4E02-interacting partner from Arabidopsis

Many well characterized nematode effector proteins are known to interact with host proteins to carry out their function *in planta* (Hewezi and Baum 2012). To determine whether *Hs*4E02 interacts with any proteins within Arabidopsis, the *Hs*4E02 protein, without the signal peptide, was used as bait in a yeast two-hybrid screen against three separate prey libraries. Each of the three prey libraries contains proteins expressed in Arabidopsis roots during different stages of *H. schachtii* infection (J2, J3, and J4 stage) (Hewezi, Howe et al. 2008). After screening all three prey libraries, 123 positive colonies were identified, which showed an interaction with the Hs4E02 bait. Prey plasmids were isolated and sequenced from each colony, to identify which protein was present in each prey vector. After discarding known false positives, a final candidate list of 33 Arabidopsis proteins was identified. The isolated prey vectors for each of these candidates was co-transformed into yeast with the *Hs*4E02 bait as well as non-specific negative controls to assess the specificity of the interaction. Three separate Arabidopsis genes recapitulated a positive interaction that was specific for *Hs*4E02 (Table 1). Of the three interacting candidates, only one, i.e., Resistant-to-Dehydration 21A (RD21A), was represented by more than one prey clone. A total of 13 RD21A prey clones were isolated from separate yeast colonies from all three yeast libraries. An alignment of the 13 RD21A clones revealed that they were different lengths, spanning

different regions of the RD21A protein (Fig. 3). It was apparent that all of these different clones shared the same 85 amino acid stretch of the RD21A protein (R237-K321), which resides within the 216 residue cysteine protease catalytic domain (L137-I352) within the RD21A protein (Fig. 3). This alignment indicates that *Hs*4E02 is likely to interact specifically with a region within the catalytic domain of the RD21A papain-like cysteine protease.

Discussion

In the current study we set out to characterize the function of the putative effector protein *Hs*4E02. Previous studies have shown that *Hs*4E02 contains a functional NLS that targets it to the plant nucleus (Elling, Davis et al. 2007). However, exogenous expression of *Hs*4E02 in Arabidopsis, as well as host-derived RNAi targeting of its transcript, failed to show an effect on host susceptibility to *H. schachtii* infection (Patel, Hamamouch, et al. 2008). Despite these unexpected results, 4E02 still stands out as a likely effector protein that has a role in the nematode-plant interaction.

In this study, we produced five separate Arabidopsis lines that constitutively expressed *Hs*4E02 (Fig. 1). Contrary to previously published data, we found that two of the five lines showed a significant increase in susceptibility to *H. schachtii* infection (Fig. 2). Additionally, the line with the highest level of Hs4E02 expression (line 1-3) also displayed the greatest increase in nematode susceptibility. These results indicate that *Hs*4E02 expression may have an effect on the nematode-plant interaction in a dose dependent manner. However, the correlation between *Hs*4E02 expression level and susceptibility is not consistent across all five transgenic lines. Lines 5-1 and 11-1 show equivalent levels of

*Hs*4E02 expression (Fig. 1). Yet, line 5-1 shows a significant increase in nematode susceptibility, while line 11-1 shows no difference compared to the wild type (Fig. 2). The inconsistency across all *Hs*4E02 expressing lines, from the current and previous studies, has led to uncertainty as to whether *Hs*4E02 expression truly has an effect on nematode susceptibility. At present, we cannot definitively say whether the increase in susceptibility is a result of *Hs*4E02 expression, or merely a chance effect of the transgene insertion site. What is clear is that low levels of *Hs*4E02 expression do not produce a measurable effect on host susceptibility. However, it is possible that future investigations into the functions of *Hs*4E02 using higher expressing transgenic lines could find a more consistent susceptibility phenotype.

Despite the inconsistency of the *Hs*4E02 phenotype there is further evidence showing that *Hs*4E02 performs an effector function *in planta*. Furthermore, there are several variables that can confound the results of the susceptibility assays that have been performed with *Hs*4E02. One such confounding variable is the degree of redundancy within an effector family. Effector proteins, from both nematodes and other plant parasites, are often part of multi gene families within a single genome (Abad, Gouzy et al. 2008; Haas, Kamoun et al. 2009; Raffaele, Farrer et al. 2010). It is thought that these effector gene families perform redundant functions within a host plant. The presence of multiple redundant copies of *Hs*4E02 could have interfered with the efficacy of host derived RNAi targeting this transcript. Small polymorphisms in the coding sequence of different protein isoforms could reduce the effect of host-derived RNAi. It is also conceivable that *Hs*4E02 functions in conjunction with other nematode effector proteins to carry out its function. In which case expressing *Hs*4E02 without its cognate partner would be unlikely to elicit a phenotype. Still

other confounding variables could have arisen from expressing a nematode protein in an exogenous plant environment. This could result in posttranslational modifications that do not occur when *Hs*4E02 is expressed within the nematode. In short, the negative results seen in susceptibility assays thus far do not exclude *Hs*4E02 from having an effector function.

Working on the presumption that *Hs*4E02 has an effector function we set out to determine whether *Hs*4E02 interacts with proteins from Arabidopsis. Using a yeast two-hybrid assay we were able to identify three separate Arabidopsis genes that showed an interaction with *Hs*4E02 in yeast (Table 1). Two of the candidate genes (AT3G45960) and (AT3G54440) were represented by one prey clone each. Though these were considered as potential host interacting proteins, their ontologies and subcellular localizations did not fit with the nuclear localization of *Hs*4E02. AT3G45960, which encodes Arabidopsis expansin-like a3 (ATEXLA3), has roles in plant cell wall modifications, while AT3G54440 encodes a glycoside hydrolase with a role in carbohydrate metabolism within the chloroplast. Though neither of these two candidates stands out as proteins with overt functional significance in the nematode-plant interaction, we cannot discount the possibility that they do interact with *Hs*4E02 during infection. As such, future studies should investigate whether these interactions occur *in planta*.

In contrast to the first two candidates, the third (AT1G47128) was isolated a total of 13 times from all three yeast libraries used in our assay. AT1G47128 encodes a papain-like cysteine protease known as Responsive to Dehydration 21A (RD21A). It is well established that cysteine proteases of this class have functional roles in senescence and plant-parasite interactions (van der Hoorn 2008). In fact, two recent studies have shown that RD21A has pro-death function, and mutations of this gene result in altered susceptibility to necrotrophic

pathogens (Shindo, Misas-Villamil et al. 2012; Lampl, Alkan et al. 2013). Other studies have identified homologous papain-like cysteine proteases that are required for pathogen-triggered immune responses in multiple plant species (Gilroy, Hein et al. 2007; Deslandes, Bernoux et al. 2008). Still other studies have shown that papain-like cysteine proteases are directly targeted and inhibited by effector proteins from multiple different pathogens (Rooney, Van't Klooster et al. 2005; Bozkurt, Schornack et al. 2011; Bar-Ziv, Levy et al. 2012). Taken together, this body of evidence shows that RD21A and other papain-like cysteine protease are well-conserved immune modulators that are targeted by multiple plant pathogens. With its established role in plant-pathogen interactions, we think that RD21A is a likely functional target of *Hs*4E02.

At first glance, the fact that RD21A is a vacuolar associated protein makes it hard to imagine that it could interact with the nuclear localized *Hs*4E02. However, this type of interaction is not unprecedented. A recent study of another vacuolar localized paralog (RD19) showed that this papain-like cysteine protease is targeted by the effector protein PopP2, from *Ralstonia solanacearum*. Furthermore, once PopP2 interacts with RD19, the complex is relocalized to the plant nucleus (Deslandes, Bernoux et al. 2008). It is tempting to speculate that *Hs*4E02 could interact with and relocalize RD21A in a similar manner. Indeed, this hypothesis fits with recent data that has shown that certain nematode effectors localize to the nucleus only after interacting with host proteins first (Hewezi and Baum 2012).

Inspection of the 13 RD21A clones isolated from our yeast two-hybrid assay lent more credence to the hypothesis that this protein interacts specifically with *Hs*4E02. A protein alignment of the 13 clones revealed that together they only share an 85-residue stretch of the RD21A protein in common (Fig. 3). This short stretch of amino acids lies

within the catalytic protease domain of RD21A and includes two of the three catalytic residues within the active site (van der Hoorn 2008). Additionally, a review of the literature revealed that this Hs4E02 interacting stretch overlaps a region homologous to the site bound by the viral protein V2 in an orthologous papain-like cysteine protease (Cyp1) (Bar-Ziv, Levy et al. 2012). Together, this data suggest that *Hs*4E02 binds specifically to a region within the active site of RD21A, and makes it likely that this is a functional interaction and not a random occurrence. Given the fact that RD21A is only active after cleavage of both its N-terminal inhibitor domain and its C-terminal granulin domains (Yamada, Matsushima et al. 2001), specific interaction with the catalytic domain is important for a functional interaction.

As of yet, the interaction between RD21A and *Hs*4E02 has only been confirmed in yeast cells. Future functional studies of *Hs*4E02 should confirm this interaction *in planta* using either bimolecular fluorescence complementation or co-immunoprecipitation. Once this interaction between *Hs*4E02 and RD21A is confirmed *in planta*, there are still several questions that need to be answered to determine the functional significance of this interaction. The question: Does *Hs*4E02 perform a virulence function?. In this study, our *Hs*4E02-expressing lines have not shown a consistent change in nematode susceptibility. However, since mutations in RD21A have been shown to affect the growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum*, infecting our *Hs*4E02 lines with these necrotrophic pathogens could reveal a susceptibility phenotype. An alternative approach would be to deliver *Hs*4E02 as an effector in a known avirulent interaction, perhaps using *Pseudomonas syringae* Dc3000, to see if the protein effects the induction of the hypersensitive response. The second question: Does RD21A play a role in the plant-nematode interaction? A

straightforward approach to answering this question would be to test RD21 knock-out mutants and overexpression lines for their susceptibility to nematode infection. Positive results in these experimental approaches would provide convincing evidence that Hs4E02 binds and modulates the function of RD21A *in planta*.

In summary, using a yeast two-hybrid system we have found that the putative effector protein *Hs*4E02 interacts with the active site of the papain-like cysteine protease RD21A from Arabidopsis. Based on previous data showing the importance of RD21A in other pathosystems, we hypothesize that this interaction has functional significance in the plant-nematode interaction. Future studies will be needed to investigate this possibility.

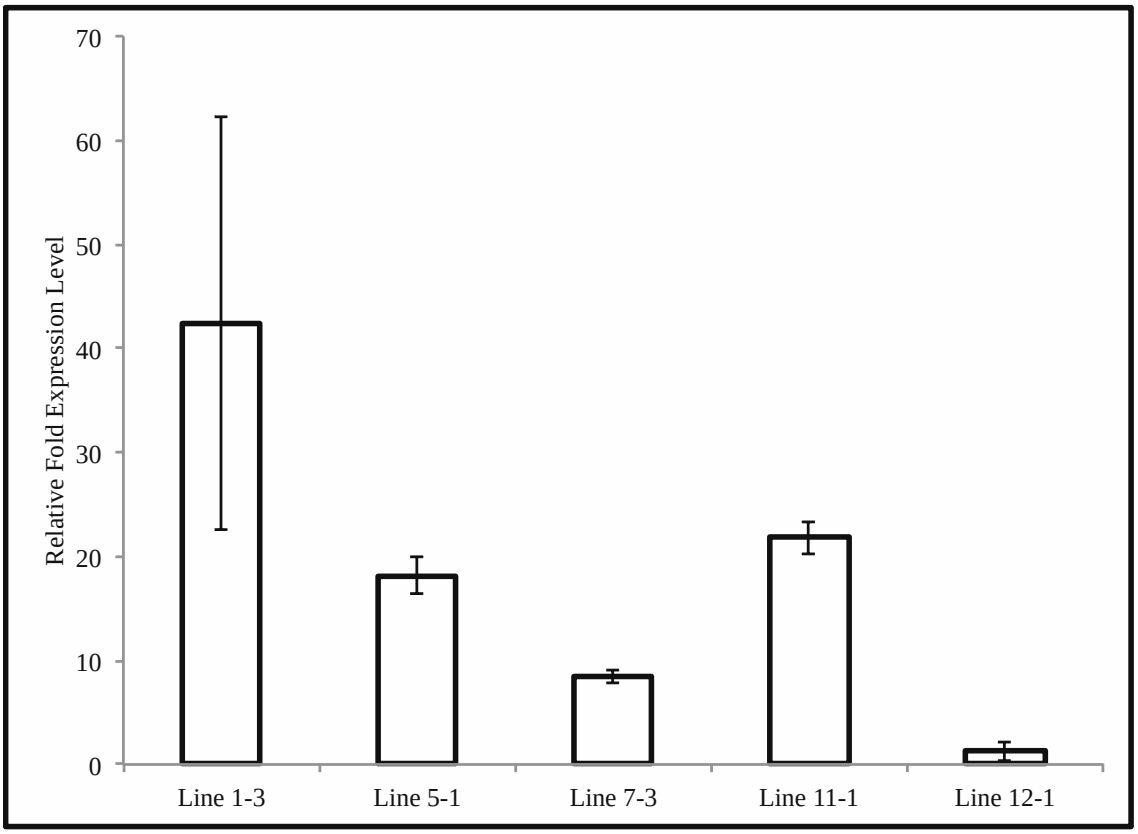

Figure 1. qRT-PCR expression analysis of Arabidopsis lines expressing *Hs*4E02. Five independently transformed non-segregating lines were assessed for their relative expression of the *Hs*4E02 transgene.

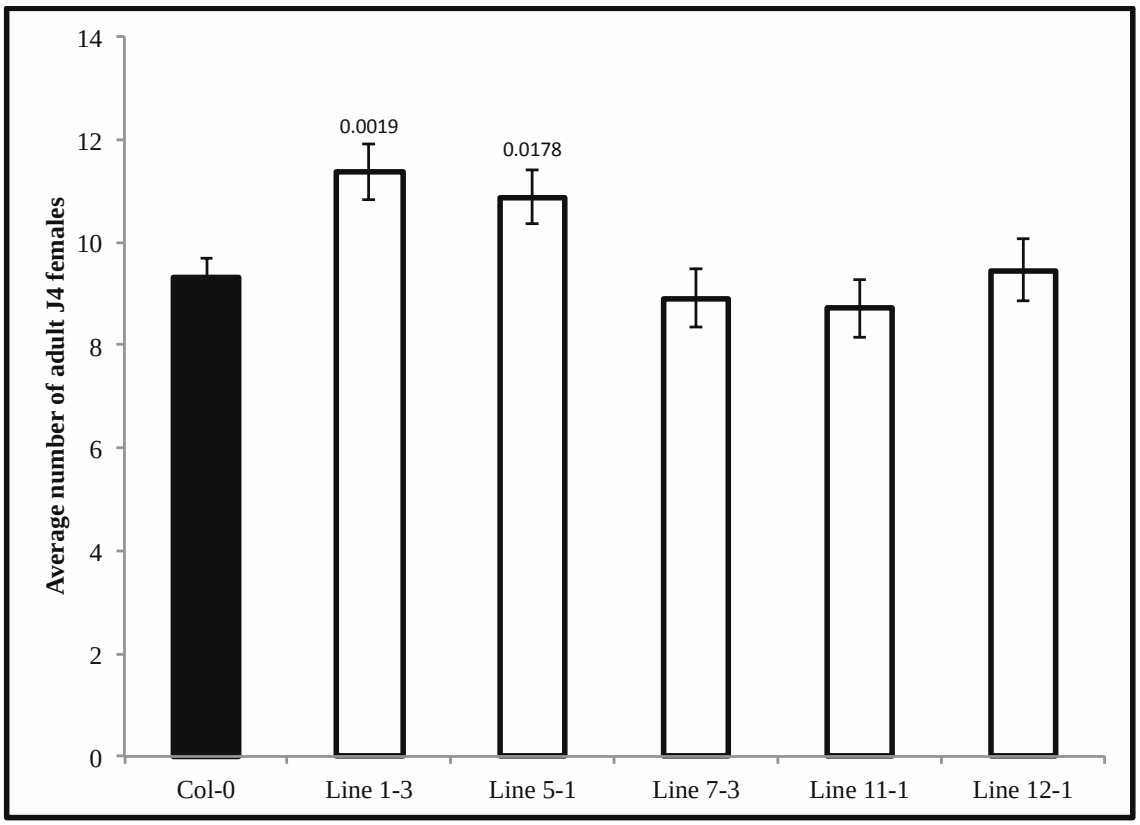

Figure 2. Assay for susceptibility of *Hs*4E02-expressing lines to *H. schachtii* infection. Two lines showed a significant increase in their susceptibility to nematode infection compared to Col-0 controls (p-values above bars), while three other lines showed no significant difference.

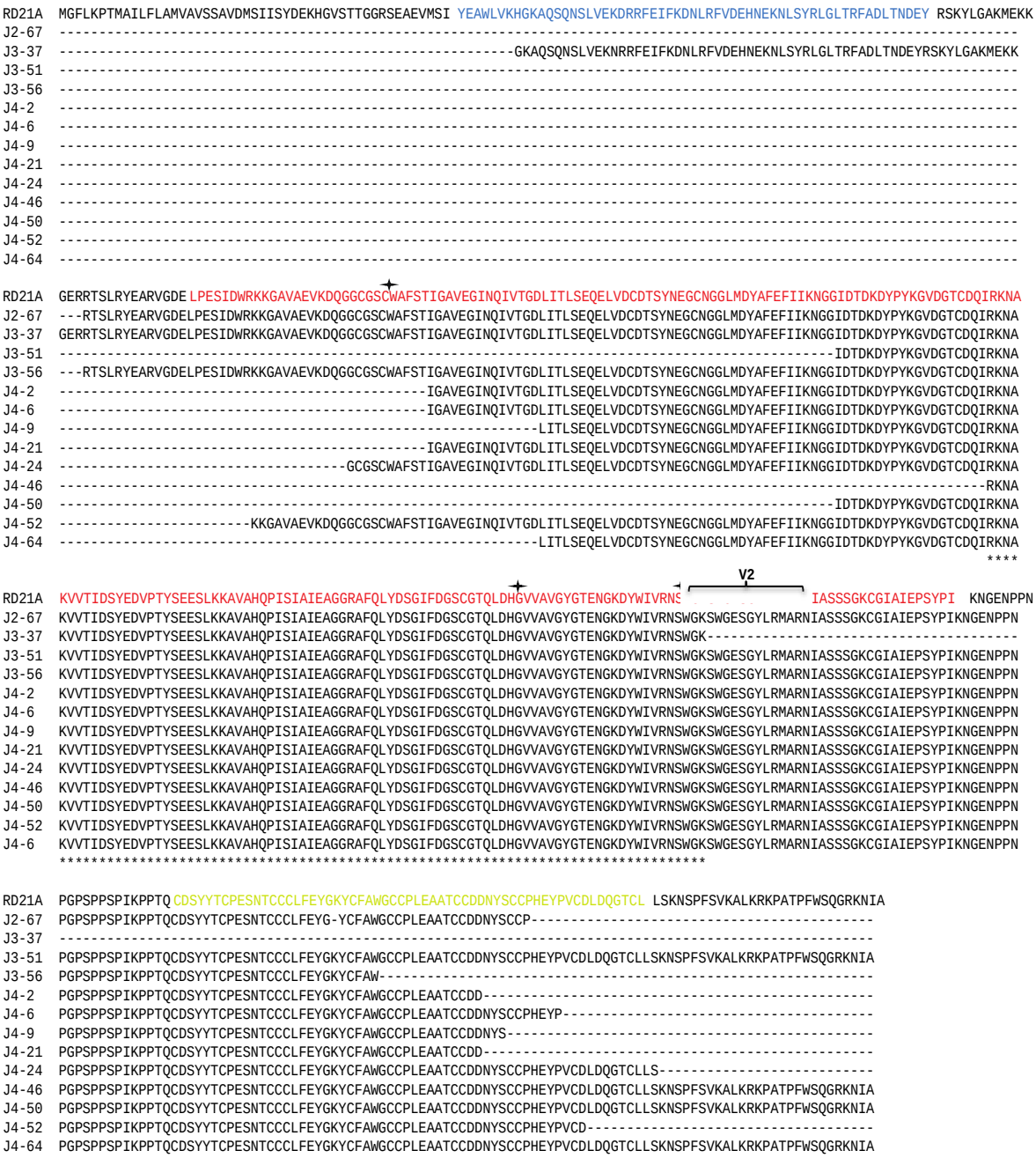

Figure 3. Alignment of RD21A yeast two-hybrid clones with the full-length protein. The thirteen RD21A prey clones, which showed a specific interaction with the Hs4E02, shared a common 85 amino acid stretch representing the putative *Hs*4E02-binding site (*). This putative binding site resided within the catalytic domain of RD21A (RED). This binding site also contains two of the three catalytic residues within the RD21A active site (star), and overlaps a region homologous to the 15 residue binding site of V2 (from Tomato Yellow Leaf Curl Virus) in a homologous papain-like cysteine protease from *Nicotiana benthamiana.* This putative *Hs*4E02-binding site did not overlap the N-terminal inhibitor domain (BLUE) or the C-terminal granulin domain (YELLOW) of RD21A.

Table 1. *Hs*4E02 Interaction candidates identified in our yeast two-hybrid screen. Descriptions of each of the individual genes that showed specific interactions with the *Hs*4E02 bait in yeast. * The number of prey clones from each interaction candidate and the yeast two-hybrid library from which they were isolated (J2, J3, or J4).

Arabidopsis gene ID	Description	Prey clones*		
		J2	J3	J4
AT3G45960	*Arabidopsis thaliana* expansin-like A3 (ATEXLA3): cell wall modification	0	0	1
AT3G54440	glycoside hydrolase family 2 protein: carbohydrate binding and carbohydrate metabolic processes within the chloroplast	1	0	0
AT1G47128	Responsive to dehydration 21A (RD21A): peptide ligase and protease activity, involved in plant stress response and immunity	1	3	9

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CHAPTER 3. *HS*25A01 PERFORMS AN EFFECTOR FUNCTION DURING THE INTERACTION BETWEEN *HETERODERA SCHACHTII* AND ITS HOST PLANT *ARABIDOPSIS THALIANA*

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Abstract

Cyst nematodes are a pervasive threat to many crop plants around the world. In an effort to combat these parasites, much effort has been focused on characterizing the effector proteins that allow them to infect their plant hosts. A family of putative effector proteins composed of three members (*Hg*25A01, *Hg*30G12, and *Hg*4G05), has previously been isolated form the esophageal gland cells of the soybean cyst nematode (*Heterodera glycines*). In this study we have identified an orthologous member of this protein family (*Hs*25A01) within the closely related species *H. schachtii.* Overexpression of *Hs*25A01 in Arabidopsis produced plants with increased susceptibility to *H. schachtii* and increased root length. The *Hs*25A01 protein interacts with multiple Arabidopsis proteins in yeast as well as *in planta*. We conclude that *Hs*25A01 represents a secreted effector protein that alters development within the plant cell, and performs a virulence function for the nematode parasite.

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Introduction

The soybean cyst nematode (*Heterodera glycines*) causes extensive monetary losses in yield each year (Koenning, Overstreet et al. 1999). The success of this pathogen relies on its ability to engage in a complex interaction with its host plant, and at the interface of this interaction are effector proteins. These effector proteins are known to be secreted by the nematode into host plant tissues using a hollow needle-like structure known as a stylet (Hewezi and Baum 2012). Once in plant tissues, many nematode effector proteins interact with and manipulate host plant factors, ultimately resulting in the formation of an elaborate feeding site within the root tissue known as a syncytium. The syncytium acts as a nutrient sink in the plant, diverting energy away from normal plant processes to feed the growing nematode through adulthood and reproduction. The nematode's effector proteins underlie the parasite's ability to form the syncytium and to live off of host plant tissues. Understanding how these effector proteins function within the plant can lead to new ways to combat these pests as well as a better understanding of the host-parasite interaction.

The effector proteins secreted through the nematode's stylet are produced in the esophageal gland cells (one dorsal and two subventral) (Hussey 1989). These three gland cells empty their proteinaceous content into the nematode's esophagus, from where it is then released through the stylet. Many putative effector proteins have been isolated from the esophageal gland cells of cyst nematodes, and several have been shown to perform a virulence function for the nematode. Multiple cell wall modifying enzymes such as cellulases, pectate lyases, and cellulose-binding proteins are secreted from the esophageal gland cells (Smant, Stokkermans et al. 1998; Hewezi, Howe et al. 2008). These proteins are thought to facilitate the drastic changes associated with the formation of the syncytium. A

functional chorismate mutase protein is secreted from the nematode's esophageal gland cells, and has been shown to alter the shikimate pathway *in planta* (Bekal S 2003). Other cyst nematode effectors have been shown to mimic CLAVATA3/ESR (CLE) signaling peptides within the plant, and consequently affect plant growth (Olsen and Skriver 2003; Wang, Mitchum et al. 2005; Lu, Chen et al. 2009). These well-characterized cyst nematode effectors have revealed important details of the plant-nematode interaction, and yet they only represent a small portion of the cyst nematode's secretome, which currently stands at over 60 protein constituents (Gao, Allen et al. 2001; Wang, Allen et al. 2001; Gao, Allen et al. 2003). The vast majority of these putative effectors remains uncharacterized and has no predicted function.

In this study we have characterized a small gene family of putative effectors that we collectively refer to as 25A01. Three distinct isoforms of the 25A01 protein family (*Hg*25A01, *Hg*30G12, and *Hg*4G05) were previously isolated from *H. glycines* gland-specific cDNA libraries (Gao, Allen et al. 2001). These clones encode a predicted N-terminal secretion signal, and were all shown to be expressed specifically within the dorsal gland cell of *H. glycines* during parasitic stages, indicating that all three proteins are likely to be secreted into host plant tissues. Though each of these three proteins was isolated individually, a more in depth analysis revealed that they share 86-95% amino acid identity, and comprise a protein family within *H. glycines* (Fig. 1). Despite their abundance within *H. glycines*, attempts to infer the functions of these proteins using computational tools have been unproductive, revealing that this protein family has no significant homologs in the NCBI non-redundant protein database (BLASTp) (Altschul, Gish et al. 1990) and is predicted to contain no functional domains (Interpro-scan) (Quevillon, Silventoinen et al. 2005).

Interestingly, a microarray experiment probing for the expression of specific *H. glycines* effector proteins showed that *Hg*25A01 was specifically up-regulated during the parasitic-second stage juvenile (J2) stage of nematode development (Ithal, Recknor et al. 2007). This stage of development coincides with the early establishment of the syncytium within the host plant, indicating that *Hg*25A01 could play a role in formation of the feeding site.

Taken together, these data indicate that the 25A01 protein family represents a group of unique proteins specifically secreted from the esophageal glands of *H. glycines* during the early stages of nematode infection. We hypothesized that the 25A0l gene family contains secreted proteins that carry out a virulence function for the nematode *in planta.* In this study, we have isolated a homologous clone of the 25A01 protein family from the closely related sugar beet cyst nematode *H. schachtii*, and investigated the role this protein plays in *H. schachtii's* infection of the model plant *Arabidopsis thaliana.* We have determined that *Hs*25A01 effects the cyst nematode-plant interaction; increasing plant susceptibility to nematode infection as well as effecting plant growth.

Materials and Methods

In situ hybridization of effector transcripts

Specific forward and reverse primers were designed for the coding sequence of each of the *Hs*25A01 transcript. These primers were used to amplify an amplicon 90 bp in length from cDNA pools generated from *H. schachtii*. This amplicon was used as a template in a unidirectional PCR reaction to produce single stranded sense and anti-sense digoxigenin (DIG)-labeled probes for each candidate effector transcript. Unidirectional PCRs were performed in 25µl volumes using a DIG-nucleotide labeling kit (Roche). *In situ*

hybridizations were performed on mixed parasitic stages of *H. schachtii* as previously described (de Boer, Yan et al. 1998). Nematodes were fixed in a 2% formaldehyde solution. Fixed nematodes were permeabilized by hand cutting with a razorblade on a glass slide in combination with a partial proteinase-K digestion (20mg/mL, 30 min RT). DIG-labeled probes were hybridized to permeabilized tissue over night at 50°C. Hybridized probes within the nematode were detected using and anti-DIG antibody conjugated to alkaline phosphatase and it's substrate. Samples were then visualized using a Zeiss Axiovert 100 inverted light microscope.

In planta subcellular localization

The *Hs*25A01 coding sequence was amplified without the signal peptide sequence using specific primers containing the restriction sites *Eco*RI and *Eag*I on the forward and reverse primers respectively (Forward primer: 5'-TAT AGA ATT CCC GAC TCC TTA TGA TGC TGA ATC GGA ATC TTC TGA AT-3' and reverse primer: 5'-TAT ACG GCC GCC GTT AGT GCT AGG GCC GGC GAA GTT GG-3'). The attached restrictions sites were used to directionally clone the amplified *Hs*25A01 fragment into the multiple cloning site of the pRJG23 vector (Grebenok, Pierson et al. 1997). The ligation produced a construct containing *Hs*25A01 fused with the coding sequence of GFP and a GUS reporter gene under the control of a double *CaMV* 35S promoter. The construct was sequenced using Sanger technology. The *Hs*25A01 fusion construct was attached to 1.6μm gold particles, and bombarded into onion epidermal cells using Biolistic Particle Delivery System (Bio-Rad) as described in previous studies (Elling, Davis et al. 2007). Bombarded onion epidermal peels were incubated in the dark for 24 hours at 26°C. Fluorescent GFP reporter was assessed and

photographed using an Zeiss Axiovert 100 inverted microscope. Bombardment was repeated 3 times in independent experiments.

Generation of transgenic Arabidopsis

The *Hs*25A01 coding sequence was amplified without the signal peptide sequence using specific primers containing the restriction sites Bam HI and Sac I on the forward and reverse primers respectively (Forward primer: 5'-TAT GGA TCC ATG CCG ACT CCT TAT GAT GCT GAA TCG GAA TCT TCT GAA T-3' and reverse primer: 5'-TAT AGA GCT CCT ACA GCC GCA GCA GCA GAA GTT TCT CCG-3' restriction sites underlined). The attached restrictions sites were used to directionally clone the amplified *Hs*25A01 fragment into the multiple cloning site of the pBI121 binary vector. This created a T-DNA cassette containing the *Hs*25A01 coding sequence under the control of the *CaMV* 35S promoter. The construct was confirmed using Sanger sequencing, and transformed into *Agrobacterium tumefaciens* strain C58 using the freeze thaw method (An 1988). *A. tumefaciens* transformed with the *Hs*25A01 expression construct were used to transform *Arabidopsis thaliana* ecotype Columbia-0 using the previously described floral dip method (Clough and Bent 1998). T1 seeds were screened on MS media containing 50mg/L kanamycin, to select for the presence of the *NPTII* selection marker. Transgenic lines were self fertilized and selected to the T3 generation, and segregation was used to determine which lines were stable carriers of the transgene.

Quantitative Real-Time-RT-PCR

Total RNA was isolated from three separate biological replicates of each *Hs*25A01 expressing line and wild type (ecotype columbia-0), using the perfect pure RNA fibrous tissue extraction kit (5 Prime) according to the manufacturer's instructions. DNase treatment was performed with the DNase I provided with the RNA extraction kit according to the manufacturer's instructions. Gene specific primers for the *Hs*25A01 coding sequence and Arabidopsis actin (AT1G49240) were designed (*Hs*25A01 for primer:5'-GCG CCG AAA AGT GAA CAG ACG C-3' *Hs*25A01 rev primer: 5'-CGC CTT TGG CTG CTT GTC TTC C-3'). One ng of DNase treated RNA and the appropriate primer pairs [10mM] were used in a 15uL reaction for cDNA synthesis and PCR reaction with component mixtures from a One-step RT-PCR kit (Quanta) according to the manufacturer's instructions. The PCR reactions were run in an I-Cycler (Bio-Rad) using the following program: 50°C for ten min, 95°C for five min, and 40 cycles of 95°C for 30 sec and 60°C for 30 sec. Following PCR amplification, the reactions were subjected to a temperature ramp to create the dissociation curve, determined by changes in fluorescence measurements as a function of temperature, by which the nonspecific products can be detected. The dissociation program was 95°C for one min, 55°C for ten sec, followed by a slow ramp from 55°C to 95°C. Four technical replicates of each reaction were performed, averaged, and then normalized to the internal control (actin). Expression levels of the transgene were calculated a using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The baseline was calculated using the non-specific background detected in the reactions using the *Hs*25A01 primer set with the wild type RNA samples.

Root length assay

Seeds from each of the three non-segregating lines as well as wild type (Columbia-0) were surface sterilized and planted into four-well polystyrene rectangular plates (Thermo Scientific) containing MS media. Eight separate plates were used containing at least 15 seeds from each line, each line was planted in each relative well position twice to negate potential effects of well position on growth. Plates were placed vertically and incubated at 23°C under 16-h-light/8-h-dark conditions. After 10 days root lengths were measured in a blinded fashion as the distance between the crown and the tip of the root in three independent experiments. At least 100 combined data points were collected for each line. Statistics for the difference between the mean root length of each transgenic line and wild type controls were calculated using a two-tailed Student's t-test.

Infection assay

Seeds from all three *Hs*25A01 expressing Arabidopsis lines and wild type (Columbia-0) controls were surface sterilized. Individual sterilized seeds were planted in individual wells of 12-well culture plates (Falcon) containing modified Knop's medium in a randomly blocked experiment using 60 seeds from each line. Plates were grown at 23°C under 16-h-light/8-h-dark conditions. Two week old seedlings were inoculated with ~250 pre-parasitic J2 *Heterodera schachtii* per seedling that were surface sterilized using a previously described protocol (Baum, Wubben et al. 2000). Inoculated plants were grown under the same conditions for an additional three weeks. After which, each plant was individually scored for the number of adult J4 female nematodes. P-values for the difference in the average number of adult females between *Hs*25A01 expressing lines and wild type were calculated using a least squares mean model (nematode=plate genotype) (SAS).

Yeast two-hybrid assays

A yeast two-hybrid (Y2H) screening was performed as described in the BD Matchmaker Library Construction and Screening Kits (Clonetech). The *Hs*25A01 coding sequence was amplified without the signal peptide using specific primers containing the restriction sites *Eco*RI and *Bam*HI on the forward and reverse primers respectively (Forward primer: 5'-TAT AGA ATT CCC GAC TCC TTA TGA TGC TGA ATC GGA ATC TTC TGA AT-3' and reverse primer: 5'-TAT AGG ATC CCT ACA GCC GCA GCA GCA GAA GTT TCT CCG-3' restriction sites underlined). The attached restrictions sites were used to directionally clone the amplified *Hs*25A01 fragment into the multiple cloning site of the pGBKT7 bait vector to produce a fusion construct of *Hs*25A01 bound to the GAL4 DNA binding domain (BD). This construct was verified by sequencing, and transformed into *Saccharomyces cerevisiae* strain Y187 to create the bait strain. The previously generated prey libraries using cDNA made from the roots of Arabidopsis infected with *H. schachtii* at three, seven, and 14 days after infection (Hewezi, Howe et al. 2008). The bait strain was mated separately with the three prey libraries in the compatible *S. cerevisiae* strain AH109. Screening and subsequent co-transformation analysis of interacting prey vectors was carried out according to Clonetech protocols, using the empty bait vector as well as lamin C containing bait vector as negative controls.

Bimolecular fluorescence complementation assay

The *Hs*25A01 coding sequence was amplified without the signal peptide using specific primers containing the restriction sites *Eco*RI and *Bam*HI on the forward and reverse

primers respectively (Forward primer: 5'-TAT AGA ATT CAC CGA CTC CTT ATG ATG CTG AAT CGG AAT CTT CTG AAT-3' and reverse primer: 5'-TAT AGG ATC CCT AGT TAG TGC TAG GGC CGG CGA AGT TGG-3' restriction sites underlined) for directional cloning in pSTAT-nYFP-C1 vector. This created a fusion protein of *Hs*25A01 and the n-terminal domain of the eYFP protein, which was confirmed by sequencing. The amplified coding sequence of AT1G08110 with the restriction sites *Xho*I and *Eco*RI (Forward primer: 5'-TAT ACT CGA GAA ATG GCG TCG GAA GCG AGG GAA TCA CC-3' and reverse primer: 5'-TAT AGA ATT CTC AAG CTG CGT TTA CGG TAG TAG TTC CGA TAG TC-3' restriction sites underlined). AT1G08110 was directionally cloned tin pSTAT-cYFP-C1 vector to produce a fusion protein with the c-terminal domain of the eYFP, and was confirmed by sequencing. A fusion protein of AT5G28830 in pSTAT-cYFP-C1 was used as a negative control to confirm the specificity of the BiFC interaction. The construct was delivered to onion epidermal cells using previously described particle bombardment method(Hewezi, Howe et al. 2008). Bombarded tissues were incubated at 26°C in the dark. A positive protein-protein interaction, seen as a fluorescent GFP signal, was assessed and photographed using a Zeiss Axiovert 100 inverted microscope. Bombardment was repeated three times in independent experiments.

Results

Isolating 25A01 homolog from *Heterodera schachtii*

PCR primers based on the three known *H. glycines* isoforms of 25A01 were used to amplify and clone fragments from *H. schachtii* cDNA. Sequence analysis of these clones revealed several different isoforms, none of which contained a stop codon, indicating a likely

sequence divergence at the 3'-end of the transcript. To overcome this difference, 3' race was used to isolate a complete 630 bp clone that contained a stop codon and a 3' poly-A tail (*Hs*25A01). *Hs*25A01 showed 73-77% amino acid identity with all *H. glycines* isoforms of 25A01 (Fig. 1). To be sure that *Hs*25A01 shares the same gland-specific expression pattern as its *H. glycines* orthologs, we performed in situ hybridization on *H. schachtii* tissue using an anti-sense probe specific for *Hs*25A01. Like the 25A01 isoforms from *H. glycines*, *Hs*25A01 showed specific expression within the dorsal esophageal gland cells of *H. schachtii* (Fig. 2)

*Hs*25A01 localizes to the cytoplasm in planta

Effector proteins secreted by plant-parasitic nematodes are known to be localized to specific subcellular compartments and to perform diverse functions within a host cell (Elling, Davis et al. 2007). Knowing where an effector localizes within the host cell can provide clues about the role it plays in promoting virulence. Subcellular localization can also help identify interacting host factors. To identify the subcellular localization of *Hs*25A01, the coding sequences (CDS) was fused to GFP and β-glucuronidase (GUS) reporter protein and placed under the control of the *CaMV* 35S promoter. This construct was bombarded into onion epidermal cells, and fluorescence microscopy revealed a GFP reporter signal localized within the cytoplasm after 24 hours (Fig. 3A) as well as a cytoplasmic GUS reporter signal detected after 48 hours (Fig. 3B). This result indicates that *Hs*25A01 localizes to the cytoplasm *in planta*. This finding supports our previous i*n silico* predictions (PSORTII) and suggests that *Hs*25A01 functions within the cytoplasm of the host plant cell.

Ectopic expression of *Hs*25A01 in Arabidopsis

Producing stably transformed host plants expressing a nematode effector CDS has allowed researchers to assess the effects these proteins have on nematode parasitism (Wang, Mitchum et al. 2005; Hewezi, Howe et al. 2008; Hewezi, Howe et al. 2010). Additionally, expression of certain nematode effector CDSs within plants also has produced morphological phenotypes that likely result from their interactions with endogenous plant factors. To analyze the potential role of *Hs*25A01 in *H. schachtii* infection, we expressed the CDS without the signal peptide in *A. thaliana* ecotype Columbia-0 (Col-0) under the control of the *CaMV* 35S promoter. Three non-segregating T3 *Hs*25A01-expressing lines were selected and tested for transgene expression using quantitative real-time RT-PCR (qRT-PCR) (Fig. 4). The *Hs*25A01 transcript was detected in all three non-segregating lines and could not be detected in untransformed Col-0 controls. Quantification of the relative transcript abundance revealed variability in the expression level in each non-segregating line. More specifically, line 15-3 showed significantly higher expression than the other two lines. The construction of stably transformed lines expressing the *Hs*25A01 protein allowed us to detect *in planta* phenotypes caused by this putative effector protein.

Constitutive expression of *Hs*25A01 stimulates root growth of Arabidopsis and increases plant susceptibility to *H. schachtii* infection

To investigate the impact of *Hs*25A01 expression on root development, a comparative root growth experiment was performed using the three *Hs*25A01-expressing lines grown simultaneously alongside wild type Col-0 controls. Root lengths were recorded 10 days after planting. All three *Hs*25A01-expressing lines had average root lengths significantly longer than those of Col-0 controls (p-value <0.001, two-tailed students t-test) (Fig. 5). Moreover,

the degree of root length increase in each transgenic line positively correlated with its level of *Hs*25A01 expression. In other words the line with the longest average root length (Line 15-3) also had the highest expression of *Hs*25A01 mRNA, and the line with the shortest average root length had the lowest level of transgene expression. These data indicate that the nematode *Hs*25A01 protein has a biological function *in planta* that promotes root growth.

To determine whether *Hs*25A01 has a virulence function, the three *Hs*25A01-expressing lines were used in nematode susceptibility assays alongside untransformed Col-0 used as controls. The number of J4 females was counted at 21 days post inoculation and used to determine the susceptibility level of each line. All three *Hs*25A01-expressing lines showed a significant increase (P-value<0.05, LSM test) in the number of J4 females compared to Col-0 controls (Fig. 6). These data indicate that *Hs*25A01 performs a virulence function for the nematode, increasing the susceptibility of the host plant to nematode infection.

*Hs*25A01 binds promiscuously to proteins from Arabidopsis

In order to perform a virulence function, effector proteins are known to interact with and modulate host proteins (Hewezi and Baum 2012). Previously characterized nematode effectors have been found to interact with a wide array of plant proteins, interfering with the normal functions of these host factors and promoting successful infection by the parasite (Hewezi and Baum 2012). To identify host proteins that interact with *Hs*25A01, the protein without a signal peptide was used as bait in a Y2H screen against prey libraries containing transcripts from Arabidopsis. Each plant library was composed of transcripts isolated from *A. thaliana* infected with *H. schachtii* at various stages of development (J2, J3, and J4). After screening 15.62×10^6 yeast colonies, 173 colonies were identified by their ability to grow on

selective drop out media (SD-Leu, -Trp, -His, -Ade). Prey plasmids from each positive colony were isolated and sequenced, which allowed us to identify 51 individual clones. Several of these clones were discarded because they contained peptide sequences from previously known false positives. To confirm the specificity of the interaction with *Hs*25AO1, each prey vector was co-transformed with the *Hs*25AO1-bait vector, empty bait vector, and bait vector containing human lamin C protein. Specific interactions were confirmed for 11 individual prey clones. To further confirm these interactions, the full-length coding sequences for eight of these candidates were cloned into prey vectors. Subsequent yeast co-transformation revealed six full-length and three partial clones of various lengths that showed specific interactions with *Hs*25A01 (Table 1). These successful and specific interactions indicate that *Hs*25A01 may bind to multiple plant proteins.

The protein-protein interactions that occur within yeast cells may differ from the interactions that occur in a heterologous environment such as a plant cell. Therefore, to confirm the interaction *in planta*, Bimolecular Fluorescence Complementation (BiFC) was conducted. Even though the cytoplasmic interaction between *Hs*25AO1 and a selected candidate interacting protein (AT1G08110) was confirmed *in planta* (Fig. 7A), fluorescence was also observed when a non-specific gene (AT5G28830) was used as a negative control (Fig. 6B). This same interaction was replicated in multiple co-bombardments. Based on these results, we cannot conclude whether these protein-protein interactions are specific. However, future investigations using the interaction partners in multiple fusion conformations could elucidate which interactions are truly specific.

Discussion

*Hg*25A01 and its protein isoforms (*Hg*30G12 and *Hg*4G05) represent a unique family of putative effector proteins secreted from the dorsal esophageal gland cells of *H. glycines*. In order to characterize the potential roles that this family of proteins may play in cyst nematodes infection, we cloned an orthologous protein from the closely related cyst nematode species *Heterodera schachtii*. Unlike *H. glycines*, *H. schachtii* is able to successfully infect Arabidopsis, making it a much more tractable system for studying the plant-cyst nematode interaction. The full-length clone isolated from *H. schachtii* (*Hs*25A01) shares 73-77% amino acid identity with the three *H. glycines* isoforms (Fig. 1). *In situ* hybridization of the *Hs*25A01 transcript reveled that this protein also shares the same dorsal gland cell expression pattern as the isoforms from *H. glycines* (Fig. 2). These strong similarities between this *H. schachtii* clone and those from *H. glycines* make it likely that *Hs*25A01 is part of the same putative effector family. These protein family members likely carry out analogous functions in both species during infection of their respective host plants. It is worth noting that although *Hs*25A01 was the only complete clone isolated from *H. schachtii*, other incomplete clones were also amplified. These incomplete clones showed small sequence divergences akin to those seen between the other members of the 25A01 family in *H. glycines*. This observation indicates that *H. schachtii* contains other 25A01 protein family members. The presence of multiple and diverse 25A01 family members in both species of cyst nematode could be an indication that this protein family is under increased diversifying selection. Interestingly, increased diversifying selection has recently become a generally accepted feature of effector proteins secreted from other plant parasites (Dodds 2010). The close contact that effector proteins have with host factors forces them to diversify quickly to avoid detection by the plant immune system. Without an in depth

genome wide comparison it is difficult to say for certain that the 25A01 protein family is under increased selection pressure. However, the presence of multiple diverse members of this protein family fits the paradigm of how effector proteins evolve, and is another piece of evidence supporting the idea that this is a true family of effector proteins. Future investigations of the 25A01 family will be needed to identify all of the members of this family, as well as determine how broadly these proteins are conserved amongst other species of cyst nematode.

In order for a protein to be a true effector it must have a function within the host plant that promotes infection by the invading pathogen. In this study we have shown that *Hs*25A01 carries-out just such a function. Indeed, transgenic Arabidopsis expressing *Hs*25A01 showed a statistically significant increase in susceptibility to *H. schachtii* infection compared to the wild type Col-0 controls (Fig. 6). These results indicate that *Hs*25A01 performs a function *in planta* that enhances the nematode's ability to infect. These data provide strong evidence that *Hs*25A01 is a true effector protein with a functional role in the host-pathogen interaction.

In addition to affecting the plant's susceptibility to cyst nematode infection, we have also shown that *Hs*25A01 causes morphological changes when expressed *in planta*. All three Arabidopsis lines expressing *Hs*25A01 showed significantly increased root length compared with wild type controls (Fig. 5). Furthermore, the average root-length for each line positively correlated with the relative expression level of the transgene. The data clearly show that *Hs*25A01 changes the normal development of the host root, and the trend may indicate that it does so in a dose-dependent manner. The increase in root length and the concomitant increase in nematode susceptibility may lead to the hypothesis that increased root length

allows for increased nematode infection rates. However, this conclusion is not supported by previous studies of the *H. schachtii*-Arabidopsis interaction. Previous studies have found that cyst nematode infection does no correlate with root-length (Wubben, Su et al. 2001; Hewezi, Howe et al. 2008; Hewezi, Maier et al. 2012). Moreover, the penetration rate of J2 *H. schachtii* has been shown to be the same between wild-type and transgenic Arabidopsis roots with long root phenotypes (Hewezi, Howe et al. 2008; Hewezi, Maier et al. 2012), and the size of the nematode's syncytium has also been shown to be unrelated to root-length (Jin, Hewezi et al. 2011). This body of evidence leads us to conclude that the increase in nematode susceptibility observed in *Hs*25A01-expressing lines is not a result of the longer root length but is due to a virulence function carried out by the effector within the plant cell.

In order to produce the susceptibility and morphological phenotypes observed in *Hs*25A01-expressing lines, this effector protein must be causing developmental changes within the plant root that enhance the nematode's ability to infect. This conclusion is supported by observations in previous studies showing that the expression of *Hg*25A01 specifically increases during the early parasitic J2 stage of the nematode's lifecycle (Ithal, Recknor et al. 2007). This early stage of development coincides with the establishment of the feeding site, when the nematode must secrete effectors that facilitate the development of the syncytium. Indeed, other nematode proteins that are thought to support syncytium development show a similar expression pattern (Hewezi, Howe et al. 2008; Lee, Chronis et al. 2011). This same type of early up-regulation of the *Hs*25A01 transcript supports the conclusion that the effector may be enacting some developmental change within the root that enhances feeding site development.

To bring about developmental change within the plant, *Hs*25A01 must be interacting with some factor within the host plant. In an attempt to identify host protein(s) that are targeted by *Hs*25A01 we used this protein as the bait in a Y2H screen against prey libraries prepared from *H. schachtii*-infected Arabidopsis. After screening ~15.62×10^6 yeast colonies we were able to identify six full-length and three partial length candidate Arabidopsis proteins that showed a positive protein-protein interaction with *Hs*25A01 (Table 1). It is important to acknowledge that protein-protein interactions that take place within a yeast cell don't necessarily represent the same interactions that occur in a different biochemical environment (i.e. the plant cell) (Stephens and Banting 2000). As such, it is vital to confirm that *Hs*25A01 can successfully interact with the candidate Arabidopsis proteins *in planta*. In an attempt to confirm these interactions, we performed BiFC in onion epidermal cells, using *Hs*25A01 fusion construct co-bombarded with complimentary constructs containing the potential interacting proteins. Though one of the candidate proteins displayed positive interactions with *Hs*25A01 *in planta,* we also observed a positive interaction with two non-specific negative control proteins included in our BiFC assay (Fig. 7). This result indicates that the *Hs*25A01 fusion protein used in our BiFC experiments binds promiscuously to many proteins. As such, the positive results seen between *Hs*25A01 and our Arabidopsis proteins cannot reliably be considered specific.

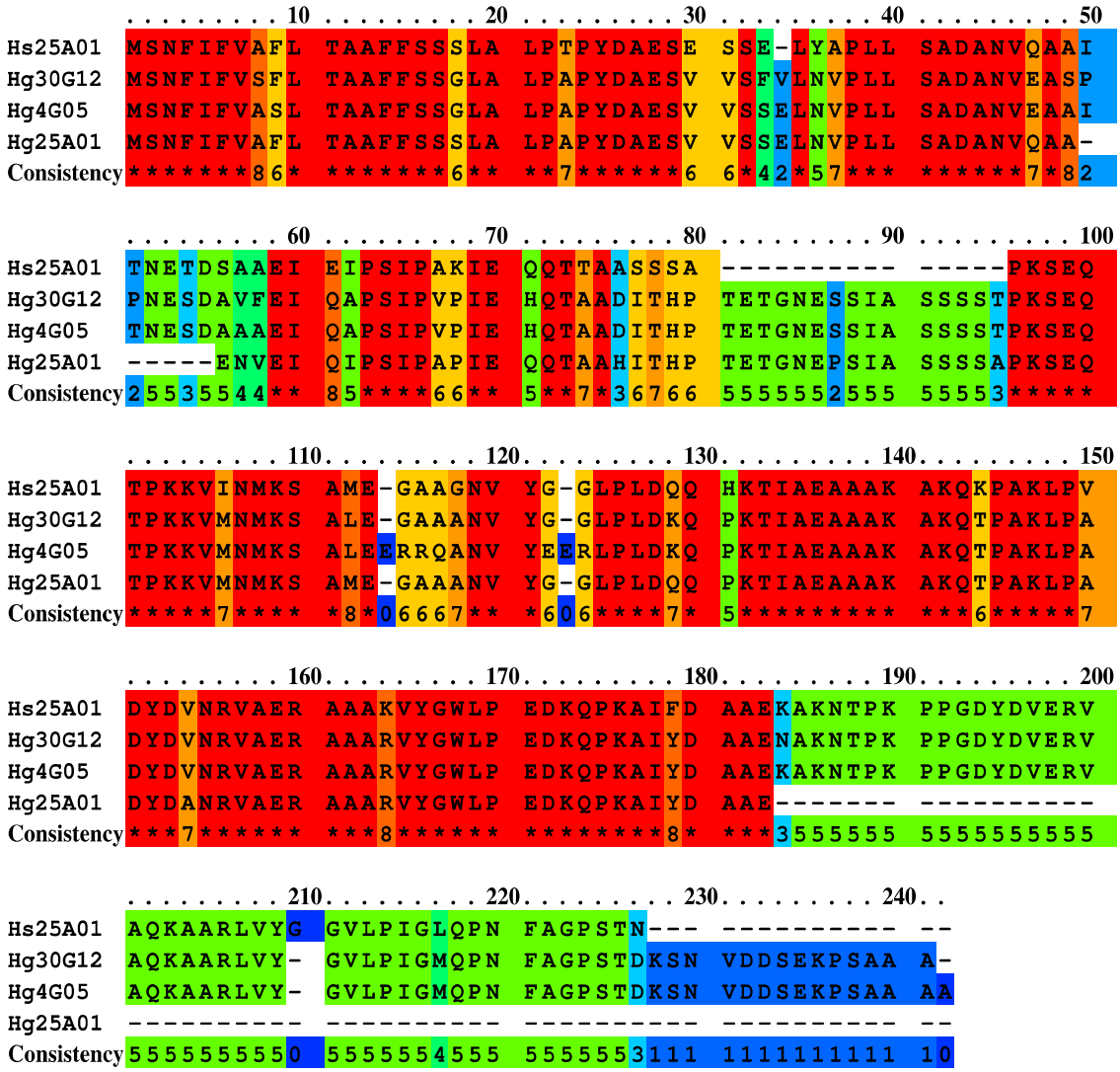
The non-specific BiFC results do not negate the validity of the interactions discovered in our Y2H screen. The complimentary fusion proteins used in BiFC assays have the potential to alter the affinity of protein-protein complexes (Kerppola 2009), and the promiscuous binding observed for the Hs25A01 fusion protein could be the result of such a disruption. Despite the inconclusive BiFC results, several of the interaction candidates from

our Y2H look like promising leads. Indeed, several of the identified candidates have been associated with plant diseases. Six of the nine candidates were identified as differentially expressed in a microarray experiment comparing Arabidopsis roots with and without *H. schachtii* infection (Table 1) (Szakasits, Heinen et al. 2009). Research into the potential functions of these plant proteins has revealed that several of these candidates have the potential to function in plant defense. One such candidate gene (AT5G13930) encodes a chalcone synthase, which is a key enzyme in the flavonoid biosynthetic pathway that produces important plant defense compounds, including salicylic acid and phytoalexins (Dao, Linthorst et al. 2011). If *Hs*25A01 was to interfere with the function of this chalcone synthase, it could conceivably affect the plant's ability to defend against nematode infection. Recently a homolog of another candidate gene identified in our Y2H screen eukaryotic initiation factor 2 (AT5G01940), was shown to play a role in the innate immune response in wheat and was shown to be specifically induced by the stripe rust pathogen *Puccinia striiformis* (Zhang, Hu et al. 2013). Yet another candidate interaction protein (AT1G78100) encodes an F-box protein. F-box-containing proteins direct specific proteins to the E3 ubiquitin ligase complex for ubiquitination and eventually degradation (Gagne, Downes et al. 2002). E3 Ubiquitin ligases have been shown to play important roles in plant defense as well as plant growth (Zheng, Miller et al. 2011; Li, Dai et al. 2012), and have been shown to be specifically targeted by effectors secreted by fungal pathogens (Park, Chen et al. 2012). Though we have not conclusively linked any of these candidate proteins to *Hs*25A01, any or all of them could conceivably be functional interacting partners of *Hs*25A01, and future research should take these candidates into consideration.

Though we were not able to confirm our Y2H results using BiFC, there are still several experimental approaches that could identify which plant factors *Hs*25A01 interacts with to carry out its effector function. One such alternative approach would be to use co-immunoprecipitation. This approach could utilize the same Arabidopsis lines expressing *Hs*25A0l that have already been used to assess nematode susceptibility and root length. Cell lysates from the *Hs*25A0l expressing lines could be probed using an antibody specific for *Hs*25A01. Any proteins bound to *Hs*25A01 would be simultaneously precipitated with our antibody-bound effector and could then be identified using mass spectrometry. One major drawback to this approach is the need for the production of an antibody. Another approach to identifying the functional interaction factors of *Hs*25A01 could utilize next generation RNA sequencing technology. By sequencing transcripts expressed in *Hs*25A01-expressing Arabidopsis lines alongside wild type controls it would be possible to see the changes in gene expression levels caused by the presence of the *Hs*25A01 protein. Comparing these data to our Y2H data could narrow the list of potential interaction candidates and point to those that are directly affected by the presence of *Hs*25A01. In addition to potentially identifying a differentially expressed protein that directly interacts with *Hs*25A01, this approach could have an added advantage of providing a global view of the plant gene expression altered by this effector. This broader profile could identify entire pathways within the plant that have been altered by *Hs*25A01 and perhaps provide a better understanding of how it enhances the nematode's ability to infect. Either of these approaches could provide important evidence for how *Hs*25A01 functions within Arabidopsis.

In summary, we have found compelling evidence suggesting that *Hs*25A01 performs an effector function *in planta*, increasing the susceptibility of the plant to *H. schachtii*

infection and at the same time inducing developmental changes within the host root. In light of what is already known about the orthologs of this protein in *H. glycines*, we think it is likely that the 25A01 family of proteins represents a group of effector proteins that have evolved specifically within cyst nematodes to enhance their ability to infect their host plants. Future investigations can build upon this work by confirming the conserved functionality of this family of proteins by determining the exact mode of action that these proteins use to carry out their effector function.



Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved

Figure 1. Amino acid alignment of the four full-length members of the 25A01 protein family cloned from both *H. glycines* and *H. schachtii* (PRALINE multiple alignment software). Consistency numbers denote the degree to which each residue position and its chemical properties are conserved within all four proteins. What do the colors mean?

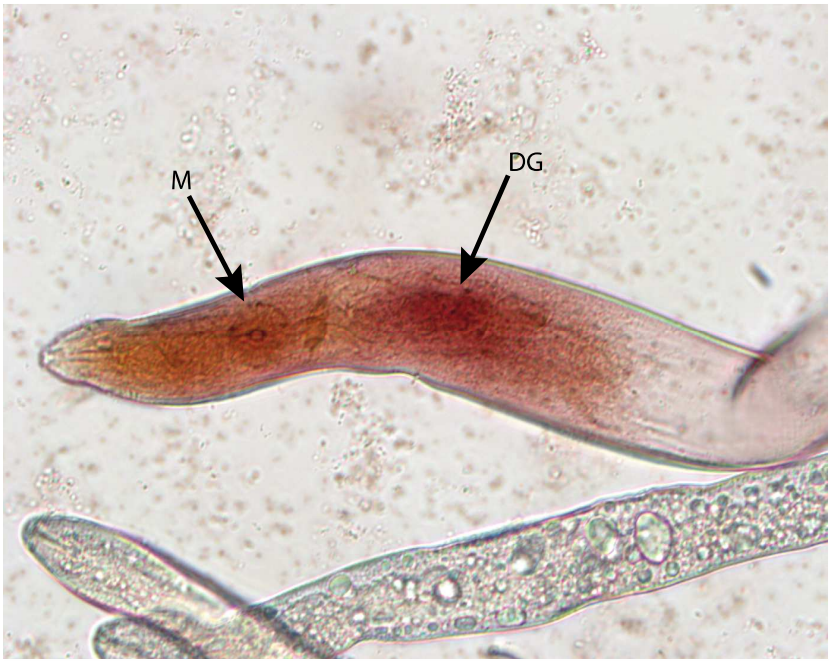


Figure 2. *In situ* hybridization of the *Hs*25A01 transcript within *H. schachtii* tissue. A DIG-labeled anti-sense probe, specific for the *Hs*25A01 transcript, was used to localize the *Hs*25A01 transcript within the dorsal gland cell (DG) of *H. schachtii*, which is located on the posterior side of the metacarpus (M).

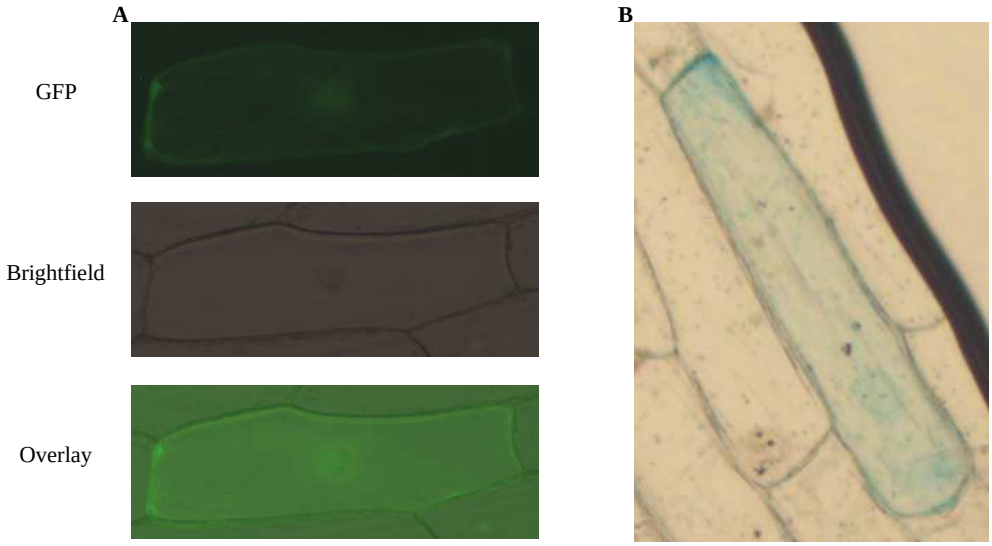


Figure 3. Sub-cellular localization of the *Hs*25A01 without a signal peptide in onion epidermal cells. A) Bombardment of the *Hs*25A01:GFP:GUS fusion constructs into onion epidermal cells produced a fluorescent signal that was visible within the cytoplasm. B) The cytoplasmic localization of the *Hs*25A01 fusion was also confirmed using the GUS reporter system.

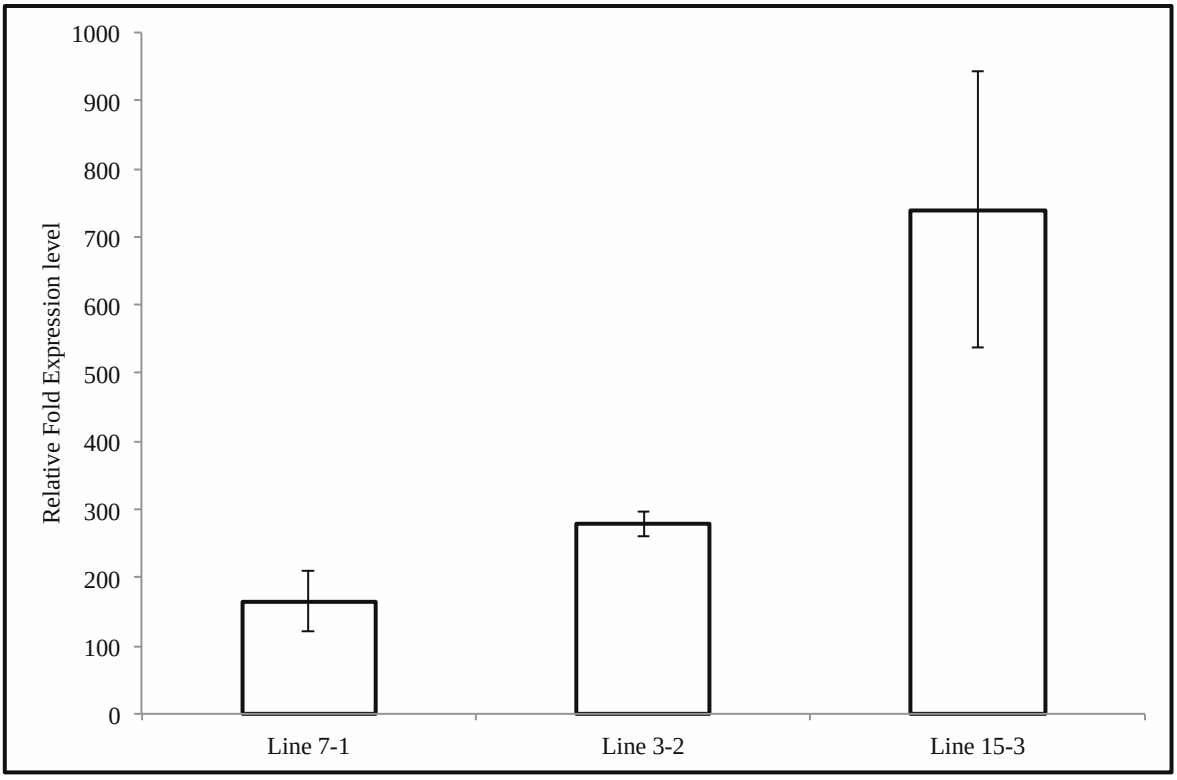


Figure 4. Comparison of the relative expression level of the *Hs*25A01 transcript within three independently transformed non-segregating T3 lines. Expression of the *Hs*25A01 transcript could be detected within the roots of all three lines, while no specific product could be detected within untransformed controls.

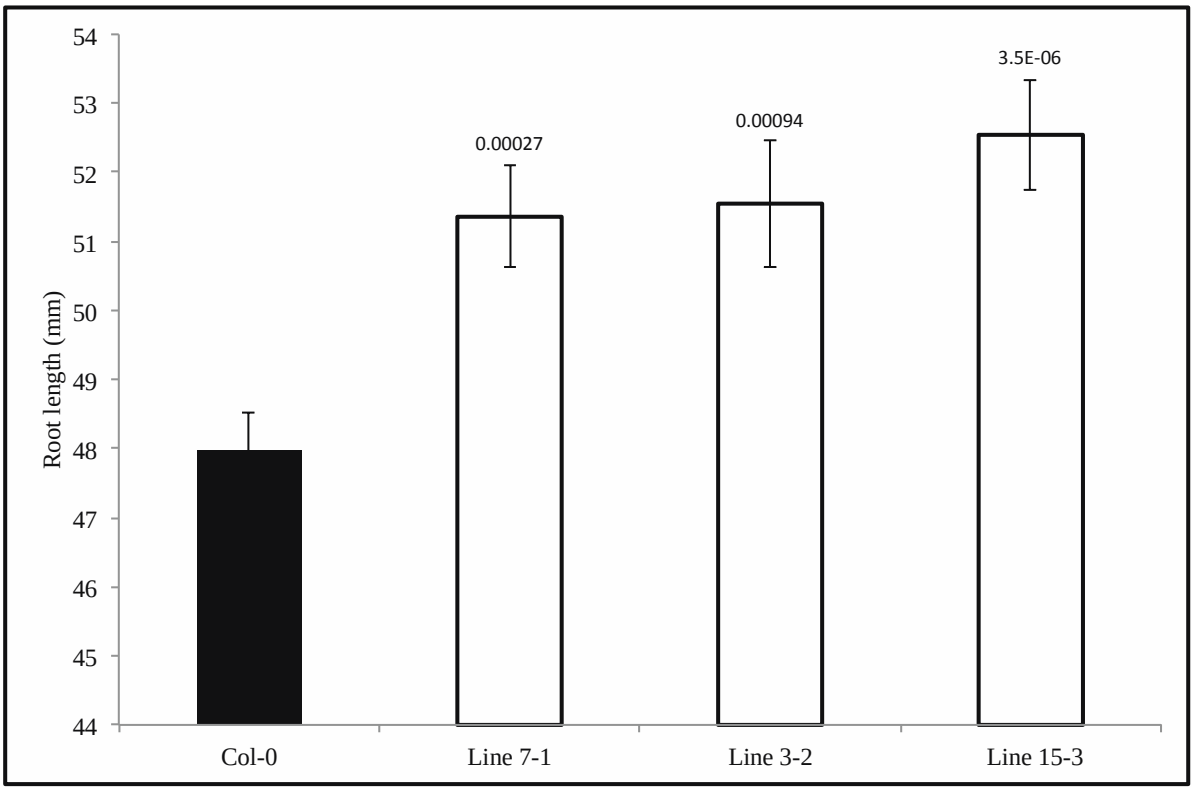


Figure 5. Comparison of the average root growth of *Hs*25A01-expressing lines compared to Columbia-0 control. Each transgenic line displayed significantly longer roots then controls (P-values displayed above each bar). The relative effect size for each line was positively correlated with its relative level of *Hs*25A01 expression.

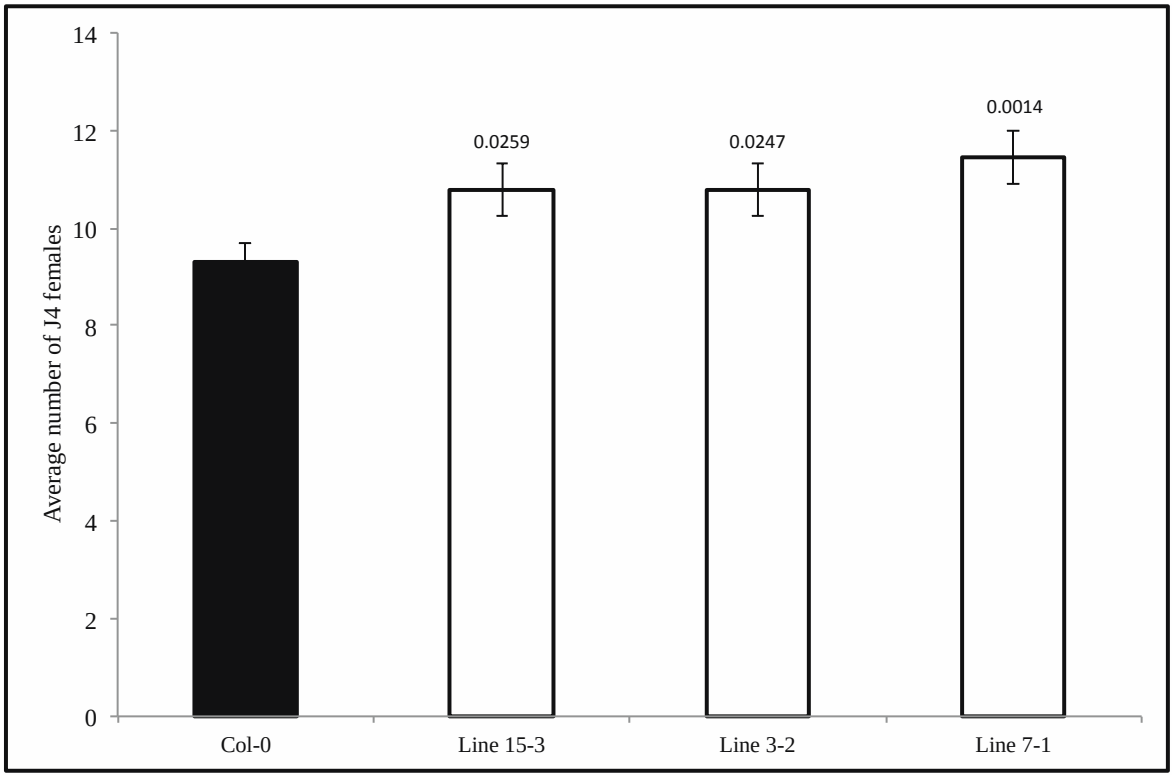


Figure 6. Susceptibility of *Hs*25A01-expressing lines to infection by *H. schachtii*. All three lines showed a significant increase (P-values above each bar) in the average number of developing J4 female nematodes compared to untransformed Columbia-0 controls.

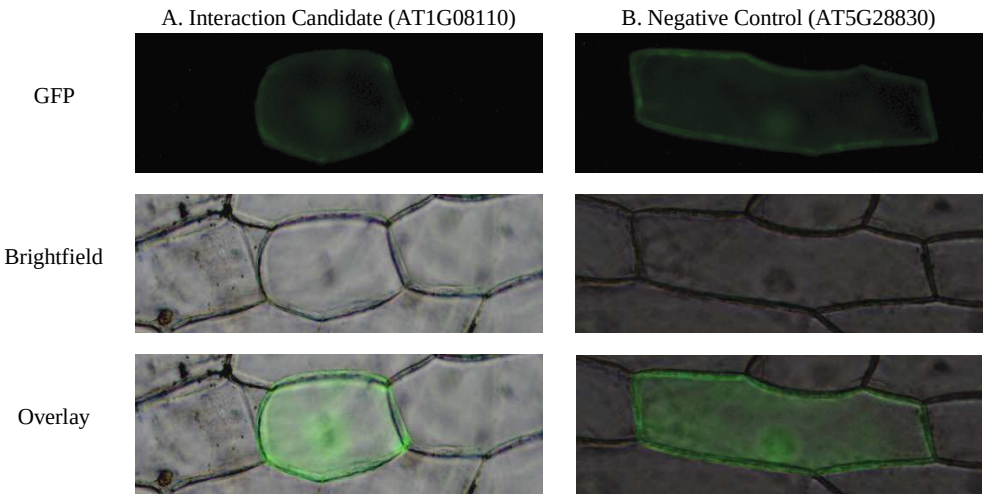


Figure 7. *in planta* BiFC assay of *Hs*25A01 with a putative interaction candidate. A) The co-bombardment of the fusion construct containing the N-terminal domain of YFP fused to *Hs*25A01 with a complementary fusion construct containing the C-terminal domain of YFP fused to the interaction candidate (AT1G08110) produced a strong fluorescent signal, indicating an interaction within the plant cell. B) The same *Hs*25A01 fusion construct was co-bombarded with the non-specific negative control protein (AT5G28830) fused to the complementary C-terminal domain of YFP. This co-bombardment also produced a fluorescent signal.

Table 1. Putative *Hs*25A01-interacting proteins from Arabidopsis. Each candidate was identified as a prey vector in a Y2H screen and then confirmed by co-transformation with the *Hs*25A01 bait vector. All clones showed enhanced growth on QDO media (SD-Leu-Trp-His-Ade) compared to empty bait vector and a non-specific negative control. The positive interaction for most of the candidates could also be detected as a blue color when the co-transformed yeast lines were grown on media containing X-alpha-galactose (X-Gal). * Qualitative expression levels within the syncytium was investigated for each Arabidopsis gene using previously published microarray data (Szakasits et al. 2009) which compared the expression levels of Arabidopsis genes within syncytia formed by *H. schachtii* with their expression levels in uninfected root cells.

Arabidopsis Gene	Gene description	Gene expression within syncytium*	Clone length	Yeast two-Hybrid	
				QDO	X-Gal
AT1G78100	F-box containing protein	Down regulated	Partial	Positive	Positive
AT1G07240	UDP-glucosyl transferase 71C5	Up regulated	Full-length	Positive	Positive
AT1G08110	lactoylglutathione lyase	Up regulated	Full-length	Positive	Positive
AT5G13930	Chalcone Synthase	Down regulated	Partial	Positive	Positive
AT4G24690	ubiquitin-associated (UBA) zinc-finger and PB1 domain-containing protein	Down regulated	Full-length	Positive	Positive
AT5G01940	translation initiation factor eIF-2 beta subunit	Up regulated	Full-length	Positive	Positive
At5G14040	mitochondrial phosphate transporter	No Difference	Full-length	Positive	Negative
AT1G33290	putative stage III sporulation protein	No Difference	Partial	Positive	Positive
AK230153	Hypothetical Protein	Not Detected	Full-length	Positive	Negative

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CHAPTER 4. MEMBERS OF THE MAJOR AVIRULENCE PROTEIN FAMILY, SECRETED FROM MELOIDOGYNE SPECIES, CONTAIN MULTIPLE CLE-LIKE MOTIFS

Modified from a paper to be submitted for publication to *Molecular Plant Pathology*

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Introduction

Cell-to-cell communication through small-secreted peptides has emerged as an important signaling mechanism in plant growth and development. One important gene family, the CLAVATA/ESR-related (CLE) gene family, encodes a large and diverse group of small signaling peptides found ubiquitously in plants (Cock and McCormick 2001; Oelkers, Goffard et al. 2008; Katsir, Davies et al. 2011). The Arabidopsis genome alone encodes 32 known CLE genes, which share similar 14 amino acid CLE motifs. These motifs are processed into 12 or 13 amino acid secreted peptides that act as ligands for Receptor-Like Kinases (RLKs), which subsequently incite developmental changes within the cell (Fiers, Golemiec et al. 2006; Ito, Nakanomyo et al. 2006). The nature of the developmental changes enacted by these small peptides varies depending on their amino acid sequence, how they are processed, as well as the expression patterns of the CLE genes within the plant (Strabala, O'Donnell P et al. 2006; Whitford, Fernandez et al. 2008; Jun, Fiume et al. 2010). The best-studied CLE protein, CLAVATA3, modulates stem-cell proliferation in the shoot and root

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[2]Intellectual and editorial contributions
[3]Intellectual and research contributions
[4]Intellectual contributions
[5]Intellectual contributions
[6]Intellectual contributions
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apical meristems (Fiers, Golemiec et al. 2006; Ito, Nakanomyo et al. 2006). While other CLE proteins have been shown to activate developmental pathways in the plant vasculature (Ito, Nakanomyo et al. 2006; Kinoshita, Nakamura et al. 2007; Katsir, Davies et al. 2011). Furthermore, certain CLE peptides have been shown to function in both the apical meristems and the vasculature (Whitford, Fernandez et al. 2008). Though it has been established that CLE signaling influences plant growth and development, the exact roles that most CLE genes play in modulating these processes remains only partially understood.

In an intriguing example of co-evolution of plants and parasites, it has become evident over the last decade that cyst nematodes (*Heterodera* and *Globodera spp.*), which are important sedentary plant-parasitic roundworms, secrete CLE ligand mimics as effector proteins into their host plants (Olsen and Skriver 2003). Interestingly, sedentary plant-parasitic nematodes developmentally reprogram plant cells to induce the formation of novel plant cell types that serve as feeding cells to the nematodes. In the case of cyst nematode parasitism, these feeding cells fuse and form a multinucleated syncytium (Golinowski, Sobczak et al. 1997). Similar to endogenous CLE proteins, cyst nematode CLE effectors are processed into functional 12 and 13 amino acid peptides after being secreted *in planta* and have been shown to enact plant developmental changes that facilitate parasitism. (Wang, Mitchum et al. 2005; Lu, Chen et al. 2009; Wang, Lee et al. 2010; Replogle, Wang et al. 2011). Indeed, cyst nematode CLE effector genes can rescue Arabidopsis CLAVATA3 mutant phenotypes (Wang, Mitchum et al. 2005; Lu, Chen et al. 2009; Wang, Lee et al. 2010; Wang, Replogle et al. 2011), suggesting that these effectors can mimic CLE-dependent signaling *in planta*.

All sedentary plant-parasitic nematodes deliver effectors into their host plant tissues. These proteins alter normal host development to induce the formation of highly specialized feeding cells (Hewezi and Baum 2012). The ability to engage in this type of sophisticated plant-parasite interaction has evolved in several groups of nematodes, the most prominent of which are the root-knot nematodes (*Meloidogyne* spp.), a large assembly of taxa that contains the world's most notorious and damaging plant-parasitic nematodes. As with cyst nematodes, root-knot nematodes induce the formation of modified feeding cells (called giant-cells) within the plant, though with etiology and structure distinct from that of the syncytia induced by cyst nematodes. Despite the analogous parasitic strategies of root-knot and cyst nematodes, only a few common effectors have been shown to be secreted by both groups (Gao, Allen et al. 2001; Huang, Gao et al. 2003; Hassan, Behm et al. 2010). Given the importance, variability, and abundance of CLE-mimicking effectors in cyst nematodes, it is surprising that no obvious and abundant ligand mimics have yet been identified in root-knot nematodes.

Here we report that the previously described family of proteins secreted from root-knot nematodes, the Major Avirulence Proteins (MAPs), contain CLE-like motifs. These findings suggest that root-knot nematodes may use ligand mimics to tap into plant signaling pathways in a manner similar to cyst nematodes.

Results

We are continuously mining *M. incognita* sequence data to confirm new effector identities. While scrutinizing a group of newly confirmed *M. incognita* effectors (unpublished data) we found that two proteins (designated Minc04584 and Minc00344 by the

M. incognita genome sequencing consortium (Abad, Gouzy et al. 2008; 2009) contain repetitive 14 amino acid motifs with sequence similarities to known CLE peptides. Interestingly, Minc04584 and Minc00344 belong to the MAP gene family containing the previously reported Major Avirulence Protein (MAP-1) secreted from *M. incognita* (Castagnone-Sereno, Semblat et al. 2009; Tomalova, Iachia et al. 2012). MAP-1 is the type member of the gene family and was identified as being differentially present between virulent and avirulent lines of *M. incognita* (Semblat, Rosso et al. 2001). While MAP proteins are thought to function in the interaction between *M. incognita* and its plant hosts, their exact mode of action remains unknown. Furthermore, while multiple members of this gene family have been reported in multiple *Meloidogyne* species, the presence of CLE-like repeats has been overlooked.

The similarity between our two newly confirmed MAP-family effectors and CLE motifs prompted us to investigate the extent to which CLE–like motifs are conserved in the MAP protein family. Previous studies have identified eight MAP-family members from *M. incognita* (Minc00365, Minc10365, Minc10366, Minc00158, Minc00344, Minc04584, MAP-1.2, CAC27774.1) (Semblat, Rosso et al. 2001; Castagnone-Sereno, Semblat et al. 2009). Our analysis was extended to also include four MAP proteins from *Meloidogyne javanica*, which were available in the NCBI protein database (CAP59537.1, CAP59538.1, CAP59536.1, and CAP59535.1). We also included a previously reported *Meloidogyne* effector protein (16D10) that appears to have a single conserved CLE domain, although its function has been shown to be very different from that of the cyst nematode CLE effectors (Huang, Dong et al. 2006). Using the MEME software suite (Bailey, Boden et al. 2009) we screened all of the 12 *Meloidogyne* MAP proteins and 16D10 for the presence of common

motifs. Using a 12 amino acid search window, the MEME software identified similar CLE-like motifs conserved in all of the MAP family proteins (Fig. 1A). This motif was repeated a variable number of times within each of the MAP family members. While Minc10365 contained only a single CLE-like motif, nine CLE-like repeats were found in Minc00365. In all, we discovered a total of 43 separate CLE-like motifs among the 12 MAP proteins. Many of these motifs were identical, even when compared between the proteins from *M. javanica* and *M. incognita*. After removing redundant copies, a total of 14 unique CLE-like variants were identified and were named MAP.V1 to MAP.V14 (Fig. 1B).

CLE motifs are inherently hard to identify. The 178 known CLE proteins from plants can only be grouped by the common 14 amino acid CLE motif, and within this motif there is no single amino acid that is conserved throughout the entire gene family. Indeed, studies of plant CLE motifs have identified only 6 well-conserved residues (invariant residues) that are considered hallmarks of the CLE family (Oelkers, Goffard et al. 2008). Because of the central location and relatively high conservation of glycine within the CLE motif, this residue is used as a reference point for locating the other invariant residues (R_{-5},P_{-2},G_{0},P_{+1},P_{+3},H_{+5}).

To quantify the similarities between the MAP CLE-like motifs and known CLE peptides, MAP.V1 through V14 were aligned with known CLE motifs from plants as well as from cyst nematode species (Fig. 1B). Examination of the alignment revealed that 5 of the 6 invariant residues found in CLEs (R_{-5},P_{-2},G_{0},P_{+1},P_{+3}) are also present in the MAP CLE-like motifs to varying degrees. Furthermore, all 43 identified CLE-like motifs contain at least three of the six invariant residues. The central highly conserved glycine (G_{0}) and proline (P_{+1}) in plant CLEs are also present in all 43 CLE-like motifs in the MAPs. 35 of the CLE-like motifs also contain the invariant proline (P_{-2}). The invariant arginine (R_{-5}) of most plant

CLEs, is consistently found one residue back in 40 of the MAP motifs (R_{-6}). This is consistent with the positioning of arginine (R_{-6}) in CLE39 from *Medicago truncatula* and several other plant CLEs (Oelkers, Goffard et al. 2008). The invariant histidine (H_{+5}) is the only residue that is not present in any of the *Meloidogyne* CLE-like motifs. In total, 27 of the motifs in the MAP proteins contain five of the six 'invariant' CLE residues (Fig. 1B). This alignment shows that sequence divergence within MAP CLE-like motifs resembles the natural variation found in known CLE motifs from plants and cyst nematodes. The presence of the prolines (P_{-2} and P_{+1}) are of particular significance. Both of these residues have been shown to be hydroxylated or arabinosylated *in planta*, and these post-translational modifications have been shown to enhance the interactions between CLE peptides and their cognate RLKs (Kondo, Sawa et al. 2006; Ohyama, Shinohara et al. 2009)

In addition to the CLE domains, the CLE effector proteins secreted from cyst nematodes also contain variable domains (VDs), which share little or no sequence homology with plant CLE proteins or with other cyst nematode CLE effectors. Despite their lack of sequence conservation, VDs are documented as having regulatory functions that are required for the CLE effectors to function *in planta.* Indeed, previous research has shown that VDs function in trafficking of CLE effectors to the host apoplast, processing of mature CLE peptides, and may also determine host specificity (Wang, Lee et al. 2010; Guo, Ni et al. 2011). In the MAP proteins, we observed that there is additional repetitive sequence outside the CLE-like domains where the amino acids tended to be less well conserved. Therefore, we searched the MAP proteins and 16D10 for the presence of variable domain like sequences, which could corroborate the hypothesis that Meloidogyne MAP effectors function in a manner similar to known cyst nematode CLE effectors. We identified two separate MAP

family motifs that show sequence similarity with the variable domains of cyst nematodes, while no such domains are present in 16D10. Curiously, one MAP motif was found only in *Heterodera* CLE protein VDs, while the other one was found only in *Globodera* CLE VDs. We named these two conserved motifs *Heterodera* Variable Domain Like Motif (HVLM) and Globodera Variable Domain Like Motif (GVLM) respectively. HVLM is a stretch of 15 amino acids (G78-P92 within *Heterodera* CLE effectors) in the variable domains of all reported *Heterodera* CLE effectors. HVLM occurs multiple times within 7 of the 12 MAP proteins searched (Minc00365, Minc00158, Minc00344, MAP-1.2, CAC27774.1, CAP59537.1, CAP59538.1), and was interspersed between the CLE-like motifs (Fig. 2A). Sequence alignment of the HVLMs from MAPs with those found in *Heterodera* VDs revealed that five of the 15 residues are perfectly conserved, and an additional five residues have conserved properties (Fig. 2B) ($\leq$ 0.5 Gonnet PAM 250 matrix).

GVLM is a 19 amino acid stretch that occurs in the variable domains of *G. rostochiensis* CLE effectors. We found GVLM directly downstream of the CLE-like repeats in nine of the twelve MAP proteins (Minc10365, Minc10366, Minc00158, MAP-1.2, CAC27774.1, CAP59537.1, CAP59538.1, CAP59536.1, and CAP59535.1) (Fig. 2A). When comparing the GVLMs from the MAP proteins with those from the *G. rostochiensis* VDs, we found that seven GVLM residues are perfectly conserved and an additional five residues have conserved properties (Fig. 2C) ($\leq$ 0.5 Gonnet PAM 250 matrix).

Of the 12 MAP proteins, only MAP-1 from *M. incognita* and one MAP protein from *M. javanica* have been subjected to limited functional characterization. MAP-1 has been shown to be secreted from infective nematodes into plant tissues (Semblat, Rosso et al. 2001; Vieira, Danchin et al. 2011). Surprisingly, in *M. incognita* the MAP-1 protein was shown to

be secreted from the amphids, chemosensory organs in the nematode head region (Semblat, Rosso et al. 2001; Vieira, Danchin et al. 2011), while the *M. javanica* gene was shown to be specifically expressed in the subventral esophageal glands of *M. javanica* (Adam, Phillips et al. 2009). These esophageal gland cells are the main sources of secreted effector proteins from both root-knot and cyst nematodes (Gao, Allen et al. 2001; Huang, Gao et al. 2003). To determine where the other members of this protein family are expressed, we performed *in situ* hybridization on mixed developmental stages of *M. incognita* juveniles using probes specific for three MAP family members (Minc00158, Minc00344, and Minc04584). We were able to localize these three MAP family members specifically to the subventral glands of J2 nematodes (Fig. 3). Given the specific expression in the esophageal gland cells, the MAP family proteins are likely to be secreted through the nematode's stylet into host plant cells during parasitism.

Discussion

Given the fundamental roles of CLE signaling in plant development, it would not be surprising to find that root-knot nematodes have evolved a mechanism to co-opt CLE-dependent signaling pathways into their mode of parasitism. Previous attempts have been made to identify CLE-motifs in root-knot nematodes, but the small size of the CLE-like motifs in MAPs, as well as their atypical arrangement, could easily cause them to be overlooked. Indeed, our discovery of these motifs could be called fortuitous, and more in depth studies may reveal as yet undiscovered CLE motifs in other root-knot nematode effectors. It is intriguing to think that CLE-mimicry could be responsible for some of the drastic developmental changes seen in roots infected with *Meloidogyne* species. *Meloidogyne*

species infect a wide range of host plants, and the ubiquitous presence of CLE signaling proteins in diverse plant species makes these proteins an ever-present target for mimicry. Modulating CLE signaling in infected root cells could help to establish feeding sites and may help the nematode evade plant immune responses. Though the exact functional role of the MAP CLE-like motifs will require further investigation, it is clear that these proteins are playing an important role in facilitating parasitism. The secretion of MAP proteins from both the amphids (Semblat, Rosso et al. 2001; Vieira, Danchin et al. 2011) and the subventral glands (Adam, Phillips et al. 2009; this study) of infective second-stage juveniles (J2) suggests that these proteins may be involved in the early recognition stage between the host plants and the nematode and lends credibility to the hypothesis that these proteins are acting as ligand mimics binding extracellular host receptors. Indeed, immunodetection of the MAP-1 protein showed that it accumulates along the giant cell wall as well as within the apoplast (Vieira, Danchin et al. 2011).

The repetitive region of the MAP-1 gene, which contains the CLE-like motifs, was recently amplified from thirteen *Meloidogyne* species out of the twenty one that were searched (Tomalova, Iachia et al. 2012). These CLE-like regions within the MAP-1 protein are nearly devoid of mutations between species, indicating that they have a virulence function that has enhanced parasite fitness and enforced strong purifying selection to conserve these sequences. Furthermore, the presence of functional and diverse CLE mimics in cyst nematodes is a testament to the functional importance that the CLE signaling pathway can have in plant parasitism.

Though the majority of CLE proteins from plants contain a single CLE motif on their C-terminus, several have been documented as having multiple tandem CLE repeats, similar

to the *Meloidogyne* MAPs (Oelkers, Goffard et al. 2008). In nematodes, CLE effector proteins secreted by the potato cyst nematode (*Globodera rostochiensis*) contain CLE motifs that are organized in tandem repeats, and these effectors have recently been shown to be processed into functional CLE peptides *in planta* (Guo, Ni et al. 2011). It is plausible that the tandem CLE-like motifs seen in the MAP family proteins could be processed in a similar manner, in which individual motifs are cleaved and secreted to interact with cognate extracellular RLKs. Similar to *G. rostochiensis*, the existence of multiple non-identical CLE-like motifs in MAP proteins may reflect an evolutionary adaption to enable root-knot nematodes to infect a wide range of host species. In this context, one can expect that such motifs can function by mimicking host factors more effectively in one particular host plant than in others.

In contrast to the tandem CLE motifs found in *G. rostochiensis* effectors, the CLE-like motifs in the MAP proteins have additional repetitive sequences between each motif. It is conceivable that this additional sequence could have functions similar to the variable domains found in cyst nematode and plant CLE proteins. The variable domains of CLE proteins are thought to play roles in the processing of active CLE peptides and have been shown to effect host specificity in cyst nematode CLE effectors (Jun, Fiume et al. 2008; Wang, Lee et al. 2010). Consistent with this line of evidence, the HVLM was identified within the MAP proteins that have multiple CLE-like motifs, and is interspersed between individual CLE-like motifs (Fig. 2A). This type of arrangement could allow separate MAP CLEs to have their own processing signal. While there is no direct evidence to prove that the HVLMs in the MAP proteins function in CLE-like processing, the fact that they are closely

associated with the CLE-like motifs and share sequence homology with the variable domain region of cyst nematode CLEs, suggest that they may have regulatory roles.

The presence of CLE mimicking effectors in both cyst and root-knot nematode species begs the question: how did these genes arise? Outside of the three motifs, there is very little sequence conservation between the flanking protein sequences from *Heterodera*, *Globodera*, and the MAP proteins from *Meloidogyne.* This suggests that these CLE-like motifs may well have arisen through convergent evolution, where the nematodes independently evolved features similar to host factors. Another striking possibility is that these CLE proteins were acquired through horizontal gene transfer (HGT) from host genomes. A recent study identified multiple HGT events that have taken place in plant-parasitic nematodes and identified a large repertoire of cell wall-degrading enzymes that were acquired from bacteria (Danchin, Rosso et al. 2010). Indeed, this same study showed that some of the MAP proteins have regions outside their tandem CLE-like repeats that have similarities with expansins from fungi (Danchin, Rosso et al. 2010), though no expansin activity has yet been reported. With the nematode's propensity for HGT and the similarities that MAP proteins share with plant proteins, it's reasonable to assume that CLE mimicking effectors could represent yet another instance of HGT in nematodes.

In summary, CLE ligand mimics are known to be secreted by cyst nematodes to facilitate successful infection of their host plants. Here we have identified CLE-like motifs, as well as VLMs, in the MAP family proteins secreted from root-knot nematodes, a family of proteins that was previously known to be involved in the host-parasite interaction. We have shown that several members of this gene family are expressed in the esophageal gland cells, which produce many known nematode effectors, including the CLE-mimics found in cyst

nematodes. With the evidence at hand we hypothesize that the MAP family of proteins function as CLE-mimics, with roles analogous to the CLE-mimicking effectors secreted from cyst nematode species. The presence of CLE-mimicking effectors in both cyst and root-knot nematodes highlights a common host node that is targeted by two evolutionarily diverse groups of nematodes, and highlights the CLE signaling pathway as a node that may have evolved in other phytonematodes as well.

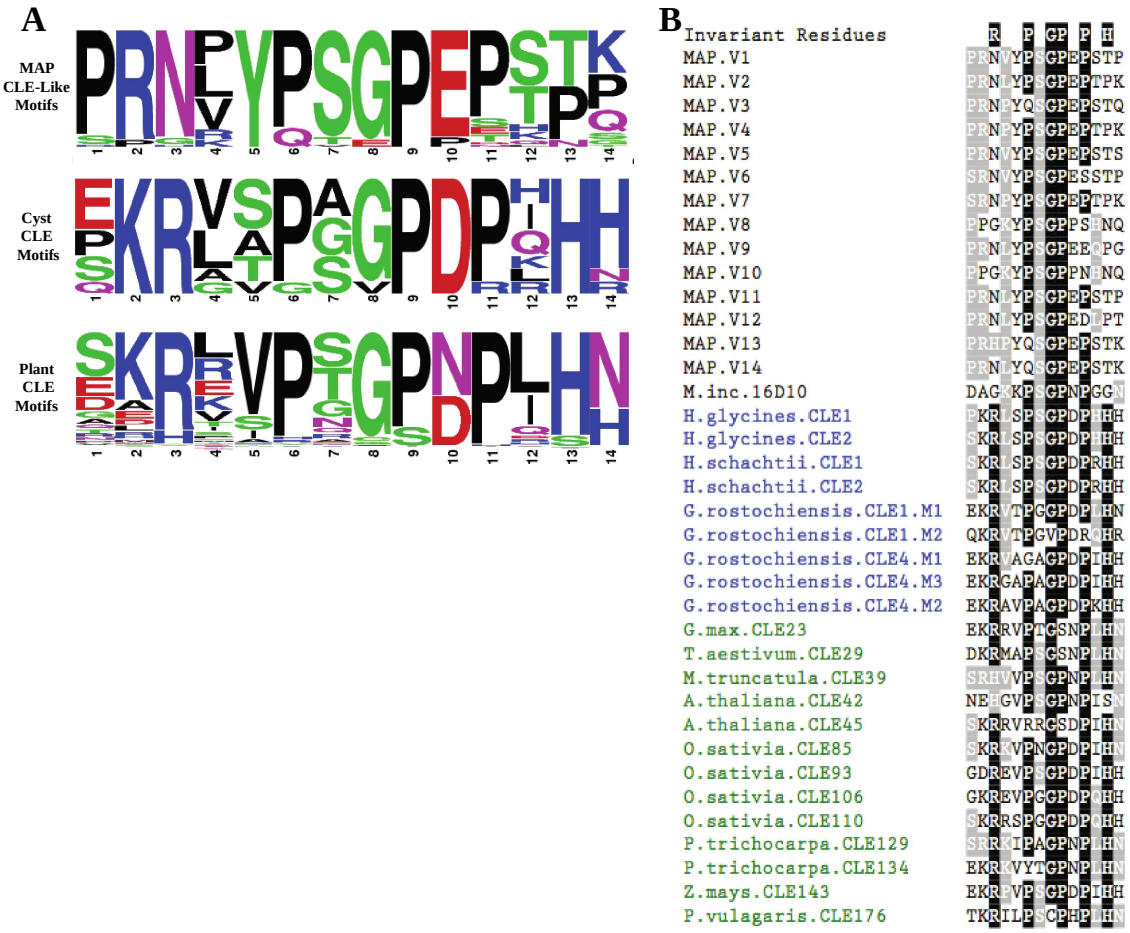

Figure 1. Comparison of CLE-like motifs in the MAP protein family to known CLE motifs in Cyst nematode and plant proteins A) Sequence logo comparison of the 43 individual CLE-like motifs identified in MAP proteins with the Sequence logos 9 known CLE motifs from cyst nematodes and 179 CLE from plants B) Sequence alignment of the CLE-Like motifs identified in the MAP proteins (MAP.V1-14) (black) and those identified in cyst nematodes (blue), plants (green), and the 16D10 effector protein from *M. incognita*. Residues that match invariant residues are highlighted in black, and other residues found in both CLEs and MAP proteins are highlighted in grey.

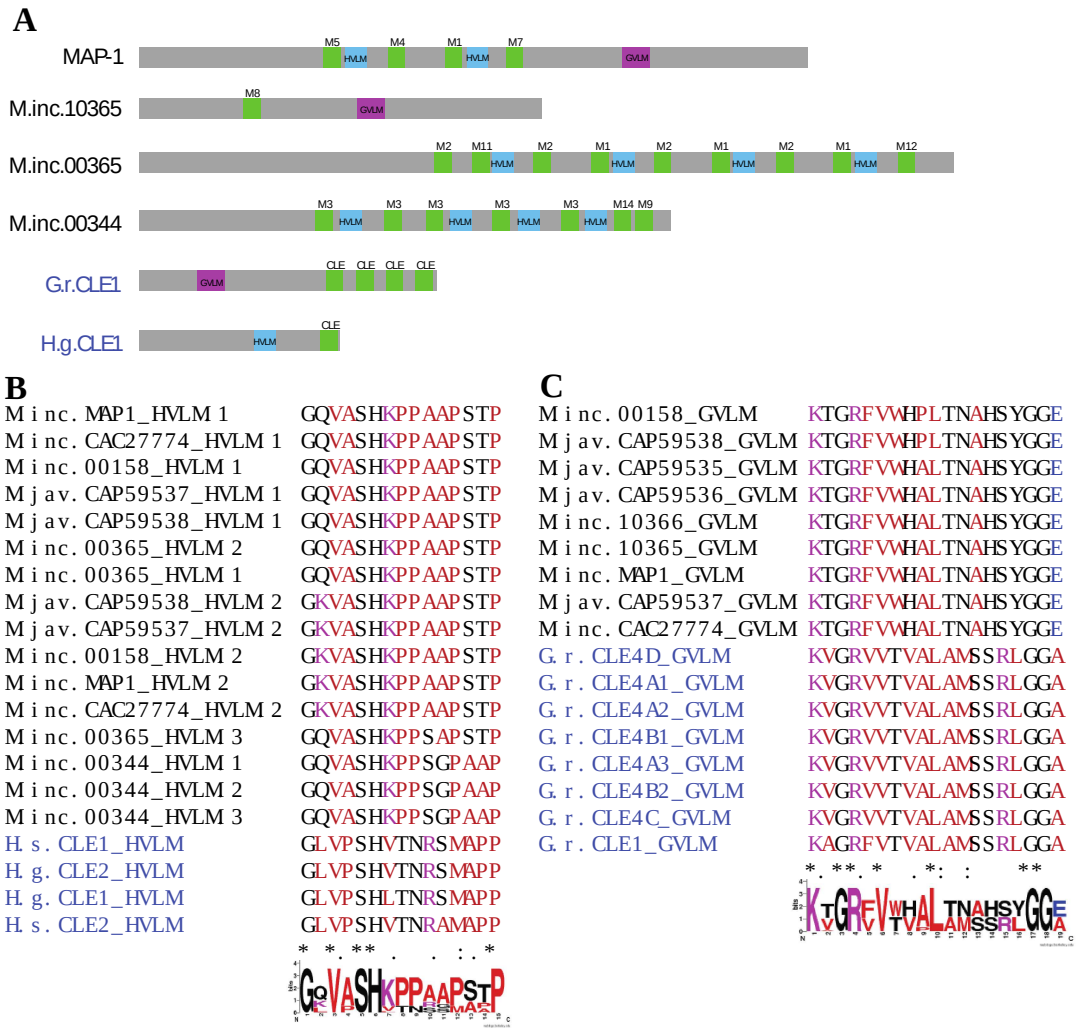

Figure 2. Identification of the HVLM and GVLM in MAP proteins and their distribution within MAP family proteins. A) Scaled representation of the relative locations of HVLM (Blue), GVDLM (Purple), and CLE-like domains (Green) within MAP proteins and cyst nematode CLE effectors. B) Sequence alignment of all HVLMs from 7 MAP proteins and those found in the CLE effectors of *Heterodera spp.* (Blue) along with a sequence logo highlighting the conserved residues among all motifs. C) Sequence alignment of all GVLMs from 9 MAP proteins and those found in the CLE effectors of *Globodera spp.* (Blue) along with a sequence logo highlighting the conserved residues among all motifs.

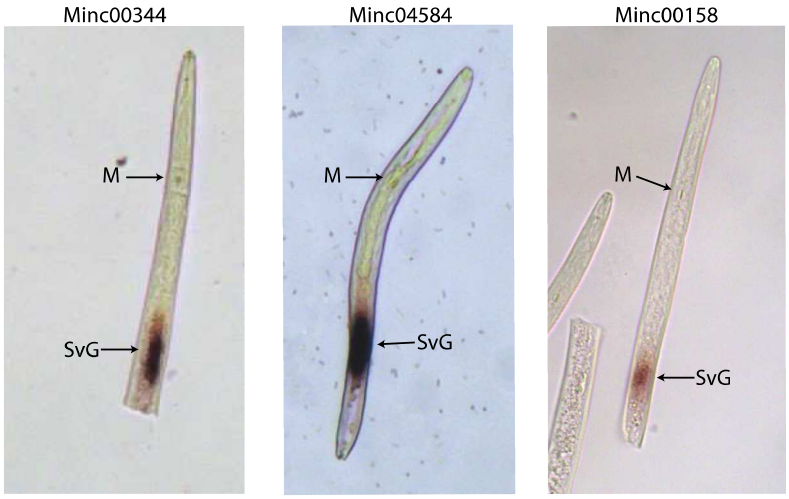

Figure 3. *In situ* hybridization of transcripts from three MAP family members. Labeled anti-sense probes specific for three separate MAP family members (Minc00344, Minc0458, and Minc00158) were used to localize the expression of their respective transcripts within *M. incognita*. All three MAP family members showed specific expression within the subventral gland cells (SvG), which are located posteriorly relative to the metacorpus (M).

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CHAPTER 5. MINING NOVEL EFFECTOR PROTEINS FROM THE ESOPHAGEAL GLAND CELLS OF *MELOIDOGYNE INCOGNITA*

Modified from a manuscript to be submitted for publication to *The Plant Journal*

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Abstract

M. incognita is one of the most damaging plant parasites on earth. Identifying and characterizing the effector proteins involved in the interaction between *M. incognita* and its host plant is an important step towards finding new ways to combat this pest. In this study we have identified 18 putative effector genes that have the potential to facilitate *M. incognita* parasitism and reproduction in host plant roots. These genes encode secreted proteins that are expressed specifically in the secretory gland cells of the nematode, indicating that they are available to interact with plant host factors. Many of the transcripts encoding these putative effectors are specifically up regulated during different stages of the nematode's life cycle, indicating that they may have a role at specific stages during *M. incognita* parasitism. Additionally, the predicted proteins show little to no homology to known proteins from free-living nematode species, indicating that they may well have evolved rapidly to evade the host immune system and may have functions specific for parasitism. By contrast, several of these

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[2]Intellectual and editorial contributions
[3]Intellectual and bioinformatic contributions
[4]Intellectual and research contributions
[5]research contributions
[6]Intellectual contributions
[7]Intellectual contributions
[8]Intellectual, editorial, financial contributions, and primary corresponding author

genes are part of gene families within the *M. incognita* genome as well as that of *M. hapla*, which points to an important role that these putative effectors are playing in both parasites. With the discovery of these putative effectors we have increased our knowledge of the effector repertoire utilized by root-knot nematodes to infect, feed, and reproduce on their host plants. Future studies investigating the roles these proteins play *in planta* will help us to mitigate the effects of this damaging pest.

Introduction

The southern root-knot nematode (*Meloidogyne incognita)*, is one of the most economically devastating plant pathogens in the world, and is able to infect nearly every cultivated crop species (Sasser and Freckman 1986; Koenning, Overstreet et al. 1999). The paucity of effective control strategies for this widely problematic pest makes it crucial to understand the factors that underlay its ability to infect plants, so that novel control strategies can be developed.

M. incognita engages in a complex interaction with its host plant. In order to survive, the parasite must penetrate a host plant root, overcome host defenses, develop an elaborate feeding site within the host (referred to as giant-cells), and maintain these giant-cells as it grows and reproduces. To perform these vital steps, the nematode secretes a cocktail of effector proteins that interact with and manipulate host plant factors. The functions that these effectors perform *in planta* facilitate the nematode's ability to infect its host (Hewezi and Baum 2012).

Previously characterized effector proteins from *M. incognita* have been shown to have diverse functions when secreted into the host plant. One such effector protein, 16D10,

has been shown to interact with a plant SCARECROW-like transcription factor, and in doing so modulates host development and increases the susceptibility of the host to *M. incognita* infection (Huang, Dong et al. 2006). *M. incognita* also secretes a calreticulin effector protein which accumulates in the plant apoplast (Jaubert, Milac et al. 2005) and has recently been shown to suppress basal immunity within its plant host to facilitate parasitism (Jaouannet, Magliano et al. 2012). Another group of effectors that is secreted from both root-knot and cyst nematodes (*Heterodera* and *Globodera spp.*) show homology to chorismate mutases and have been shown to alter plant development when expressed *in planta* (Bekal S 2003; Doyle and Lambert 2003). And yet another effector protein, *Mj-NULG*, was recently isolated from the closely related species *Meloidogyne javanica*. This effector was shown to be specifically up-regulated during feeding site development and localizes to the nucleus of the giant-cells, where it plays an important role in facilitating nematode parasitism (Lin, Zhuo et al. 2012). These recent characterization studies highlight the important roles these effectors play in specific plant subcellular compartments and at specific times during the parasites life cycle. However, the majority of *Meloidogyne* effector proteins remain undiscovered and uncharacterized. This stresses the need for a more complete knowledge of the *M. incognita* effector repertoire and a better understanding of how individual effectors function within host tissues.

Past efforts to identify effectors have utilized whole worm ESTs as well as proteomics approaches and have found a number of candidates (Bellafiore, Shen et al. 2008; Roze, Hanse et al. 2008). The most successful approach however has incorporated the isolation and sequencing of mRNA isolated specifically from the esophageal region of the nematode, which contains three gland cells (one dorsal and two subventral) that secrete

nematode effectors (Huang, Gao et al. 2003). This type of tissue-specific sequencing has been successfully implemented in both animals and plants to identify low abundance transcripts that would have been missed when sequencing transcriptomes of the entire organism (Brandt 2005; Chemello, Bean et al. 2011). This approach in conjunction with Sanger sequencing methods has identified 37 putative *M. incognita* effector proteins to date (Huang, Gao et al. 2003). Though this is an extensive list, the fact that there are currently 64 known effector proteins from cyst nematodes leads us to believe there should be a comparable number of effectors in *M. incognita*.

In an effort to search for previously undiscovered effectors from *M. incognita*, gland cell-specific mRNA was isolated and sequenced using Roche 454 technology, allowing for significantly greater read coverage than previous approaches. The resulting transcript data were analyzed using a combination of *in silico* and molecular approaches to identify 18 *M. incognita* genes encoding putative effector proteins that are expressed specifically in the esophageal gland cells.

The secreted proteins encoded by these *M. incognita* genes have no annotated function and no orthologs in free-living organisms, yet many are members of gene families and are conserved within the genome of *Meloidogyne hapla*, suggesting a conserved and specific function within the two parasites. Quantitative real-time RT-PCR allowed us to identify several unique expression profiles for these genes during the sedentary parasitic stages of *M. incognita* infection. Furthermore, subcellular localization revealed that the majority of these putative effectors localize to the cytoplasm *in planta*. Together our data suggest that these 18 *M. incognita* genes encode secreted proteins with specific roles at different stages of parasitism, and that the majority likely function within the cytoplasm of

the giant-cells. The discovery of these new putative effectors has expanded the known *M. incognita* secretome and laid the groundwork for a greater understanding of the molecular basis of nematode parasitism.

Materials and Methods

Microaspiration of gland cell region and mRNA extraction

M. incognita was grown on greenhouse tomato roots (*Lycopersicon esculentum* cv. Marion). Parasitic stage nematodes (J2-J4) were extracted from roots, surface sterilized, and imbedded in 0.7% agarose. A glass micropipette containing 10µL of mRNA extraction buffer was used to aspirate the gland cell cytoplasm from individual nematodes. In total two pools of gland cell cytoplasm, each derived from 50 individual nematodes, were collected and stored at -80°C until mRNA extraction as previously described(Huang, Gao et al. 2003). Poly(A) RNA was extracted separately from both the aspirated cytoplasm pools using Dynabeads Oligo $(dT)_{25}$ magnetic beads (Dynal, Lake Success, NY, U.S.A), and eluted with DEPC-treated ddH_20 as previously described(Gao, Allen et al. 2001). First-strand cDNA synthesis was then performed on 4µL of the pooled mRNA sample in a 10µL reaction, which also included 0.5µL 3'-RACE cDNA Synthesis Primer (10mM, Clontech Laboratories, Palo Alto, CA, U.S.A.), 0.5µL of SMART II oligonucleotide (10µM, Clonetech), 2.0µL of 5Xfirst strand buffer, first strand buffer, 1.0µL DTT (20mM), 1.0µL of dNTP (10mM), and 1.0µL Superscript II (200u/µL, GIBCO-BRL, Grand Island, NY, U.S.A). The SMART oligonucleotide system was used to enrich for full-length cDNA. The reactions were incubated for 1.5 hours at 42°C, after which 90µl of TE buffer (10mM Tris [pH7.6], 1mM EDTA) was added. LD-PCR was then performed using a 100µl reaction containing first-

strand reaction solution (10µl), 2µl dNTP (10mM), 10µl of TaqPlus Long low salt buffer, 1µl TaqPlus Long polymerase (Statagene, La Jolla, CA, U.S.A.), and 2µl of Nested Universal Primer (Clonetech). PCR was performed with a hot start followed by 24 cycles of 94°C (20sec), 65°C (30sec), and 72°C (6min). Negative controls using DEPC water were performed at each reaction step above.

454 sequencing and assembly

The gland cell derived cDNA library was split into two separate pools, one of which was nebulized to reduce the fragment size, and both were sequenced together using the Roche 454 platform (University of Iowa, Iowa City, IA). Sequencing produced 1.7 million total reads with an average length of 281bp. After removing short and low-complexity reads (Sequence Cleaner) 623 thousand reads were assembled using Newbler v2.5.

Sequence analysis and identification of candidate effectors

Isotigs were translated (Prot4EST V3.0b), and the Protein predictions were then compared against all the known proteins from both *M. incognita* and *M. hapla* genomes using a cut off of 95% ID over 75% of the length of the isotig (BLASTp) (Altschul, Gish et al. 1990). After the known proteins were Identified, they were analyzed using OrthoMCL algorithm, which in brief uses an all against all blast strategy to sort proteins from different organisms into groups of likely orthologs and paralogs (Chen, Mackey et al. 2006). By grouping the identified RKN proteins with the total known proteins from 18 other selected species (five free living nematode species, five parasitic nematodes, five Parasitic Helminths, the fly, and two host plant species) proteins which had orthologs in free living nematode

species were excluded, while those that grouped exclusively with proteins from parasites and/or host plant species were selected as candidates. As an added measure the effector candidates were then compared (BLAST) against free-living nematodes to assure that no consequential homology (over 35 bits) was present. Remaining candidates were analyzed for the presence of a signal peptide as well as the absence of a trans-membrane domain outside of the first 60 amino acids of the N-terminus (Phobius) (Kall, Krogh et al. 2004). This first list of previously annotated secreted candidates consisted of 91 full-length proteins.

In situ hybridization of effector transcripts

Forward and reverse primers were designed specifically for the coding sequence of each of the 91 candidate genes. These primers were used to amplify an amplicon 150-300 bp in length from cDNA pools generated from *M. incognita*. This amplicon was used as a template in a unidirectional PCR reaction to produce single stranded sense and anti-sense digoxigenin (DIG)-labeled probes for each candidate effector transcript. Unidirectional PCRs were performed in 25µl volumes using a DIG-nucleotide labeling kit (Roche). In situ hybridizations were performed on mixed parasitic stages of *M. incognita* as previously described (de Boer, Yan et al. 1998). Established parasitic populations of nematodes were extracted from the roots tomato (*Lycopersicon esculentum cv. Rutgers*) by maceration of the infected tissue followed by progressive sieving as previously described (de Boer, Yan et al. 1999). These parasitic stages were supplemented with freshly hatched pre-parasitic J2 stage *M. incognita* juveniles. Mixed stage nematodes were fixed in a 2% formaldehyde solution. Fixed nematodes were permeabilized by hand cutting with a razorblade on a glass slide in combination with a partial proteinase-K digestion (20mg/mL, 30 min RT). DIG-labeled

probes were hybridized to permeabilized tissue over night at 50°C. Hybridized probes within the nematode were detected using and anti-DIG antibody conjugated to alkaline phosphatase and it's substrate. Samples were then visualized using a Zeiss Axiovert 100 inverted light microscope.

Developmental expression patterns of putative effectors

To assess the developmental expression profile of our positive candidates we designed RT-PCR primers specific from 13 unique transcripts (one representative from each paralogous gene family). Four biological replicates of *M. incognita* were separately extracted at each of 5 time points during parasitism (Eggs, pre-parasitic J2, 3DPI, 7DPI, 14DPI, and 21DPI). Total RNA was extracted from each sample using the Perfect Pure RNA fibrous tissue extraction kit (5 prime), all according to the manufacturer's instructions. For quantitative RT-PCR approximately two ng of *M. incognita* mRNA was used for cDNA synthesis and PCR amplification in 15µl reactions using a one-step RT-PCR kit (Quanta) according to the manufacturers protocol. QRT-PCR reactions were run using an iCycler RT-PCR thermal cycler (Bio-Rad), using the following program: 50C for ten min, 95C for 5 min, and 40 cycles of 95C for 30 sand 60C for 30 s. Four replicated reactions were run for each primer pair for each mRNA sample. QRT-PCR data was analyzed using the iCycler IQ Optical Systems Software version 3.0a (Bio-Rad). Melting curves of all PCR reactions were assessed to assure specific product amplification. *M. incognita* actin and tubulin primer pairs were used as internal controls to normalize gene expression levels at each time point. Following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), relative fold changes were calculated using gene expression levels in the egg as a base line. P-values for each time point

were calculated using both the Tukey multiple comparisons test (95% family-wise confidence level) and Bonferroni pairwise comparisons. A significant difference (p-value<0.05) was considered biologically significant only if it was consistently seen in both the actin and tubulin normalizations.

Sub-cellular localization

Full-length coding sequences of 12 of putative parasitism genes were amplified from *M. incognita* cDNA, and cloned into the pGEMt-EZ vector (Promega) for sequencing. Confirmed clones were used as templates in PCR reactions to amplify fragments with out the signal peptide along with appropriate restriction sites for cloning into the pSAT6-eYFP vectors, obtained from ARBC. Cloning into the pSAT6-eYFP vectors created YFP fusion proteins under the control of the CaMV 35S promoter, which were confirmed by sequencing. Confirmed vectors were bombarded into onion epidermal cells via Biolistic bombardment as previously described (Elling, Davis et al. 2007). Bombarded onion cells were incubated for 16-24 h in the dark, then visualized using a Zeiss Axiovert 100 inverted light microscope.

Results

Sequencing and assembly

The cytoplasm of esophageal gland-cells was microaspirated from 50 *M. incognita* nematodes at different parasitic stages to isolate total RNA. The mRNA was then purified and used to generate two pools (one nebulized and one un-nebulized) of cDNA using the SMART system (CloneTech). Each cDNA pool was sequenced using Roche 454 technology, which produced a combined total of 1.7 million reads, with an average length of 281 bp.

Reads with insufficient complexity to facilitate assembly were removed (SeqClean software), leaving 623,000 clean reads. Out of these 623,000 high quality reads, which were assembled using Neubler V2.5 software produced 17,741 isotigs (unique transcripts), which were predicted to be contained within 14,443 isogroups (unique genes) alongside 72,397 singletons. To identify the full-length transcripts associated with the isotigs we compared the 17,741 isotigs with the 20,359 proteins predicted from the *M. incognita* genome (Abad, Gouzy et al. 2008) using BLASTx with a minimum cut-off of 90% identity over 75% of the isotig length (Altschul, Gish et al. 1990). We found that 5,700 gland isotigs (4,548 isogroups) had strong sequence homology to 3,758 known *M. incognita* proteins from the M. incognita genome (Fig. 1A). These full-length proteins were subjected to several criteria to identify novel effector candidates (see below).

In silico filtering of effector candidates

Most nematode effectors have evolved to become specific to the parasitic lifestyle, and are unlikely to have orthologs in free-living species. This has been demonstrated by the lack of sequence homology between the previously identified effector proteins from cyst and root-knot nematode parasites, and free-living species (Huang, Gao et al. 2003). To determine which of our 3,758 gland-derived candidate proteins had orthologous sequences that are specific to parasitic species and absent in free-living species, we utilized a dataset previously generated using OrthoMCL (Abubucker, Martin et al. 2011). This dataset contained the known proteomes from seven helminth parasites (*M. incognita, M. hapla, Trichinella spiralis, Brugia malayi, Pristionchus pacificus*, *Schistosoma japonicum,* and *Schistosoma mansoni),* two host plants (*Glycine max, Arabidopsis thaliana*), as well as proteins from nine

free-living species (*Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Mus musculus*, *Homo sapiens* and five *Caenorhabditis* species) (Chen, Mackey et al. 2006; Abubucker, Martin et al. 2011). The OrthoMCL algorithm clustered these 18 proteomes into 38,776 groups of orthologous and paralogous proteins. Of all the groups, 2,419 contained orthologous proteins only from parasites or host plant species, and lacked proteins from free-living *S. cerevisiae*, *Drosophila*, and *Caenorhabditis* species. We identified 4,073 proteins from *M. incognita* that were contained in these 2,419 parasite-specific groups. These 4,073 proteins, in addition to 5,180 *M. incognita* proteins that were not clustered with any other proteins, were combined (9,253 proteins) and compared to our 3,758 gland-expressed candidate proteins. This comparison resulted in the identification of 1,080 gland-derived proteins that seem to have evolved specifically for parasitic life-styles (Fig. 1B).

One of the hallmark characteristics of nematode effector proteins is the presence of a secretion signal peptide on their N-terminus that targets the mature protein to the secretory pathway, as well as the absence of an internal transmembrane domain that would retain the protein in membranes of nematode cells. Therefore, we analyzed 1,080 gland-derived proteins for the presence of a secretion signal and the absence of an internal transmembrane domain (Kall, Krogh et al. 2004). This selection procedure resulted in the identification of 91 candidate effector genes (Fig. 1C). This list of effector candidates was then subjected to RNA *in situ* hybridization to determine whether they are expressed specifically in the esophageal gland cells.

Because esophageal gland cells are directly connected through the esophagus to the nematode stylet, secreted proteins that are exclusively expressed in and secreted from these gland cells are believed to have biological functions in the nematode–plant interactions. To

determine the gland-specific expression patterns of the 91 candidate effector genes, we generated specific antisense digoxigenin (DIG)-labeled probes for each candidate and hybridized them to fixed *M. incognita* nematodes at different parasitic life stages. Out of the 91 candidates tested, probes for 14 individual candidate effectors were found that specifically hybridized to mRNA accumulating within the subventral (ten probes) or dorsal (three probes) gland cells of *M. incognita* (Fig. 2 and Table 1). Interestingly, another candidate protein (Minc00801) showed strong and specific expression in the rectal glands of J4 female *M. incognita* (Fig. 2Q). Since the rectal glands are known to form the egg mass matrix (Maggenti and Allen 1960), this candidate is potentially secreted into plant tissues and worthy of further analysis. In total, 14 candidates were identified as genes that are likely to have effector roles in *M. incognita* parasitism of host plants (Table 1).

Sequence analysis of these 14 candidate effector genes revealed that two of these proteins, Minc10418 and Minc08073, had 98% amino acid identity, indicating that they are likely paralogous members of a gene family within *M. incognita*. Two other proteins, Minc00344 and Minc04584, were previously identified as members of the MAP-1 gene family, which are thought to play roles at the early stages of interaction between *M. incognita* and its host plant (Semblat, Rosso et al. 2001; Castagnone-Sereno, Semblat et al. 2009). Two additional proteins, Minc02097 and Minc18033, showed near perfect identity with the putative esophageal gland cell effector proteins 35A02 and 19F07, respectively (Huang, Gao et al. 2003). Finally, eight of the 14 candidate effectors showed no significant sequence similarity to any proteins in the NCBI non-redundant protein database, and therefore were designated novel effectors.

The 14 candidate effectors were also analyzed for the presence of known motifs and domains using InterPro Scan (Quevillon, Silventoinen et al. 2005). Only one putative effector (Minc01696) was predicted to contain a known functional protein domain. This protein contained a region of 337 amino acids with strong sequence similarity (9e-27) to TTK dual-specificity protein kinases from multiple metazoan species. This region was located 17 residues from the predicted signal peptide cleavage site and was predicted to contain a complete protein kinase catalytic domain (PS50011 InterProScan, Score=29.997). This putative effector also contained an additional 210 residues on its C-terminus, which showed no homology to any known protein (Table 1).

Putative effectors have paralogs in *M. incognita* and orthologs in related parasites

Pathogen effectors that play essential roles in parasitism are often represented by multiple paralogous copies within their respective genome (Haas, Kamoun et al. 2009; Raffaele, Farrer et al. 2010). These paralogs are thought to have redundant and/or specific functions, and may promote plasticity in effector function. To determine whether the newly mined putative effectors have paralogs, the protein sequences of these 14 putative effectors were used as queries in blast searches against the known proteins from the *M. incognita* genome. Interestingly, four of these proteins showed significant blast hits (BLASTp >150bits, 50%ID) to other proteins with predicted signal peptides, making them good effector candidates as well.

To determine if these new paralogs had been detected in the previous *in situ* hybridization, the probes used to identify the original parasitism genes were compared to the coding sequences of the newly identified paralog candidates. One paralogous pair,

Minc18861 and Minc11817, showed perfect coding sequence homology. Indicating that the original probe used was able to cross-hybridize with, and localize, both transcripts within the gland cells. The other three pairs of paralogs showed significant polymorphisms in the region of the original *in situ* hybridized probe. To assure that all of the secreted paralogs were indeed expressed specifically in the secretory gland cells, new probes were designed specifically to hybridize to each paralog. When these probes were used in *in situ* hybridization on mixed stage *M. incognita* tissue, all three paralogous genes were shown to be specifically expressed in the same esophageal gland cells as their originally discovered paralog (Table 1, Fig. 2). This raised the total number of newly discovered putative effectors to 18 genes within *M. incognita*.

Genome comparison of *M. incognita* with the closely related root-knot nematode *M. hapla* revealed that both genomes contain orthologous sequences (Bird, Williamson et al. 2009)**.** To determine whether the newly identified putative effectors have orthologs in *M. hapla*, each was blasted against the predicted proteins from the *M. hapla* genome (Opperman, Bird et al. 2008). Importantly, nine of the *M. incognita* effectors had homologs (BLASTp >35bits, 50%ID) in *M. hapla*, which were also predicted to contain secretion signal peptides. These proteins are likely part of orthologous effector families that carry out similar functional roles both in *M. hapla* and *M. incognita* parasitism.

Putative effectors localize to the cytoplasm *in planta*

The *in planta* subcellular localization of nematode effectors provides clues to their site of action and the identity of potential host targets. To determine the cellular compartments where the newly identified nematode effectors would localize, the full-length

coding sequence (minus signal peptides) were fused to GFP and GUS reporter genes under the control of the cauliflower mosaic virus (*CaMV*) 35S promoter. These constructs were delivered into onion epidermal cells by biolistic bombardment. All of the fusion proteins showed cytoplasmic localization in the plant cells (Fig. 3). These data are consistent with the cytoplasmic localization prediction using Psort II software (Yu, Wagner et al. 2010).

Putative effectors are differentially expressed at different stages of *M. incognita* parasitism

Nematode effector genes are known to be developmentally regulated, with increasing mRNA abundances during the parasitic stages. The developmental expression profiles of 13 of the newly identified parasitism genes were quantified using real-time RT-PCR in eggs, pre-parasitic J2, and infective *M. incognita* stages at 3, 7, 14, and 21days post infection (DPI) (Fig. 4). All 13 putative effector genes displayed the lowest detectable expression levels in eggs. In contrast, the expression patterns during other developmental stages varied widely, with different effectors showing peak expression during the early parasitic stages (3DPI) or during late parasitic stages (14-21DPI), supporting the hypothesis that these effectors play diverse roles in host parasitism (Fig. 4).

Discussion

Previous studies have successfully identified putative effector proteins that are specifically expressed in the esophageal gland cells of *M. incognita* and other plant-parasitic nematodes (Gao, Allen et al. 2001; Huang, Gao et al. 2003). However, the complexity of the plant-nematode interaction indicates that the *M. incognita* effector repertoire is more extensive than previously described. To expand the known effector repertoire of *M. incognita*

we isolated mRNA from the esophageal gland cells, and applied Roche 454 sequencing technology to obtain high read coverage. We then took advantage of the availability of the whole genome sequence of *M. incognita* (Abad, Gouzy et al. 2008) to identify full-length coding sequences of our gland-derived reads. This approach facilitated the bioinformatic identification of candidate effectors based on homology models and the presence of an N-terminal signal peptide sequence. Combining this bioinformatic approach with an *in situ* hybridization screen resulted in the identification of 18 putative effector genes, whose expression was specifically localized to the esophageal gland cells of *M. incognita.*

As in previous studies, the majority of these proteins are unique to *M. incognita*, showing no homology to proteins in the non-redundant database and containing no detectable functional domains. Only a few of the effector proteins secreted from any parasite are widely conserved, and more often than not, they are exclusively produced by one pathogen or a group of closely related pathogens. Indeed, recent studies of effectors from both fungal and oomycete pathogens have shown they are under increased diversifying selection pressure to avoid the host immune system (Haas, Kamoun et al. 2009; Schirawski, Mannhaupt et al. 2010). As a result of this increased selection pressure, effector proteins quickly evolve to become specific to certain parasites and tend to show little homology with other proteins. The unique nature of effector proteins often makes it difficult to predict their functions and stresses the need for functional characterizations of each individual effector.

Despite the lack of sequence homology for most of the putative effector proteins, one positive candidate, Minc01696, was predicted to contain a complete kinase domain (InterPro scan and CDD). This predicted kinase domain has high homology to the unique dual-specificity kinase domains of the MPS1 family of protein kinases. MPS1 kinases are

conserved in most eukaryotes, and members of this family have been shown to play important roles in modulating mitosis (Liu and Winey 2012). In fact, it has been recently shown that increased levels of MPS1 in human cancer cells mediated the formation of aneuploid nuclei (Daniel, Coulter et al. 2011). In light of the fact that the giant-cells formed by *Meloidogyne* species are also characterized by multiple aneuploid nuclei (Jones and Payne 1978; Huang 1985), we speculate that Minc01696 could function analogously to the MPS1 kinases in plants, perhaps facilitating aneuploidy during the formation of the giant-cells. This hypothesis is further supported by our qRT-PCR data showing that Minc01696 exhibits specific and significant up regulation at 3 dpi (Fig. 4A), which coincides with the initial formation of the giant-cells. More in depth characterization will be needed to determine whether or not Minc01696 functions as an MPS1 kinase *in planta.*

Two other putative effectors, Minc04584 and Minc00344, previously have been shown to be members of the MAP gene family (Castagnone-Sereno, Semblat et al. 2009). Members of this gene family are thought to play roles in the early interaction between root-knot nematodes and their host plants. More specifically, the first MAP family member (MAP-1) was isolated as a candidate avirulence protein and was shown to be secreted from the amphids of *M. incognita,* as the nematode migrates throughout the host root (Semblat, Rosso et al. 2001). Though the exact function of this gene family has yet to be described, the MAP-1 transcript has recently been isolated from twelve different *Meloidogyne* species (Tomalova, Iachia et al. 2012) indicating that it has an indispensible function for parasitism across *Meloidogyne* species. In contrast to the amphid localization previously described for the MAP-1 protein (Semblat, Rosso et al. 2001; Vieira, Danchin et al. 2011), we found that both Minc04584 and Minc00344 are strongly expressed in the subventral gland cells of *M.*

incognita (Fig. 2M and 2N). Considering that Minc04584 and Minc00344 are distantly related to MAP-1 relative to the rest of the family members could explain the different secretion schemes of MAP protein family members. The fact that the MAP family proteins seem to be secreted both from the amphids and the stylet further establishes these family members as important factors in the parasitism of host plant.

Although the majority of effector molecules tend to be unique to specific parasites and not widely conserved in free-living organisms, there are certain families of effectors that are well conserved within related groups of parasites. The large effector repertoire of the plant pathogen *Ralstonia solanacearum* contains 3 effector gene families that are broadly conserved across different strains of the bacteria (Poueymiro and Genin 2009). These conserved gene families are thought to play basal roles in facilitating parasitism in a wide variety of hosts. Previous analysis of cyst and root-knot nematode effector repertoires has revealed that many effectors are also organized into gene families (Gao, Allen et al. 2001; Huang, Gao et al. 2003; Abad, Gouzy et al. 2008). In our study, we found that ten of the 17 putative effector genes are organized in gene families within the *M. incognita* genome. For a parasite with a wide host range, like *M. incognita*, it may be advantageous to have different effector variants that function best in different host plant species.

Our study has also shown that these gene families can be used to identify other effector candidates. By searching the *M. incognita* genome we were able to identify paralogs of our initial *in situ*-positive candidates. These paralogs turned out to be positive effector candidates that weren't identified in our original screen. Indeed, every identified paralog with a signal peptide was also shown to be expressed specifically in the esophageal gland cells. Using the putative effector genes from *M. incognita*, we were also able to identify seven

orthologous proteins in the *M. hapla* genome as effector candidates. These findings validate the use of this approach in future studies to identify other effector candidates in *M. incognita* as well as other closely related root-knot nematode species. Conservation of these orthologous gene family members in multiple root-knot nematode species indicates that they may have fundamental roles in facilitating nematode parasitism. It would be very interesting to study whether these effector proteins also have conserved orthologs in other parasites. A more comprehensive analysis of all root-knot nematode species could identify the most conserved, and presumably the most indispensible, effector families within the *Meloidogyne* genus.

Recent experimental data have provided evidence of extracellular, cytoplasmic, and nuclear targeting of root-knot nematode effectors, suggesting diverse functional activities in the infected host cells (Tytgat, Vanholme et al. 2004; Elling, Davis et al. 2007; Hewezi, Howe et al. 2008). Knowing where effectors localize *in planta* could help guide future analysis of their function. Previous studies have used bioinformatic predictions to localize root-knot nematode effectors to specific host subcellular compartments (Huang et al., 2003; Bellafiore et al., 2008). Here, we studied the subcellular localization of the newly identified effectors (Fig. 3). All candidate effectors displayed a cytoplasmic localization, despite the fact that some of these effectors contained predicted nuclear localization signals (Psort II). It is possible that these effectors could be redirected towards specific subcellular compartments via host interacting factors. This type of effector interaction has been observed for certain cyst nematode effector proteins (Hewezi and Baum 2012).

Nematode effector genes are often differentially regulated over the course of the parasite's life cycle. An effector gene that is specifically expressed during a certain stage of

infection is likely to play a specific role during that stage of infection. Quantifying the expression profiles of all identified candidate effector genes over the course of *M. incognita* infection of tomato plants revealed distinct stage-specific expression patterns. More specifically, two effector candidates (Minc01696 and Minc10418/Minc08073) showed significant up regulation at 3 dpi (Fig. 4A and 4B). This early time point coincides with the initiation of the giant-cells, suggesting that these genes may have functional roles during the establishment of the feeding site. Four additional transcripts (Minc2097, Minc00344, Minc13292, and Minc03328) showed a consistent up-regulation during the first three parasitic time points (3 dpi, 7 dpi, and 14 dpi), followed by a distinct down regulation at 21 dpi (when adult females begin to reproduce) (Fig. 4C-F). This pattern of expression is consisted with a potential involvement of these putative effectors in early and late events of parasitism but is dispensable for nematode reproduction. Interestingly, the opposite trend was observed for the candidate effector Minc00801, which exhibits a relatively low expression during the first five time points followed by obvious up-regulation at 21 dpi. The high expression abundance of this effector specifically at 21 dpi suggests a role in egg laying and perhaps the reproductive success of the nematode (Fig. 4L). Consistent with this hypothesized function, the mRNA of this candidate effector was specifically localized to the rectal glands of the adult female nematodes. This was intriguing because the rectal glands produce a gelatinous matrix (GM) which surrounds the nematode's eggs (Maggenti and Allen 1960). In addition to enhancing the viability of the eggs in the soil, the GM is implicated in forming a pore in the surrounding plant tissue that allows the egg mass to escape the root (Orion and Franck 1990; Orion, Kritzman et al. 2001). In this context, a previous study identified a protein with cellulose-binding activity, which was secreted from

the vagina of *M. incognita* (Vieira, Danchin et al. 2011). These observations indicate that proteins secreted in the GM could be involved in egg laying and perhaps the reproductive success of the parasite.

In conclusion, the use of next generation 454 sequencing technology has allowed us to sequence the gland cells of *M. incognita* on an unprecedented level. The novel effector proteins identified in this study have significantly expanded the effector repertoire of *M. incognita*, and set the stage for a more complete understanding of how this parasite infects its host plants.

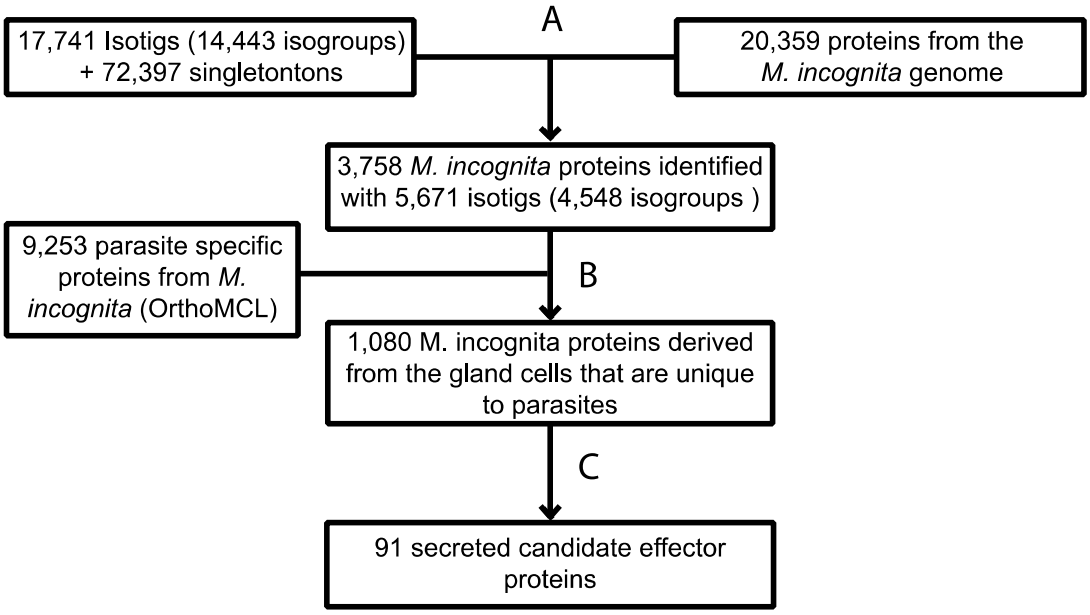

Figure 1. diagram of *in silico* filtering methods used to identify effector candidates A) The isotigs assembled from the gland cells specific sequences were compared to the known protein coding sequences from the *M. incognita* genome using a nucleotide Blast (minimum cut off of 90% identity over 70% of an Isotig's length). B) *M. incognita* proteins represented in our gland specific sequences were compared to known parasite specific *M. incognita* proteins (previously predicted using OrthoMCL (Abubucker, Martin et al. 2011). C) Identified *M. incognita* proteins were analyzed for the presence of an N-terminal secretion signal and the absence of a transmembrane domain (Phobius).

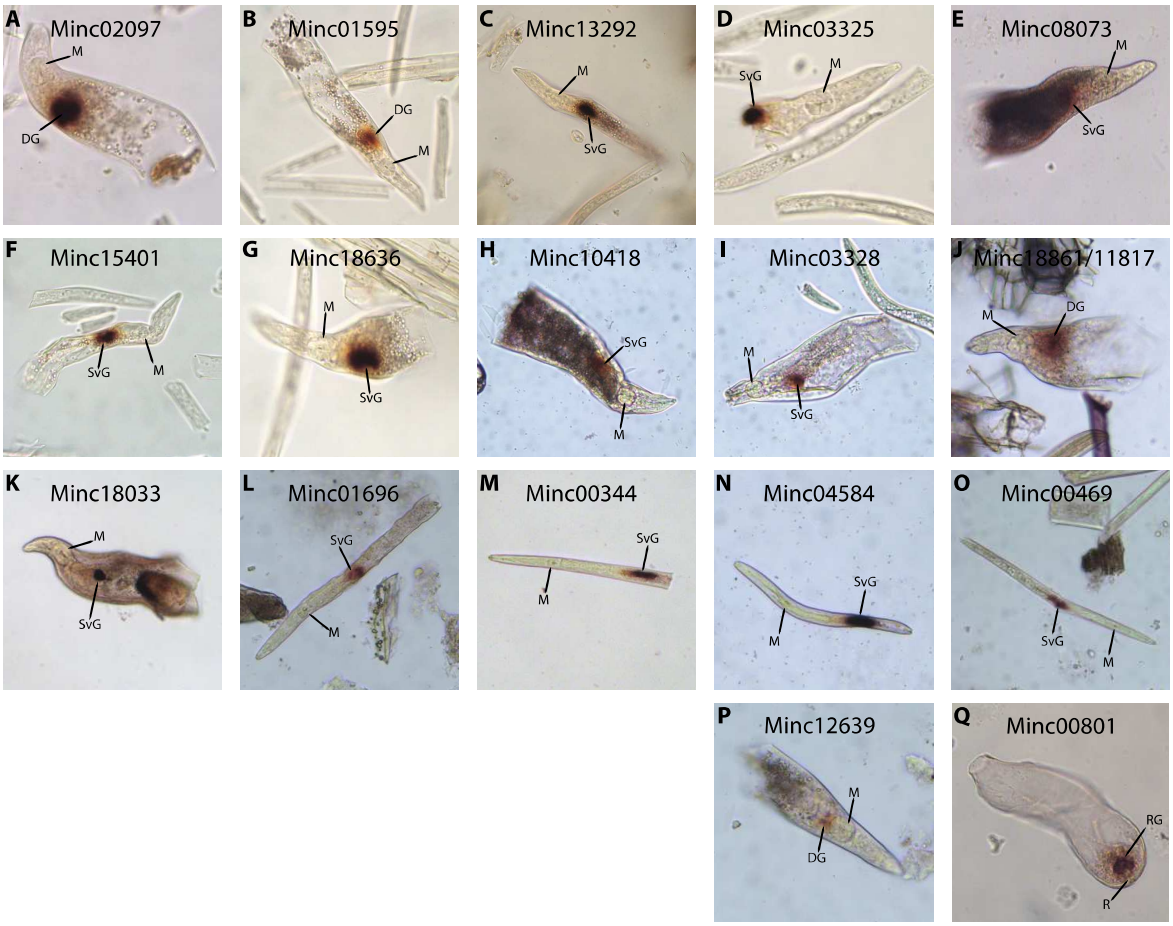

Figure 2. Detection of DIG-labeled probes hybridized to transcripts expressed within the secretory gland cells of the *M. incognita*. A total of 16 probes detected transcripts from 17 genes expressed specifically within the Dorsal (DG) or subventral (SvG) esophageal gland cells specific hybridization (A-P). 10-30 nematodes were hybridized with each probe, and specific gland expression was determined by the shape, size, and proximity of the stained region relative to the metacarpus (M). One probe specific for Minc00801(Q) produced specific staining in the rectal glands (RG) attached to the rectum (R) of J4 female nematodes.

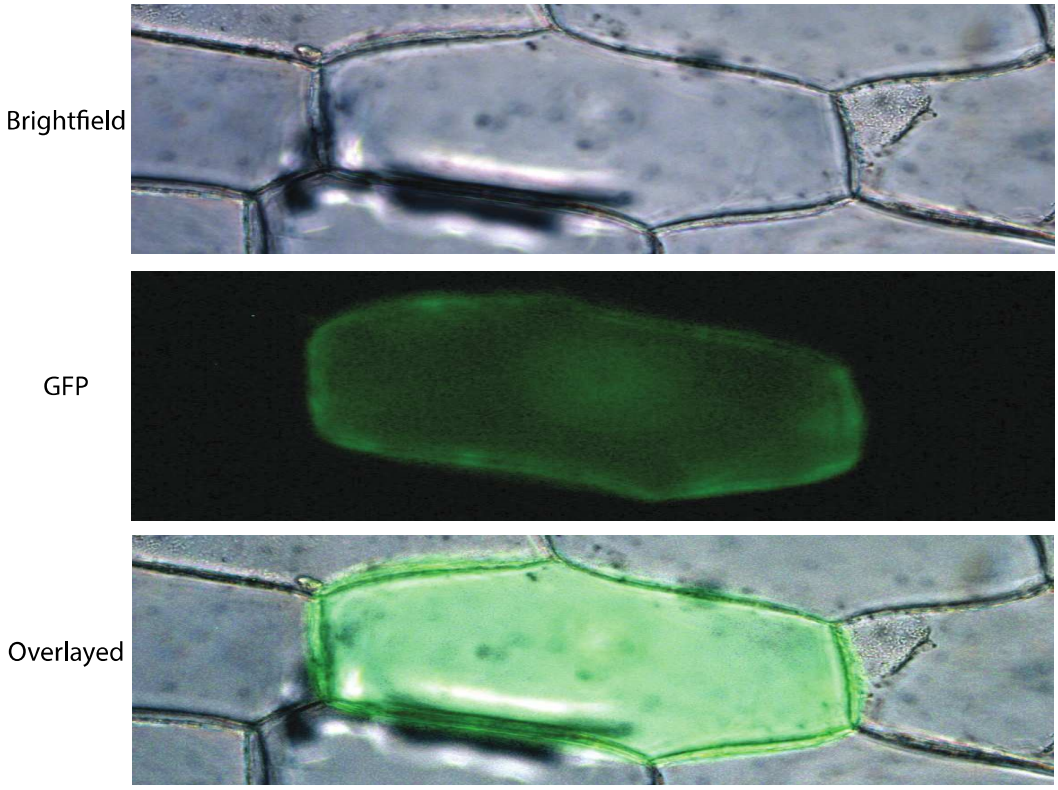

Figure 3. Sub-cellular localization of effector-GFP fusion proteins in onion epidermal cells. N-terminal GFP fusion constructs were made for twelve of the putative parasitism proteins cloned from *M. incognita*. All twelve, including Minc00469 (Above), produced a cytoplasmic GFP signal when bombarded into onion epidermal cells.

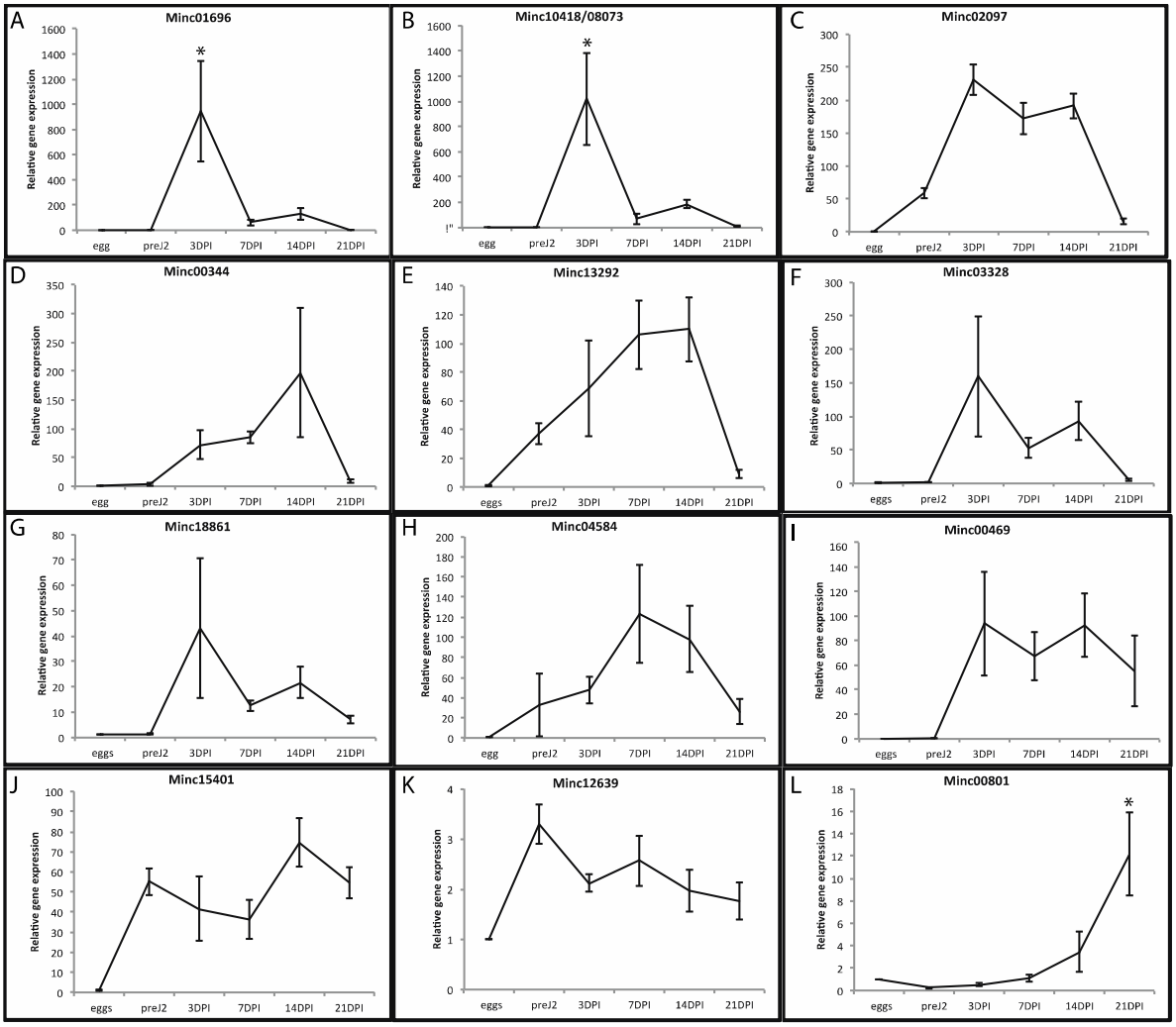

Figure 4. qRT-PCR expression analysis of putative parasitism gene over the life-cycle of *M. incognita*. A-B) Two putative parasitism genes that show a significant increase in expression at 3DPI (* = p-value <0.05). C-F) Four putative parasitism genes that show relatively high expression during the parasitic stages of *M. incognita* (3DPI, 7DPI, and 14DPI), and show a significant decrease during the adult stage at 21DPI. G-K) Five putative parasitism genes that show a relatively consistent expression pattern across all four infected time points (3DPI-21DPI). L) Minc00801, the rectal gland specific transcript, shows a significant increase in expression during the adult stage of *M. incognita* 21DPI (* = p-value <0.05).

Table 1. Summary of the 18 putative effector genes identified in this study. Column A) amino acid similarity to paralogous genes in the *M. incognita* genome (BLASTp % identity and bit score). Column B) amino acid similarity with orthologous proteins containing signal peptides in the M. hapla genome (BLASTp % Identity and bit score). Column C) highest homolog in the NCBI-nr database (BLASTp minimum cutoff >35 bits). Column D) protein domains predicted using InterPro Scan software. Column E) gland specific expression found in either the dorsal gland (DG), the subventral glands (SvG), or the rectal glands (RG) using in situ hybridization.

M.Incognia **CDS (bp)**	***M. incognita*** **paralogs (%ID, bit score)**	***M. hapla*** **orthologs (%ID, bit score)**	**NCBI homology (%ID, bit score)**	**Predicted domains**	**Gland localization**
Minc13292 (1,605)	Minc03325 (80%, 721)	N/A	N/A	N/A	SvG
Minc18861 (453)	Minc11817 (100%,313)	Contig1084.frz3.gene4 (68%, 105)	N/A	N/A	DG
Minc08073 (2,133)	Minc10418 (98%, 1389)	N/A	N/A	N/A	SvG
Minc00801 (1,086)	N/A	Contig125.frz3.gene5 (79%, 62.8)	N/A	N/A	RG
Minc02097 (1,599)	Minc01595 (92%, 933)	N/A	Putative esophageal gland cell protein 25 [*M. incognita*] (97%, 1060)	N/A	DG
Minc18033 (1,314)	N/A	Contig518.frz3.gene7 (64%, 412)	Putative esophageal gland cell protein 17 [*M. incognita*] (99%, 884)	N/A	SvG
Minc01696 (1,731)	N/A	Contig83.frz3.gene1&gene2 (95%, 32)	Dual specificity protein kinase TTK [*Rattus norvegicus*] (30%, 123)	Protein Kinase	SvG
Minc00344 (1,158)	Minc04584 (90%, 312)	Contig252.frz3.gene17 (52%, 167)	Putative avirulence protein MAP-1 [M. incognita](47%, 185)	N/A	SvG
Minc00469 (198)	N/A	N/A	N/A	N/A	SvG
Minc15401 (951)	Minc18636 (94%, 249)	Contig1554.frz3.gene5 (71%, 155)	N/A	N/A	SvG
Minc10418 (2,130)	Minc08073 (98%, 1385)	N/A	N/A	N/A	SvG
Minc12639 (303)	N/A	Contig1246.frz3.gene18 (94%, 75b)	N/A	N/A	DG
Minc03328 (1,449)	N/A	N/A	Unnamed protein product [*Trypanosoma congolense* IL3000] (25%, 38.1)	N/A	SvG
Minc04584 (630)	Minc00344 (82%, 327)	Contig252.frz3.gene17 (66%, 142)	Putative avirulence protein MAP-1 [*M. incognita*] (40%, 135)	N/A	SvG
Minc03325 (1,593)	Minc13292 (79%, 721)	N/A	N/A	N/A	SvG
Minc11817 (453)	Minc18861 (100%, 313)	Contig1084.frz3.gene4 (68%, 105)	N/A	N/A	DG
Minc01595 (1,653)	Minc02097 (87%, 923)	N/A	Putative esophageal gland cell protein 25 [*M. incognita*] (87%, 921)	N/A	DG
Minc18636 (939)	Minc15401 (87%, 385)	Contig1554.frz3.gene5 (55%, 151)	N/A	N/A	SvG

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CHAPTER 6. GENERAL CONCLUSIONS

During the course of my research I have been struck by the complexity of the interaction between sedentary endoparasitic nematodes and their host plants. These parasites have evolved the ability to manipulate plant defenses, development, and metabolism to create an entirely new plant organ that exists only to feed the nematode. What makes this feat seem all the more amazing to me is that they appear to be able initiate all of these changes using only a mixture of effector proteins. In my opinion, if we were able identify the entire repertoire of nematode effectors and understand how they function within host plants it would likely facilitate novel mitigation strategies and give us a better understanding of the pathways that nematodes manipulate within plants.

In the past fifteen years there has been a huge surge in our understanding of nematode effector proteins. Many of these new discoveries have been facilitated by the advent of next generation sequencing technologies. These technologies have promoted powerful genomic and transcriptomic approaches for identifying effectors and the effects they have within the host. Indeed, these approaches have provided a wealth of data that have begun to answer many questions about these parasites, and have highlighted even more questions.

One question that has caught my attention: ‘How well are these effector proteins conserved across the various taxa of parasitic nematodes?’ As described in the introduction, there are several characterized effector proteins that are known to be conserved in both root-knot and cyst nematodes. In fact, chorismate mutase effectors have even been found in species of migratory parasitic nematodes (Haegeman, Joseph et al. 2011). This type of broad conservation indicates that chorismate mutase likely plays a basal role in facilitating parasitism. By identifying which nematode effectors are conserved in different species of

nematode we can begin to identify which effectors are likely to function in all nematode infections, versus those that are more likely to function only in specific host plants. These data could be critical when trying to identify the host specificity determinants of different nematode populations.

Our ability to gather this type of data is currently limited only to the few specific nematode species and isolates for which we have genomic and transcriptomic data. However, initiatives are underway to expand nematode effector databases. Empowered by the lowering cost of sequencing, as well as new techniques that allow very specific isolation of nematode gland cells (Maier, Hewezi et al. 2013), these initiatives seek to identify the effector repertoires of multiple species of nematodes that have never been sequenced. In the future, these techniques could be applied to multiple field races of each species and give us a more comprehensive view of the diversity of these effector proteins. I believe that the availability of this type of meta-data will be a huge advantage when trying to identify which effectors are the most crucial to which interactions.

On the flip side of this interaction, a complementary question can be asked about plant factors: ‘what are the specific proteins or pathways within the plant that are targeted by effectors from nematodes and other plant pathogens?’ Much of the research on effector proteins has been focused on understanding how they function within the plant and what pathways within the plant they alter. If we can understand what all effectors do *in planta*, then presumably we will be able to paint a complete picture of how and why pathogens affect their plant hosts the way they do.

Despite the fact that we are nowhere near having a complete understanding of effector functions, common virulence targets have already been identified in pathogens from

different kingdoms. During my research I have come across several of these common virulence targets. One example that has really stuck in my mind is the discovery of Minc01696 from *M. incognita* (see chapter 5). This putative effector contains a dual specificity kinase domain similar to those in TTK/MPS1 kinases, which play important roles in regulating mitosis. The possibility that Minc01696 alters mitotic processes is intriguing, because one of the iconic morphological characteristics of giant-cells are multiple aneuploid nuclei. In fact, increases in plant nuclear DNA ploidy have also been observed at the interaction sites of fungal pathogens and symbionts. It has been postulated that this increase in ploidy could facilitate increased metabolic activity within plant cells (Wildermuth 2010). If the link between TTK/MPS1 kinases and mitosis can be confirmed, it could implicate this pathway as a regulatory hub that is targeted by effectors in other pathosystems as well.

I came across a similar plant regulatory hub while studying the effector 4E02 from *H. schachtii* (see Chapter 2). I found that *Hs*4E02 can interact with the active site of the plant papain-like cysteine protease RD21A. This is very interesting because RD21A and its homologs are known to initiate plant defense responses, and are targeted by effectors from diverse plant pathogens (Bar-Ziv, Levy et al. 2012; Lozano-Torres, Wilbers et al. 2012; Lampl, Alkan et al. 2013). Indeed, plant papain-like cysteine proteases are a common virulence target for multiple pathogens. Many more common virulence targets are beginning to emerge in plants, and as more information is gathered regarding these targets I think it is likely that we will be able to categorize effectors from different pathogens based on their common host targets. This will have wide-reaching effects on our ability to identify new effectors, characterize their function, and perhaps even our ability to design pest resistance in crop plants.

There are many important questions about the plant-nematode interaction that remain to be answered, but in my mind, the two that are the most critical are the ones that I have summarized in this chapter. In the relatively short time I have been a graduate student I have been fortunate to witness some big discoveries in the field of effector biology, and I am excited to see what happens in the future.

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