5-2018

Combining Translational and Functional Genomic Approaches to Augment Management Strategies of Plant Parasitic Nematodes

Sandeep Sharma Khatiwada
University of Arkansas, Fayetteville

Combining Translational and Functional Genomic Approaches to Augment Management Strategies of Plant Parasitic Nematodes

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

Sandeep Sharma Khatiwada
Assam Agricultural University
Bachelor of Science in Agriculture, 2007
GB Pant University of Agriculture and Technology
Master of Science in Molecular Biology and Biotechnology, 2010

May 2018
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Burt H. Bluhm, Ph.D.
Dissertation Chair

_____
Terry Kirkpatrick, Ph.D.
Committee Member

_____
Ken Korth, Ph.D.
Committee Member

_____
Vibha Srivastava, Ph.D.
Committee Member

_____
Ned French, Ph.D.
Ex-officio Member

ABSTRACT

Plant-parasitic nematodes pose a major threat to crop yield worldwide. Discontinued use of harmful chemicals has prompted the search for alternative management strategies that are effective yet environmentally friendly. Harpin proteins, which are derived from bacteria, and nematophagous fungi, natural predators of nematodes, are ideal for biological control of plant-parasitic nematodes. However, research on the efficacy of harpin proteins on nematodes, and biology of nematophagous fungi is minimal. Previously, a taxonomically uncharacterized nematophagous fungus designated ARF18 effectively suppressed nematode populations in soil. The overarching goal of this dissertation is to utilize applied and functional genomic approaches to augment management of plant-parasitic nematodes with the following objectives: 1) To test the efficacy of harpin protein treatments in different host-nematode interactions, 2) Utilize genome of ARF18 to provide taxonomic placement and establish it as a biocontrol agent against plant-parasitic nematodes, 3) Identify genes regulated by harpin during reniform pathogenesis, and 4) Develop target enrichment sequencing method using *Cercospora zeae-maydis* as model system to accelerate functional genomics research. Evaluation of harpin protein on soybean and cotton showed a consistent reduction in reniform populations in soybean without significant effects on plant growth. Similarly, ARF18 parasitized cysts *in-vitro,* and based on the ITS region, ARF18 grouped within *Brachyphoris*. ARF18 genome assembled into 412 scaffolds, indicating a size of 45.6 Mb with 14,461 putative protein-encoding genes. Transcriptome of soybean using Ion Torrent PGM identified numerous genes from soybean roots albeit lower sequencing depth hindered the identification of differentially expressed transcripts between different treatments. Subsequently, a target enrichment method was developed in *C. zeae-maydis* to dissect cercosporin regulation. Ease of genetic manipulation, availability of near complete genome, and

presence of easily screen able phenotype facilitated the method development in *C. zeae-maydis*. The method identified more than 80 genes that altered cercosporin production in *C. zeae-maydis*. Additionally, RNAi lines created for six genes confirmed the linkage of the phenotypes to the mutation identified. The method could be easily adapted to different organisms, especially in nematophagous fungi to accelerate gene discovery and function to advance research towards the management of different plant parasitic nematodes.

ACKNOWLEDGEMENTS

I am very highly grateful to numerous people who have helped me achieve this important milestone in my career.

First and foremost I would like to thank my advisor, Dr. Burt Bluhm, for providing me the opportunity to work in his group and his valuable feedback and advice on the conceptualization of this dissertation. Without his constant guidance and motivation, this dissertation would not have been possible.

I would also like to thank my dissertation advisory committee- Dr. Terry Kirkpatrick, Dr. Ken Korth, Dr. Ned French, and Dr. Vibha Srivastava for their constant support and supervision during my Ph.D. program. I am truly grateful for the direction you have provided for preparing this dissertation.

I would like to thank the Plant Pathology department for providing me with this wonderful opportunity and Dr. Rhoads, the Director of Cell and Molecular Biology Program, in providing constant assistance and support. I am very much indebted to my lab mates John Ridenour, Jonathon Smith, Braham Dhillon for sharing ideas and concepts for preparation of this dissertation. With their moral support and encouragement, this journey has been truly remarkable. I would also like to thank Wayne Hawkins for the help with the cercosporin screen project.

Finally, I would like to thank my Mom and Dad for believing in me and keeping patience with my Ph.D. program.

TABLE OF CONTENTS

LIST OF PUBLICATIONS (FIRST AUTHOR)

Chapter IV, Appendix: Sharma S, Zaccaron AZ, Ridenour JB, Bradshaw A, Kirkpatrick T, Cartwright K, and Bluhm BH (2017). Taxonomic resolution of the nematophagous fungal isolate ARF18 via genome sequencing. Genome Announcements. DOI: 10.1128/genomeA.00903-17

CHAPTER I

Introduction and Review of Literature

PROJECT OVERVIEW

Plant-parasitic nematodes are one of many important pathogens of crop plants. They reduce yield and productivity directly and indirectly (Abad *et al.*, 2008). Directly, nematodes damage roots, which restricts the absorption of water and nutrients. Indirectly, they cause damage by acting as vectors of viruses, or they interact with certain fungal and bacterial pathogens to aggravate disease. Some of the most important nematodes affecting crop productivity are *Meloidogyne incognita*, the root-knot nematode; *Heterodera glycines*, the soybean cyst nematode; *Pratylenchus* spp., the root lesion nematode; *Radopholus similis*, the burrowing nematode; and *Rotylenchulus reniformis*, the reniform nematode (Jones *et al.*, 2013). Conventional control of nematodes has relied heavily on chemical nematicide treatments (Cabrera *et al.*, 2015, Wang *et al.*, 2015) and crop rotation (Djian-Caporalino *et al.*, 2014), and to a lesser extent, biological control (Cheng *et al.*, 2015, Noreen *et al.*, 2015) and soil solarization (Butler *et al.*, 2014). Many chemical methods of control have been discontinued due to environmental and health concerns, or because they have proven to be ineffective (Martin, 2003, Westphal, 2011).

Nematodes are important agricultural pests.

Yield losses due to plant-parasitic nematodes have been estimated at 12.3% of global food production (Sasser & Freckman, 1986). In the United States alone, losses caused by plant-parasitic nematodes are estimated to be around 10 billion dollars (Hassan *et al.*, 2013). Maize, rice, soybean, cotton, potatoes, wheat, sugar cane, sweet potatoes, and pine trees are some of the plant species affected by these parasites. More than 4,100 species of plant-parasitic nematodes

have been identified to date, and the number is continually increasing (Decraemer & Hunt, 2006).

Although nematodes may comprise the most abundant phylum of animal taxa on earth (Blaxter *et al.*, 1998), plant-parasitic nematodes of economic importance are a comparatively small, specialized group. Although majority of plant-parasitic nematodes are root feeders, some species feed on aerial parts of plants (Jones *et al.*, 2011). Plant parasitic nematodes are obligate parasites of plants feeding exclusively on the cytoplasm of plant cells (Williamson & Gleason, 2003). Ten taxa of nematodes have been classified as highly economically important pathogens: *Meloidogyne* spp., *Globodera* and *Heterodera* spp., *Pratylenchus* spp., *Radopholus similis*, *Ditylenchus dipsaci*, *Bursaphelenchus xylophilus, Rotylenchulus reniformis*, *Xiphinema index, Nacobbus aberrans,* and *Aphelenchoides besseyi* (Jones *et al.*, 2013).

Nematode damage may go undetected due to lack of clear symptoms in the above-ground parts (Lilley *et al.*, 2007). Symptoms are often confused with those caused by other pathogens, or by water and nutrient deficiency. Additionally, the strategy of parasitism differs in different genera of plant-parasitic nematodes. Sedentary endoparasites penetrate host celsl and establish permanent feeding sites, typically a giant cell or a syncytium. In contrast, migratory endoparasites do not form specialized feeding compartments, but rather feed inter- or intra-cellularly throughout the plant during pathogenesis.

The reniform nematode *Rotylenchulus reniformis* is a serious pathogen of multiple crop species. Reniform nematode is prevalent in tropical and subtropical regions of the world, and is particularly common in the southern USA. Reniform nematode has been documented to infect over 350 plant species, including many agronomically important vegetables, fruits, ornamentals,

fiber crops and weeds (Robinson *et al.*, 1997). In the United States, reniform nematodes cause cotton losses estimated at $150 million annually (Robinson, 2007). Symptoms caused by reniform nematode are essentially indistinguishable from symptoms caused by water and nutrient deficiencies, and include reduced root growth and secondary root development, root necrosis, stunted growth, and foliar chlorosis.

Losses due to reniform nematodes range from 40-60%, depending on temperature, humidity, crop conditions and soil populations (Jones *et al.*, 2013). Since the reniform nematode is a sedentary semi-endoparasite, it does not penetrate completely into roots. Instead, it inserts about a third of its anterior body into roots and forms a feeding site called a syncytium (van Megen *et al.*, 2009). Yield losses remain problematic, as the development of commercially viable genetic resistance to reniform nematode has met with limited success.

Current strategies for controlling plant-parasitic nematodes

Although the existence of plant-parasitic nematodes has been known since 1743, their economic importance was not realized until the 1940s (Zasada *et al.*, 2010). At that time, the advent of soil fumigation practices began, which were widely used to control plant-parasitic nematodes for over forty years. Methyl bromide was the foremost chemical fumigant to control plant-parasitic nematodes, other soil-borne pathogens, and weeds in many high value crops. Various characteristics of methyl bromide made it ideal as a soil fumigant, including its broad-spectrum nature, volatility (which allowed it to penetrate soil deeply), and its efficacy. Unfortunately, methyl bromide posed an extreme hazard to workers, applicators, and the environment, which led to its phase-out for agricultural and most other uses (Martin, 2003, Santos *et al.*, 2006, Zasada *et al.*, 2010).

Other chemicals developed as an alternative to methyl bromide have been used successfully as pre-planting soil fumigation treatments (Zasada *et al.*, 2010). Examples include chloropicrin, metam sodium, metam potassium, and dazomet. Although none are as effective as methyl bromide, all have at least some degree of efficacy against plant-parasitic nematodes. Chloropicrin is almost as volatile as methyl bromide but has a narrower spectrum of activity, and is currently used in combination with other control methods (Duniway, 2002). Metam sodium and metam potassium exhibit considerably lower volatility, and their limited distribution in soil after application makes their efficacy inconsistent (Martin, 2003). In general, due to health and environmental risks, or limited consistency and efficacy, soil fumigants have only limited use in today's agricultural systems for controlling the plant-parasitic nematodes.

Crop rotation, one of the most important agricultural practices since ancient times (Conklin, 1961), is an effective way to manage agricultural pests and diseases, including nematodes. This practice involves rotating the host crop with a non-host to keep the population density of the pathogen from increasing to damaging levels (Rodriguezkabana & Canullo, 1992). Although this technique sounds simple, in practice it is sometimes difficult to implement due to various biological and economical constraints. The first concern is that the economic return obtained from a low-value rotation crop may not match the return from a high-value crop, even with nematodes present at economically damaging levels. Crop rotation is also generally applicable only in annual production systems, and the duration of the rotated crop, its interaction with other pests, and the availability of other management practices may not be practical.

Biofumigation is a term used to describe the use of plants (generally of the Brassicaceae family) to control plant pests. This approach utilizes plant-produced secondary metabolites such as glucosinolates that have long been known to reduce nematode populations (Morgan, 1925).

Although this management practice is environmentally friendly and relatively economical, nematode management via biofumigation has not been consistent due to various factors such as the source of the metabolite, soil type, method of incorporation of plant material, soil temperature, soil moisture, and the plant developmental stage (Matthiessen *et al.*, 2004).

Soil solarization, another cultural technique that has been utilized in some geographical locations to control plant-parasitic nematodes, was first described in 1976 (Katan *et al.*, 1976), and has been widely used as a pre-planting method to control various soil-borne pathogens. The practice of soil solarization involves using the sun to heat soil, which is covered with plastic mulch. Soil solarization, in combination with organic amendments, was highly effective in controlling nematodes (Oka *et al.*, 2007). Although this management technique may not effectively control all plant-parasitic nematode species, it can potentially be integrated with other management practices to increase control.

Genetic resistance has been one of the most effective means to control plant-parasitic nematodes (Williamson & Kumar, 2006). Nematode resistance has been identified in several crops (Rossi *et al.*, 1998, van der Voort *et al.*, 1999, Bakker *et al.*, 2003, Liu *et al.*, 2012), with varying degrees of commercial application. $Hs1^{pro\text{-}1}$, the first nematode resistance gene to be isolated, conferred resistance against the sugar beet cyst nematode, *Heterodera schachtii* (Cai *et al.*, 1997). Other nematode resistance (*R*) genes that are effective in agricultural systems include *Mi-1* and *Hero A* from tomato (Milligan *et al.*, 1998, Ernst *et al.*, 2002), *Gpa2* and *Gro1-4* from potato (Bakker *et al.*, 2003, Paal *et al.*, 2004), and *Rhg1* and *Rhg4* from soybean (Weisemann *et al.*, 1992, Kandoth *et al.*, 2011). Various other *R* genes have been successfully mapped in potato, tomato, wheat, rice, pepper, and other plant species (Williamson & Kumar, 2006).

It is important to note, however, that not all genes conveying nematode resistance are fully effective in production agriculture. For example, the *Mi* gene does not convey resistance at temperatures above 28°C, which limits its efficacy in warmer production regions (Williamson, 1998). *Hero A* has a narrow range of efficacy, conferring resistance to *G. rostochiensis* but only partial resistance to *G. pallida* (Ernst *et al.*, 2002). $Hs1^{pro\text{-}1}$, although successful in conferring nematode resistance, has a deleterious effect on yield, (Panella & Lewellen, 2007). Although resistance genes are highly effective in controlling pathogens like plant parasitic nematodes, their deployment needs to be optimized in agricultural settings.

Induced systemic resistance (ISR) is a type of non-specific resistance expressed when plants are exposed to elicitors such as cell wall fragments, plant extracts, or synthetic chemicals (Walters & Fountaine, 2009). Induced resistance is categorized into two categories: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is activated by exposure to various agents such as pathogens and chemicals. SAR is mediated through the production of salicylic acid (Spoel & Dong, 2012) and is activated against biotrophic pathogens. ISR, on the other hand, develops in response to plant colonization by beneficial microbes such as plant growth promoting rhizobacteria (PGPR) (Xiang *et al.*, 2017), yeast (Lee *et al.*, 2017) and fungi (Schouteden *et al.*, 2015, Perez-de-Luque *et al.*, 2017). The key hormones that mediate ISR are jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.*, 2014). Induced resistance can effectively suppress plant parasitic nematodes. For example, treatment of rice plants with beta-amino butyric acid (BABA) not only inhibited nematode penetration of roots, but also suppressed gall development (Ji *et al.*, 2015). Similarly, treating tomato plants with *Trichoderma harzianum* isolate T-78 significantly reduced gall formation by priming SA- and JA- dependent pathways (Martinez-Medina *et al.*, 2017). Additional approaches deployed to induce resistance in plants

against plant parasitic nematodes include biocontrol bacteria (Siddiqui & Shaukat, 2004), methyl jasmonate (Fujimoto *et al.*, 2011), and mycorrhiza (Vos *et al.*, 2013). Although induced systemic resistance has shown promise in controlling plant parasitic nematode population, their efficacy in field conditions have not been thoroughly explored.

Application of harpin proteins as inducers of plant defense

Harpin proteins, which are of bacterial origin, belong to type III secretion system and are determinants of hypersensitive response (HR) or pathogenicity (Lindgren, 1997). This was originally demonstrated through the use of *hrp* mutants, which failed to elicit hypersensitive response or pathogenesis (Lindgren *et al.*, 1986, Yang *et al.*, 2002, Sinn *et al.*, 2008). Similar results were obtained by deletion of *hpa1* or its orthologs in *Xanthomonas* spp., where reduced bacterial growth and disease symptoms were observed in host plants inoculated with mutants (Noel *et al.*, 2002, Kim *et al.*, 2004, Sgro *et al.*, 2012). The mode of action of harpin induced defense is through the activation of multiple defense-signaling pathways (Tripathy *et al.*, 2003). For example, transgenic tobacco expressing HrpN of *Erwinia amylovora* showed enhanced resistance to *Botrytis cinerea* (Jang *et al.*, 2006). Additionally, the transgenic plants also showed increased growth and development. Additional effects of harpin treatment to plant cells include disruption of membrane physiology (Pike *et al.*, 1998), inhibition of ATP synthesis (Xie & Chen, 2000), activation of MAP kinase signaling pathways (Desikan *et al.*, 2001) Plants treated exogenously with harpin generally show increased reactive oxygen species (ROS) and also the involvement of enzymes of the respiratory cycles, especially the citric acid cycle in mitochondria (Krause & Durner, 2004). Plants treated with harpin show an improvement in the overall growth of the plants (Livaja *et al.*, 2008). Additionally, plant responses to harpin treatment are due, in part, to widespread transcriptional reprogramming (Truman *et al.*, 2006). Transcriptional

changes due to harpin treatments to Arabidopsis included genes regulating cell wall biogenesis, cellular communication and signaling (Livaja *et al.*, 2008).

The general increase in the immune response due to harpin treatment is through the activation of diverse signaling pathways like the MAP kinase pathway (Desikan *et al.*, 2001), the salicylic acid pathway (Dong *et al.*, 1999) and the ethylene responsive pathway (Chuang *et al.*, 2010). Activation of such diverse defense signaling pathways demonstrates the existence of a cross-talk between various signaling pathways.

Utilizing nematophagous fungi as biological control agents against plant-parasitic nematodes

Conceptually, biological control is an effective and ecologically friendly method to control plant pathogens (Li *et al.*, 2015). Broadly defined, biological control is the use of living organisms or their metabolites to control pests that impact health and agriculture (Eilenberg *et al.*, 2001). Nematophagous fungi have intriguing potential as biological control agents against plant-parasitic nematodes. Based on their strategy of predation, nematophagous fungi can be broadly classified into three major categories: Nematode-trapping fungi, parasitic fungi, and toxic fungi (Siddiqui & Mahmood, 1996). Members of all three categories naturally prey upon nematodes and thus help keep populations in check in natural ecosystems (Yang *et al.*, 2007a).

One bottleneck to utilizing nematophagous fungi as biological control agents is a general lack of genomic resources. With the advent of next-generation DNA sequencing, *de novo* genome sequencing of economically important fungi has expanded rapidly. Interestingly there are more than 200 species of nematophagous fungi that attack and parasitize nematode eggs, juveniles, and adults (Yang *et al.*, 2007b). However, comparatively fewer genomes of nematophagous fungi have been sequenced, namely *Arthrobotrys oligospora* (Yang et al., 2011),

Pochonia chlamydosporia (Larriba et al., 2014), *Drechslerella stenobrocha* (Liu et al., 2014), *Purpureocillium* lilacinum (Prasad et al., 2015), *Monacrosporium haptotylum* (Meerupati et al., 2013), and *Hirsutella minnesotensis* (Lai *et al.*, 2014).

Nematophagous fungi have been studied for many years at the University of Arkansas. One particularly well-studied nematophagous fungus, designated ARF18 (also known as TN14) (Kim & Riggs, 1991), was highly efficacious against adults and juveniles of reniform and cyst nematodes (Timper & Riggs, 1998, Wang *et al.*, 2004). ARF18 has considerable potential to be developed as an effective biological control agent against nematodes that cause economic damage. However, the taxonomic identity of this important fungus has not been resolved, and the mechanisms through which the fungus parasitizes nematodes are poorly understood.

Molecular genetics research in nematophagous fungi

Common approaches to determine gene function include studying gene transcription, translation, and protein-protein interaction. In filamentous fungi and oomycetes, functional genomics has expanded rapidly in recent years, which has been facilitated by advancements in several key areas (Weld *et al.*, 2006). For example, transformation systems have been developed for taxonomically diverse fungi, which makes a wide range of fungal species amenable to genetic manipulation such as targeted gene deletion, creation of random mutants (Ridenour *et al.*, 2012, Vela-Corcia *et al.*, 2015, Niu *et al.*, 2016), and expression of functionally diverse reporter constructs (Gressler *et al.*, 2015). Advancements in fungal functional genomics have also been augmented by increased availability of genomic resources.

Despite the potential value of nematophagous fungi in controlling plant parasitic nematodes, research to identify mechanisms of pathogenicity in these organisms is very limited.

Recently, techniques for functional genomics have been developed for *Purpreocillium lilacinum*, including the creation of plasmids, optimization of transformation methods, and identification of selectable markers, and targeted gene disruption was achieved where in the cytochrome oxidase (COX1) gene was successfully knocked out via *Agrobacterium*-mediated transformation (Yang *et al.*, 2016). Similar knockout protocols have been developed in *Arthrobotrys oligospora* to target genes via PEG-mediated transformation of protoplasts (Liang *et al.*, 2015, Liang *et al.*, 2017). Additionally, a targeted gene knock-out system has also been developed for *Drechmeria coniospora* (Lebrigand et al., 2016).

Although the genomes of a few nematophagous fungi have been sequenced, molecular genetic studies to understand mechanisms of pathogenicity have not been performed. Studies to understand pathogenicity have mostly been confined to more descriptive approaches, such as transcriptomic or proteomic studies during nematode parasitism (Yang *et al.*, 2011, Liu *et al.*, 2014). These studies have identified enzymes such as subtilisin-like serine proteases, chitinases, and several peptidases that are implicated in virulence. However, information validating whether these enzymes are involved in pathogenesis is limited.

Cercospora zeae-maydis - a model system to develop tools for molecular genetic studies

To better understand and to potentially manipulate nematophagous fungi, it is critical to develop novel tools of molecular genetics in fungal species. An important plant pathogenic fungus can potentially serve as a beneficial target for such studies, because of existing knowledge and public resources available, and techniques and methods optimized for the species. *Cercospora zeae-maydis*, one of the causal agents of gray leaf spot, is an important pathogen of corn worldwide (Shim and Dunkle 2002). *C. zeae-maydis* is a foliar pathogen that

produces the non-host specific phytotoxin cercosporin during pathogenesis. Cercosporin was first isolated from *Cercospora kikuchii* (Kuyama and Tamura 1957), and belongs to a family of perylenequinones, that are activated by light (Bluhm et al., 2008). The toxin produces reactive oxygen species including singlet oxygen and superoxide radicals, which cause damage to the cell membrane through lipid peroxidation, loss of integrity and leakage resulting in cell death (Lousberg et al, 1979). Cercosporin is produced via a polyketide synthase pathway and is encoded by a cluster of 8 genes named *CTB1-8* (Cercosporin Toxin Biosynthesis; Chung et al., 2003). The genes in this cluster have been characterized in *Cercospora nicotianae* (Newman and Townsend, 2016), and are *CTB1*- an iterative, nonreducing polyketide synthase-encoding gene, *CTB2*- a methyltransferase, *CTB3*- a monooxygenase/ methyltransferase, *CTB4* – a major facilitator superfamily transporter, *CTB5*- an oxidoreductase, *CTB6*- ketone reductase, *CTB7*-an oxidoreductase, and *CTB8*- a zinc finger transcription factor.

We have identified the complete cluster in *C. zeae-maydis* (*CTB1-8*) required for synthesis and transport of cercosporin. The cluster resides in scaffold 12 of the genome and spans a region of around 18 Kilobases. Although the genes (CTB cluster) directly regulating the production of cercosporin in different *Cercospora* species have been identified, the factors that regulate the biosynthesis of this important secondary metabolite are poorly understood. *C. zeae-maydis* is a model system to dissect cercosporin regulation in filamentous fungi. It is easily amenable to different transformation techniques and other genetic tools. Additionally, the production of cercosporin can be screened and quantified *in-vitro* (Winfred Peck-Dorleku 2013).

Forward genetic screens have substantially advanced the identification of genes underlying phenotypes in filamentous fungi (Korn *et al.*, 2015, Pfannenstiel *et al.*, 2017). However, a key bottleneck is characterizing genomic lesions associated with the insertion of

mutagenesis cassettes. Methods developed previously include plasmid rescue (Tam and Lefebvre 1993), thermal asymmetric interlaced PCR (TAIL PCR; Dent et al. 2005), restriction enzyme site-directed amplification PCR (Gonzalez-Ballester et al, 2005), 3'- rapid amplification of cDNA ends (Meslet-Cladiere and Vallon 2012), and site finding PCR (Li et al, 2012). Each of these methods has limitations, particularly regarding throughput. Recently several methods have been utilized, such as *ChlaMme* seq (Zhang et al, 2014), using next-generation sequencing to correctly identify the site of the genomic lesion. However, the methods currently in use are limited by sequencing depth and the number of samples that can be processed simultaneously.

Target capture (also called target-enrichment) sequencing selectively enriches specific regions of genomes or transcriptomes for sequencing (Mamanova *et al.*, 2010). Enrichment of specific regions of the genome/transcriptome not only improves coverage to facilitate differential expression analyses, but also makes sequencing more economical by pooling numerous samples in a single reaction (Craig *et al.*, 2008, Cronn *et al.*, 2008, Harismendy & Frazer, 2009). The performance and feasibility of target enrichment are based on various parameters including the percentage of target regions captured, specificity to the intended target regions, uniformity of target capture, reproducibility of the experiment, cost involved in sequencing, ease of use, and the amount of DNA/RNA required as the starting material (Mamanova *et al.*, 2010). Several applications utilizing target enrichment technology have been developed for genetic research, including extracting and cloning resistance genes (Witek *et al.*, 2016) and detection of mutations in particular regions of the genome (Schmitt *et al.*, 2015). Recent approaches to refine target-enrichment technology have made it practical for use in several applications such as determination of T-DNA insertion (Inagaki *et al.*, 2015), and a more recent Southern-by-sequencing technology (Zastrow-Hayes *et al.*, 2015). However, applications of target-capture

sequencing in filamentous fungi research are limited, although its potential so serve as a powerful tool for basic and applied research in substantial.

The overarching goal of this dissertation is to identify alternative methods to control plant-parasitic nematodes and develop molecular genetics tools to augment the management strategies of plant-parasitic nematodes. The work in the chapters below aim to identify harpin and ARF18 as a potential biological control against plant-parasitic nematodes, identify harpin-induced defense response in soybean plants against reniform nematodes, and the nature of the defense response- induced constitutive or priming response, and utilize *Cercospora zeae-maydis* as a model system to develop target-enrichment sequencing to accelerate molecular genetics studies. The experiments and their results have been organized into the following chapters:

Chapter 2 describes the efficacy of harpin protein during different crop-nematode interactions. This chapter aims to identify a system that shows a response to harpin proteins in greenhouse conditions. The results from this chapter demonstrate that harpin protein helps to significantly reduce reniform population in soybean plants.

Chapter 3 aims to identify the signaling pathway induced in soybean during harpin protein treatment using RNA sequencing and development of transgenic soybean lines. This chapter would help us identify potential biomarkers that are regulated by harpin during defense response against reniform nematodes in soybean.

Chapter 4 aims to sequence and assemble the genome of the unnamed nematophagous fungal isolate ARF18 to provide taxonomic placement of the fungus and identify genes that would provide clues on its potential as a nematophagous fungus. The chapter discusses the details of the

genome sequencing, assembly and annotation and identification of putative nematode parasitism genes.

Chapter 5 aims to develop tools and methods for molecular genetics to identify genes regulating cercosporin production in *Cercospora zeae-maydis* an important pathogen of corn with a potential application in functional genomics of nematophagous fungi to augment nematode management strategies.

References

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.-C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.-N., Schiex, T., Smant, G., Weissenbach, J., and Wincker, P. (2008) Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* **26**: 909-915.

Bakker, E., Butterbach, P., van der Voort, J.R., van der Vossen, E., van Vliet, J., Bakker, J., and Goverse, A. (2003) Genetic and physical mapping of homologues of the virus resistance gene Rx1 and the cyst nematode resistance gene Gpa2 in potato. *Theoretical and Applied Genetics* **106**: 1524-1531.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T., and Thomas, W.K. (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**: 71-75.

Butler, D.M., Kokalis-Burelle, N., Albano, J.P., McCollum, T.G., Muramoto, J., Shennan, C., and Rosskopf, E.N. (2014) Anaerobic Soil Disinfestation (ASD) Combined with Soil Solarization as a Methyl Bromide Alternative: Vegetable Crop Performance and Soil Nutrient Dynamics. *Plant and Soil* **378**: 365-381.

Cabrera, J.A., Hanson, B.D., Gerik, J.S., Gao, S., Qin, R., and Wang, D. (2015) Pre-plant soil fumigation with reduced rates under low permeability films for nursery production, orchard and vineyard replanting. *Crop Protection* **75**: 34-39.

Cai, D.G., Kleine, M., Kifle, S., Harloff, H.J., Sandal, N.N., Marcker, K.A., KleinLankhorst, R.M., Salentijn, E.M.J., Lange, W., Stiekema, W.J., Wyss, U., Grundler, F.M.W., and Jung, C. (1997) Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275**: 832-834.

Cheng, F., Wang, J., Song, Z., Cheng, J.e., Zhang, D., and Liu, Y. (2015) Complete genome sequence of Bacillus thuringiensis YC-10, a novel active strain against plant-parasitic nematodes. *Journal of biotechnology* **210**: 17-18.

Conklin, H.C. (1961) The study of shifting cultivation. *Current Anthropology* **2**: 27-59.

Craig, D.W., Pearson, J.V., Szelinger, S., Sekar, A., Redman, M., Corneveaux, J.J., Pawlowski, T.L., Laub, T., Nunn, G., Stephan, D.A., Homer, N., and Huentelman, M.J. (2008) Identification of genetic variants using bar-coded multiplexed sequencing. *Nature Methods* **5**: 887-893.

Cronn, R., Liston, A., Parks, M., Gernandt, D.S., Shen, R., and Mockler, T. (2008) Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. *Nucleic Acids Research* **36**.

Decraemer, W., and Hunt, D.J. (2006) Structure and classification. *Plant nematology.*: 3-32.

Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K., and Neill, S.J. (2001) Harpin induces activation of the arabidopsis mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiology* **126**: 1579-1587.

Djian-Caporalino, C., Palloix, A., Fazari, A., Marteu, N., Barbary, A., Abad, P., Sage-Palloix, A.-M., Mateille, T., Risso, S., Lanza, R., Taussig, C., and Castagnone-Sereno, P. (2014) Pyramiding, alternating or mixing: comparative performances of deployment strategies of nematode resistance genes to promote plant resistance efficiency and durability. *Bmc Plant Biology* **14**.

Dong, H.S., Delaney, T.P., Bauer, D.W., and Beer, S.V. (1999) Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. *Plant Journal* **20**: 207-215.

Duniway, J.M. (2002) Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. *Phytopathology* **92**: 1337-1343.

Eilenberg, J., Hajek, A., and Lomer, C. (2001) Suggestions for unifying the terminology in biological control. *Biocontrol* **46**: 387-400.

Ernst, K., Kumar, A., Kriseleit, D., Kloos, D.U., Phillips, M.S., and Ganal, M.W. (2002) The broad-spectrum potato cyst nematode resistance gene (Hero) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. *Plant Journal* **31**: 127-136.

Fujimoto, T., Tomitaka, Y., Abe, H., Tsuda, S., Futai, K., and Mizukubo, T. (2011) Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (Meloidogyne incognita) in tomato plants (Solanum lycopersicum) after foliar treatment with methyl jasmonate. *Journal of Plant Physiology* **168**: 1084-1097.

Gressler, M., Hortschansky, P., Geib, E., and Brock, M. (2015) A new high-performance heterologous fungal expression system based on regulatory elements from the Aspergillus terreus terrein gene cluster. *Frontiers in Microbiology* **6**.

Harismendy, O., and Frazer, K.A. (2009) Method for improving sequence coverage uniformity of targeted genomic intervals amplified by LR-PCR using Illumina GA sequencing-by-synthesis technology. *Biotechniques* **46**: 229-231.

Hassan, M.A., Thi Hoa, P., Shi, H., and Zheng, J. (2013) Nematodes threats to global food security. *Acta Agriculturae Scandinavica Section B-Soil and Plant Science* **63**: 420-425.

Inagaki, S., Henry, I.M., Lieberman, M.C., and Comai, L. (2015) High-Throughput Analysis of T-DNA Location and Structure Using Sequence Capture. *Plos One* **10**.

Jang, Y.S., Sohn, S.I., and Wang, M.H. (2006) The hrpN gene of Erwinia amylovora stimulates tobacco growth and enhances resistance to Botrytis cinerea. *Planta* **223**: 449-456.

Ji, H., Kyndt, T., He, W., Vanholme, B., and Gheysen, G. (2015) beta-Aminobutyric Acid-Induced Resistance Against Root-Knot Nematodes in Rice Is Based on Increased Basal Defense. *Molecular plant-microbe interactions : MPMI* **28**: 519-533.

Jones, J., Gheysen, G., Fenoll, C., Nicol, J.M., Turner, S.J., Coyne, D.L., Nijs, L.d., Hockland, S., and Maafi, Z.T., (2011) Current Nematode Threats to World Agriculture. In: Genomics and Molecular Genetics of Plant-Nematode Interactions. Springer Netherlands, pp. 21-43.

Jones, J.T., Haegeman, A., Danchin, E.G.J., Gaur, H.S., Helder, J., Jones, M.G.K., Kikuchi, T., Manzanilla-Lopez, R., Palomares-Rius, J.E., Wesemael, W.M.L., and Perry, R.N. (2013) Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology* **14**: 946-961.

Kandoth, P.K., Ithal, N., Recknor, J., Maier, T., Nettleton, D., Baum, T.J., and Mitchum, M.G. (2011) The Soybean Rhg1 Locus for Resistance to the Soybean Cyst Nematode Heterodera glycines Regulates the Expression of a Large Number of Stress- and Defense-Related Genes in Degenerating Feeding Cells. *Plant Physiology* **155**: 1960-1975.

Katan, J., Greenberger, A., Alon, H., and Grinstein, A. (1976) Solar heating by polyethylene mulching for the control of diseases caused by soil-borne pathogens [Verticillium dahliae, Fusarium oxysporum, tomatoes, eggplants, fungus diseases]. *Phytopathology* **66**.

Kim, D.G., and Riggs, R.D. (1991) Characteristics and efficacy of a sterile hyphomycete (ARF18), a new biocontrol agent for *Heterodera glycines* and other nematodes. *Journal of Nematology* **23**: 275-282.

Kim, J.G., Jeon, E., Oh, J., Moon, J.S., and Hwang, I. (2004) Mutational analysis of Xanthomonas harpin HpaG identifies a key functional region that elicits the hypersensitive response in nonhost plants. *Journal of Bacteriology* **186**: 6239-6247.

Korn, M., Schmidpeter, J., Dahl, M., Muller, S., Voll, L.M., and Koch, C. (2015) A Genetic Screen for Pathogenicity Genes in the Hemibiotrophic Fungus Colletotrichum higginsianum Identifies the Plasma Membrane Proton Pump Pma2 Required for Host Penetration. *Plos One* **10**.

Krause, M., and Durner, J. (2004) Harpin inactivates mitochondria in Arabidopsis suspension cells. *Molecular Plant-Microbe Interactions* **17**: 131-139.

Lai, Y.L., Liu, K.K., Zhang, X.Y., Zhang, X.L., Li, K., Wang, N.N., Shu, C., Wu, Y.P., Wang, C.S., Bushley, K.E., Xiang, M.C., and Liu, X.Z. (2014) Comparative Genomics and Transcriptomics Analyses Reveal Divergent Lifestyle Features of Nematode Endoparasitic Fungus Hirsutella minnesotensis. *Genome Biology and Evolution* **6**: 3077-3093.

Larriba, E., Jaime, M., Carbonell-Caballero, J., Conesa, A., Dopazo, J., Nislow, C., Martin-Nieto, J., and Lopez-Llorca, L. (2014) Sequencing and functional analysis of the genome of a nematode egg-parasitic fungus, Pochonia chlamydosporia. *Fungal Genetics and Biology* **65**: 69-80.

Lebrigand, K., He, L.D., Thakur, N., Arguel, M.J., Polanowska, J., Henrissat, B., Record, E., Magdelenat, G., Barbe, V., Raffaele, S., Barbry, P., and Ewbank, J.J. (2016) Comparative Genomic Analysis of Drechmeria coniospora Reveals Core and Specific Genetic Requirements for Fungal Endoparasitism of Nematodes. *Plos Genetics* **12**.

Lee, G., Lee, S.H., Kim, K.M., and Ryu, C.M. (2017) Foliar application of the leaf-colonizing yeast Pseudozyma churashimaensis elicits systemic defense of pepper against bacterial and viral pathogens. *Scientific Reports* **7**.

Li, J., Zou, C.G., Xu, J.P., Ji, X.L., Niu, X.M., Yang, J.K., Huang, X.W., and Zhang, K.Q. (2015) Molecular Mechanisms of Nematode-Nematophagous Microbe Interactions: Basis for Biological Control of Plant-Parasitic Nematodes. *Annual Review of Phytopathology, Vol 53* **53**: 67-95.

Liang, L.M., Gao, H., Li, J.Z., Liu, L., Liu, Z.H., and Zhang, K.Q. (2017) The Woronin body in the nematophagous fungus Arthrobotrys oligospora is essential for trap formation and efficient pathogenesis. *Fungal Biology* **121**: 11-20.

Liang, L.M., Shen, R.F., Mo, Y.Y., Yang, J.K., Ji, X.L., and Zhang, K.Q. (2015) A proposed adhesin AoMad1 helps nematode-trapping fungus Arthrobotrys oligospora recognizing host signals for life-style switching. *Fungal Genetics and Biology* **81**: 172-181.

Lilley, C.J., Bakhetia, M., Charlton, W.L., and Urwin, P.E. (2007) Recent progress in the development of RNA interference for plant parasitic nematodes. *Molecular Plant Pathology* **8**: 701-711.

Lindgren, P.B. (1997) The role of hrp genes during plant-bacterial interactions. *Annual Review of Phytopathology* **35**: 129-152.

Lindgren, P.B., Peet, R.C., and Panopoulos, N.J. (1986) Gene-cluster of *Pseudomonas syringae* pv *Phaseolicola* controls pathogenicity of bean plants and hypersensitivity on nonhost plantsGene-cluster of Pseudomonas syringae pv Phaseolicola controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *Journal of Bacteriology* **168**: 512-522.

Liu, K.K., Zhang, W.W., Lai, Y.L., Xiang, M.C., Wang, X.N., Zhang, X.Y., and Liu, X.Z. (2014) Drechslerella stenobrocha genome illustrates the mechanism of constricting rings and the origin of nematode predation in fungi. *Bmc Genomics* **15**.

Liu, S.M., Kandoth, P.K., Warren, S.D., Yeckel, G., Heinz, R., Alden, J., Yang, C.L., Jamai, A., El-Mellouki, T., Juvale, P.S., Hill, J., Baum, T.J., Cianzio, S., Whitham, S.A., Korkin, D., Mitchum, M.G., and Meksem, K. (2012) A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. *Nature* **492**: 256-+.

Livaja, M., Zeidler, D., von Rad, U., and Durner, J. (2008) Transcriptional responses of Arabidopsis thaliana to the bacteria-derived PAMPs harpin and lipopolysaccharide. *Immunobiology* **213**: 161-171.

Mamanova, L., Coffey, A.J., Scott, C.E., Kozarewa, I., Turner, E.H., Kumar, A., Howard, E., Shendure, J., and Turner, D.J. (2010) Target-enrichment strategies for next-generation sequencing. *Nature Methods* **7**: 111-118.

Martin, F.N. (2003) Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology* **41**: 325-350.

Martinez-Medina, A., Fernandez, I., Lok, G.B., Pozo, M.J., Pieterse, C.M.J., and Van Wees, S.C.M. (2017) Shifting from priming of salicylic acid- to jasmonic acid-regulated defences by Trichoderma protects tomato against the root knot nematode Meloidogyne incognita. *New Phytologist* **213**: 1363-1377.

Matthiessen, J., Warton, B., and Shackleton, M. (2004) The importance of plant maceration and water addition in achieving high Brassica-derived isothiocyanate levels in soil. *Agroindustria* **3**: 277-280.

Meerupati, T., Andersson, K.M., Friman, E., Kumar, D., Tunlid, A., and Ahren, D. (2013) Genomic Mechanisms Accounting for the Adaptation to Parasitism in Nematode-Trapping Fungi. *Plos Genetics* **9**.

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V.M. (1998) The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* **10**: 1307-1319.

Morgan, D.O. (1925) Investigations on Eelworm in Potatoes in South Lincolnshire. *Journal of Helminthology* **3**: 185-192.

Niu, J., Arentshorst, M., Nair, P.D.S., Dai, Z.Y., Baker, S.E., Frisvad, J.C., Nielsen, K.F., Punt, P.J., and Ram, A.F.J. (2016) Identification of a Classical Mutant in the Industrial Host Aspergillus niger by Systems Genetics: LaeA Is Required for Citric Acid Production and Regulates the Formation of Some Secondary Metabolites. *G3-Genes Genomes Genetics* **6**: 193-204.

Noel, L., Thieme, F., Nennstiel, D., and Bonas, U. (2002) Two novel type III-secreted proteins of Xanthomonas campestris pv. vesicatoria are encoded within the hrp pathogenicity island. *Journal of Bacteriology* **184**: 1340-1348.

Noreen, R., Ali, S.A., Hasan, K.A., Sultana, V., Ara, J., and Ehteshamul-Haque, S. (2015) Evaluation of biocontrol potential of fluorescent Pseudomonas associated with root nodules of mungbean. *Crop Protection* **75**: 18-24.

Oka, Y., Shapira, N., and Fine, P. (2007) Control of root-knot nematodes in organic farming systems by organic amendments and soil solarization. *Crop Protection* **26**: 1556-1565.

Paal, J., Henselewski, H., Muth, J., Meksem, K., Menendez, C.M., Salamini, F., Ballvora, A., and Gebhardt, C. (2004) Molecular cloning of the potato Gro1-4 gene conferring resistance to pathotype Ro1 of the root cyst nematode Globodera rostochiensis, based on a candidate gene approach. *Plant Journal* **38**: 285-297.

Panella, L., and Lewellen, R.T. (2007) Broadening the genetic base of sugar beet: introgression from wild relatives. *Euphytica* **154**: 383-400.

Perez-de-Luque, A., Tille, S., Johnson, I., Pascual-Pardo, D., Ton, J., and Cameron, D.D. (2017) The interactive effects of arbuscular mycorrhiza and plant growth-promoting rhizobacteria synergistically enhance host plant defences against pathogens. *Scientific Reports* **7**.

Pfannenstiel, B.T., Zhao, X.X., Wortman, J., Wiemann, P., Throckmorton, K., Spraker, J.E., Soukup, A.A., Luo, X.Y., Lindner, D.L., Lim, F.Y., Knox, B.P., Haas, B., Fischer, G.J., Choera, T., Butchko, R.A.E., Bok, J.W., Affeldt, K.J., Keller, N.P., and Palmer, J.M. (2017) Revitalization of a Forward Genetic Screen Identifies Three New Regulators of Fungal Secondary Metabolism in the Genus Aspergillus. *Mbio* **8**.

Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M., and Bakker, P. (2014) Induced Systemic Resistance by Beneficial Microbes. *Annual Review of Phytopathology, Vol 52* **52**: 347-375.

Pike, S.M., Adam, A.L., Pu, X.A., Hoyos, M.E., Laby, R., Beer, S.V., and Novacky, A. (1998) Effects of Erwinia amylovora harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated E-amylovora. *Physiological and Molecular Plant Pathology* **53**: 39-60.

Prasad, P., Varshney, D., and Adholeya, A. (2015) Whole genome annotation and comparative genomic analyses of bio-control fungus Purpureocillium lilacinum. *Bmc Genomics* **16**.

Ridenour, J., Hirsch, R., and Bluhm, B., (2012) Identifying Genes in Fusarium verticillioides Through Forward and Reverse Genetics. In: Plant Fungal Pathogens. Springer, pp. 457-479.

Robinson, A.F. (2007) Reniform in US cotton: When, where, why, and some remedies. *Annual Review of Phytopathology* **45**: 263-288.

Robinson, A.F., Inserra, R.N., Caswell-Chen, E.P., Vovlas, N., and Troccoli, A. (1997) Rotylenchulus species: Identification, distribution, host ranges, and crop plant resistance. *Nematropica* **27**: 127-180.

Rodriguezkabana, R., and Canullo, G.H. (1992) Cropping systems for the management of phytonematodes. *Phytoparasitica* **20**: 211-224.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E., and Williamson, V.M. (1998) The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 9750-9754.

Santos, B.M., Gilreath, J.P., Motis, T.N., Noling, J.W., Jones, J.P., and Norton, J.A. (2006) Comparing methyl bromide alternatives for soilborne disease, nematode and weed management in fresh market tomato. *Crop Protection* **25**: 690-695.

Sasser, J.N., and Freckman, D.W. (1986) A world perspective on nematology - the role of the society. *Journal of Nematology* **18**: 596-596.

Schmitt, M.W., Fox, E.J., Prindle, M.J., Reid-Bayliss, K.S., True, L.D., Radich, J.P., and Loeb, L.A. (2015) Sequencing small genomic targets with high efficiency and extreme accuracy. *Nature Methods* **12**: 423-+.

Schouteden, N., De Waele, D., Panis, B., and Vos, C.M. (2015) Arbuscular Mycorrhizal Fungi for the Biocontrol of Plant-Parasitic Nematodes: A Review of the Mechanisms Involved. *Frontiers in Microbiology* **6**.

Sgro, G.G., Ficarra, F.A., Dunger, G., Scarpeci, T.E., Valle, E.M., Cortadi, A., Orellano, E.G., Gottig, N., and Ottado, J. (2012) Contribution of a harpin protein from Xanthomonas axonopodis pv. citri to pathogen virulence. *Molecular Plant Pathology* **13**: 1047-1059.

Siddiqui, I.A., and Shaukat, S.S. (2004) Systemic resistance in tomato induced by biocontrol bacteria against the root-knot nematode, Meloidogyne javanica is independent of salicylic acid production. *Journal of Phytopathology* **152**: 48-54.

Siddiqui, Z.A., and Mahmood, I. (1996) Biological control of plant parasitic nematodes by fungi: A review. *Bioresource Technology* **58**: 229-239.

Sinn, J.P., Oh, C.-S., Jensen, P.J., Carpenter, S.C.D., Beer, S.V., and McNellis, T.W. (2008) The C-Terminal Half of the HrpN Virulence Protein of the Fire Blight Pathogen Erwinia amylovora Is Essential for Its Secretion and for Its Virulence and Avirulence Activities. *Molecular Plant-Microbe Interactions* **21**: 1387-1397.

Spoel, S.H., and Dong, X.N. (2012) How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology* **12**: 89-100.

Timper, P., and Riggs, R.D. (1998) Variation in efficacy of isolates of the fungus ARF against the soybean cyst nematode *Heterodera glycines*. *Journal of Nematology* **30**: 461-467.

Tripathy, S., Kleppinger-Sparace, K., Dixon, R.A., and Chapman, K.D. (2003) N-Acylethanolamine signaling in tobacco is mediated by a membrane-associated, high-affinity binding protein. *Plant Physiology* **131**: 1781-1791.

Truman, W., de Zabala, M.T., and Grant, M. (2006) Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *Plant Journal* **46**: 14-33.

van der Voort, J.R., Kanyuka, K., van der Vossen, E., Bendahmane, A., Mooijman, P., Klein-Lankhorst, R., Stiekema, W., Baulcombe, D., and Bakker, J. (1999) Tight physical linkage of the nematode resistance gene Gpa2 and the virus resistance gene Rx on a single segment introgressed from the wild species Solanum tuberosum subsp andigena CPC1673 into cultivated potato. *Molecular Plant-Microbe Interactions* **12**: 197-206.

van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., Holovachov, O., Bakker, J., and Helder, J. (2009) A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology* **11**: 927-S927.

Vela-Corcia, D., Romero, D., Tores, J.A., De Vicente, A., and Perez-Garcia, A. (2015) Transient transformation of Podosphaera xanthii by electroporation of conidia. *Bmc Microbiology* **15**.

Vos, C., Schouteden, N., van Tuinen, D., Chatagnier, O., Elsen, A., De Waele, D., Panis, B., and Gianinazzi-Pearson, V. (2013) Mycorrhiza-induced resistance against the root-knot nematode Meloidogyne incognita involves priming of defense gene responses in tomato. *Soil Biology & Biochemistry* **60**: 45-54.

Walters, D.R., and Fountaine, J.M. (2009) Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions. *Journal of Agricultural Science* **147**: 523-535.

Wang, G., Chen, X., Deng, Y., Li, Z., and Xu, X. (2015) Synthesis and Nematicidal Activities of 1,2,3-Benzotriazin-4-one Derivatives against Meloidogyne incognita. *Journal of Agricultural and Food Chemistry* **63**: 6883-6889.

Wang, K.N., Riggs, R.D., and Crippen, D. (2004) Suppression of *Rotylenchulus reniformis* on cotton by the nematophagous fungus ARF. *Journal of Nematology* **36**: 186-191.

Weisemann, J.M., Matthews, B.F., and Devine, T.E. (1992) Molecular markers located proximal to the soybean cyst nematode resistance gene, *Rhg*4. *Theoretical and Applied Genetics* **85**: 136-138.

Weld, R.J., Plummer, K.M., Carpenter, M.A., and Ridgway, H.J. (2006) Approaches to functional genomics in filamentous fungi. *Cell Research* **16**: 31-44.

Westphal, A. (2011) Sustainable Approaches to the Management of Plant-parasitic Nematodes and Disease Complexes. *Journal of Nematology* **43**: 122-125.

Williamson, V.M. (1998) Root-knot nematode resistance genes in tomato and their potential for future use. *Annual Review of Phytopathology* **36**: 277-293.

Williamson, V.M., and Gleason, C.A. (2003) Plant-nematode interactions. *Current Opinion in Plant Biology* **6**: 327-333.

Williamson, V.M., and Kumar, A. (2006) Nematode resistance in plants: the battle underground. *Trends in Genetics* **22**: 396-403.

Witek, K., Jupe, F., Witek, A.I., Baker, D., Clark, M.D., and Jones, J.D.G. (2016) Accelerated cloning of a potato late blight-resistance gene using RenSeq and SMRT sequencing. *Nature Biotechnology* **34**: 656-660.

Xiang, N., Lawrence, K.S., Kloepper, J.W., Donald, P.A., McInroy, J.A., and Lawrence, G.W. (2017) Biological Control of Meloidogyne incognita by Spore-forming Plant Growth-promoting Rhizobacteria on Cotton. *Plant Disease* **101**: 774-784.

Xie, Z.X., and Chen, Z.X. (2000) Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. *Molecular Plant-Microbe Interactions* **13**: 183-190.

Yang, C.H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J.W.T., Perna, N.T., and Keen, N.T. (2002) hrp genes of Erwinia chrysanthemi 3937 are important virulence factors. *Molecular Plant-Microbe Interactions* **15**: 472-480.

Yang, F., Abdelnabby, H., and Xiao, Y.N. (2016) Construction and verification of a gene knockout system in the nematophagous fungus, Purpreocillium lilacinum (Hypocreales: Ophiocordycipitaceae). *Applied Entomology and Zoology* **51**: 99-110.

Yang, J., Tian, B., Liang, L., and Zhang, K.-Q. (2007a) Extracellular enzymes and the pathogenesis of nematophagous fungi. *Applied Microbiology and Biotechnology* **75**: 21-31.

Yang, J., Wang, L., Ji, X., Feng, Y., Li, X., Zou, C., Xu, J., Ren, Y., Mi, Q., Wu, J., Liu, S., Liu, Y., Huang, X., Wang, H., Niu, X., Li, J., Liang, L., Luo, Y., Ji, K., Zhou, W., Yu, Z., Li, G., Liu, Y., Li, L., Qiao, M., Feng, L., and Zhang, K.-Q. (2011) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. *Plos Pathogens* **7**.

Yang, Y., Yang, E., An, Z.Q., and Liu, X.Z. (2007b) Evolution of nematode-trapping cells of predatory fungi of the Orbiliaceae based on evidence from rRNA-encoding DNA and multiprotein sequences. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 8379-8384.

Zasada, I.A., Halbrendt, J.M., Kokalis-Burelle, N., LaMondia, J., McKenry, M.V., and Noling, J.W. (2010) Managing Nematodes Without Methyl Bromide. *Annual Review of Phytopathology, Vol 48* **48**: 311-328.

Zastrow-Hayes, G.M., Lin, H.N., Sigmund, A.L., Hoffman, J.L., Alarcon, C.M., Hayes, K.R., Richmond, T.A., Jeddeloh, J.A., May, G.D., and Beatty, M.K. (2015) Southern-by-Sequencing: A Robust Screening Approach for Molecular Characterization of Genetically Modified Crops. *Plant Genome* **8**.

CHAPTER II

Treatment of Soybean and Cotton Seeds with Harpin Protein Demonstrates its Efficacy Against Reniform nematodes

Abstract

Plant parasitic nematodes are one of the most destructive pathogens of crop plants, causing billions of dollars losses annually. Chemicals are widely used method for controlling plant parasitic nematodes but cause widespread concern for human health and environmental hazard consequently effective alternative methods are needed for nematode. In this research, trials were conducted in the green house to study the efficacy of harpin protein treatments to soybean and cotton seeds and their effect on plant growth and on reniform and cyst nematode populations in soil. When samples were analyzed 45 days after inoculation, harpin protein treated soybean and cotton seeds showed significantly less reniform population in soil compared to control non-treated plants. However, plant root and shoot weight did not significantly differ among treatments. Similar experiments were performed with soybean-cyst pathosystem and treating seeds with harpin protein did not affect the population of cyst nematodes in soil. Additionally, soybean seeds soaked in harpin solution were analyzed with scanning electron microscopy. The harpin proteins formed a coating over the soybean seed, suggesting that harpin protein interacts with the surface of soybean seeds. This is the first report on efficacy of harpin treatments to seeds on reniform nematode population in soil. The data from the chapter suggest the possibility in utilizing harpin proteins as a component of nematode control and guide the usage of harpin proteins as an integral component for management of reniform nematodes.

1. Introduction

Plant-parasitic nematodes (PPNs) are obligate, biotrophic pathogens and one of the major pests of economically important crops globally. More than 4,100 species of plant-parasitic nematodes have been described (Decraemer and Hunt, 2006), and nematodes cause severe losses to agricultural crops worldwide. Economic damage due to plant-parasitic nematodes exceeds $150 billion annually (Abad et al., 2008). A typical nematode infestation averages more than 10 per cent annual yield loss in productivity, and losses can exceed 20 per cent in crops such as bananas (Sasser and Freckman, 1986). Plant-parasitic nematodes have evolved various strategies to interact with host plants (Gheysen and Mitchum, 2011); but have developed a common mode of infecting plants with their hollow spear-like mouthpart called the stylet. Stylets serve several purposes including infecting host roots, penetrating host cells, injecting virulence factors, and deriving nutrients from their hosts. Plant-parasitic nematodes have also evolved diverse lifestyles of infecting plants. Although, nematodes can infect different regions of the plants, root-infecting nematodes are most damaging.

Of the various plant parasitic nematode species, the three most economically important nematodes in the US are the root-knot nematode (RKN), the soybean cyst nematode (SCN), and the reniform nematode. The reniform nematode (RN), *Rotylenchulus reniformis* Linford & Oleveira, is a sedentary semi-endoparasite and is widespread in the Southern United States, including Alabama, Mississippi, and Louisiana, and has a broad host range. RN parasitizes more than 300 plant species, including vegetables, fruits, and weeds (Robinson et al., 1997; Lawrence et al., 2008). Cotton and soybean are the major crops in the Southern United States affected by RN (Stetina et al., 2014), and it is considered a major species affecting soybean yield after root knot and cyst nematode (Robinson et al., 1997). In US alone, the loss caused due to RN in cotton

is estimated $150 million (Robinson, 2007) while a loss of around 4.8 million bushels of soybean in the southern US states alone is reported (SSDW, 2015). The symptoms caused due to RN are very similar to nutrient deficiency. Although they cause reduced root growth and secondary root development, the symptoms are practically unobservable in the roots. Major symptoms of the RN infection include root necrosis, stunted growth, chlorosis in the leaves and incomplete pod filling (McGawley et al., 2011). Depending on the humidity, temperature, condition of the crops and nematode population in soil, RN can cause 40-60% loss in yields (Jones et al., 2013). The cyst nematode is an obligatory biotroph and is common throughout the world. The soybean cyst nematode is a major threat to soybean production worldwide and is responsible for an estimated loss exceeding $1.2 billion in the United States (Gardner et al., 2017). One of the most important features of this nematode is its ability to persist in the soil as cysts. The cyst nematode can survive prolonged periods without the presence of host. This makes the control of this nematode difficult even by rotation with non-hosts. Crop rotation and planting resistant varieties are widely used methods for the control of the cyst nematodes. However, the use of resistant varieties is limited and the nematode has developed different races to overcome plant resistance (Shi et al., 2015; Zhou et al., 2017). The control of this nematode has been problematic especially due to discontinuation of chemical pesticides.

Until recently, management practices of plant-parasitic nematodes relied primarily on chemicals like aldicarb and methyl bromide. However, these chemicals pose a serious concern for the human health and the environment. Additionally, chemical control strategies do not result in long-term suppression of nematodes and are not cost-effective (Molinari, 2011). Currently, chemicals like abamectin, thiodicarb, 1,3-dichloropropene, and oxamyl are being deployed to control plant parasitic nematodes (Kinloch and Rich, 2001; Lawrence and McLean, 2002; Faske

and Starr, 2006). Although strategies like soil solarization, crop rotation, resistance cultivars, resistance genes transfer, RNA interference (RNAi), biological control and regulatory approaches have been deployed as alternatives to chemical control, however, these control strategies have met with limited success. Induced immunity refers to a form of resistance developed in response to an external stimuli (van Loon et al., 1998). Induced immunity usually results in a global response in plants and acts against wide variety of plant pathogens like bacteria, fungi, viruses and nematodes (Hammerschmidt, 1999). Additionally induced systemic resistance causes enhanced photosynthesis response, increased nitrogen uptake, and less abiotic stress (Shoresh et al., 2010). Thus, use of plants own defense (natural or induced), therefore, could be developed as an alternate, effective and environmentally safe strategy to control plant parasitic nematodes.

Harpin proteins are components of type III secretion system (T3SS) of gram negative, plant-pathogenic bacteria which are encoded by hypersensitive response and pathogenicity (*hrp*) genes (Tampakaki et al., 2010). These proteins are heat stable, acidic, glycine rich and lack cysteine residues (Choi et al., 2013). With the discovery of HrpN in *Erwinia amylovora*, the causal agent of fire blight of cherry apple, it was found that these proteins could elicit a pathogen-independent response in plants (Wei et al., 1992). Harpin proteins, when applied on non-host plant species, trigger hypersensitive response and systemic response against wide range of pathogens, including bacteria, fungi, oomycetes, viruses, and insects (Dong et al., 1999; Dong et al., 2004; Reboutier et al., 2007; Che et al., 2011). However, purified forms of harpin proteins are known to induce systemic response even when sprayed on plants, which is independent of HR pathway. Previous work done in tomato, *Arabidopsis*, and tobacco have shown that harpin proteins elicit diverse immune responses, that are mediated through salicylic acid, ethylene, or

jasmonic acid (Dong et al., 1999; Kariola et al., 2003; Sohn et al., 2007; Chuang et al., 2010). Additionally harpin proteins are known to induce production of reactive oxygen species (ROS), ion-mediated pore formation, and callose deposition (Kvitko et al., 2007; Oh et al., 2007; Reboutier and Bouteau, 2008; Engelhardt et al., 2009; Haapalainen et al., 2011). Although the induction of resistance in plants by harpin proteins is well documented, the mechanistic action underlying this induction of resistance is understudied.

Though, roles of harpin proteins inducing plants defense response against diverse pathogens has been well studied but not against plant parasitic nematodes. The objective of this study was to identify the efficacy of seed treatment of harpin protein on the population of reniform and soybean cyst nematode. In this study soybean seeds treated with harpin proteins show significant reduction in reniform nematode population in soil. The experiments performed on soybean and *R. reniformis* pathosystem suggest that harpin proteins induce responses in soybean plants that is adverse to the reniform nematodes in soil and provide evidence that harpin proteins activate defense in soybean plants against the reniform nematodes. This study could potentially open new avenues for soybean growers to manage reniform nematodes and offer new approaches without using harmful chemicals.

2. Materials and methods

2.1 Nematodes culture and collection

R. reniformis was propagated and maintained on susceptible cotton (*Gossypium hirsutum*) plants in green house conditions. For establishing culture of *R. reniformis* nematodes, cottonseeds were sown in 10 cm clay pots filled with 1:1 sterilized sand and field soil. Upon germination of cotton plants, after two weeks, the pots were inoculated with mixed population of

3,500 vermiform *R. reniformis*. Soybean cyst nematodes were grown in susceptible Lee variety of soybean. Soybean seeds were sown in a mixture of sterilized sand and field soil in styro-foam cups. After germination, the pots were inoculated with eggs extracted from cysts. Plants were watered regularly, and Miracle grow (Scotts Company LLC,Marysville, OH) fertilizer (24-8-16) was applied as a source of nutrients for plants. The pots were maintained in green house conditions at temperature of 28-30 °C and constant humidity with 16:8 light dark hours for optimum growth and propagation of the nematodes.

2.2 Extraction of nematodes for inoculum preparation

The vermiform stages of *R. reniformis* were extracted from the soil samples using the density centrifugation technique (Jenkins, 1980). To collect mixed vermiform stages of nematodes, soil along with roots of cotton plants were carefully removed from pots and placed in a bucket and washed gently with running water to separate the soil from roots of plants. The soil was stirred in water for 30 seconds and allowed to stand for another 30 seconds to allow larger soil particles to settle at bottom of bucket while nematodes and smaller soil particles remain suspended in solution (Ganji et al., 2013). The nematode and soil water suspension was gently decanted through a series of sieves of pore sizes 150 µm pore and 38 µm. Nematodes and finer soil particles were collected from the 38 µm sieve by decanting with water. To further purify the vermiform nematodes from finer soil particles, density centrifugation technique was utilized. Briefly, nematode and finer soil suspension were placed in 50 ml tubes and centrifuged at 1400 rpm for 5 minutes at room temperature. Upon centrifugation, nematodes along with finer soil particles pellet at the bottom of the tubes. Supernatant was carefully discarded to avoid disturbing the pellet, containing soil particles and nematodes. The pellet was then mixed with 30% sucrose solution and stirred using a spatula ensuring that pellet was stirred from the bottom

of tubes. This suspension was spun at 1400 rpm for 3 minutes at room temperature. The finer soil particles settle at the bottom while the vermiform nematodes float in the 30% sugar solution, which was carefully decanted through 25 μm pore sized sieve to collect the vermiform nematodes. The nematodes were carefully washed with running water to remove all sugar solution and avoid killing nematodes by osmotic stress. Vermiform nematodes collected on 25 μm sieves were re- suspended in sterile water and counted under dissecting microscope to determine population density.

Eggs of soybean cyst nematodes were extracted from freshly harvested cysts previously inoculated in Lee variety of soybean. Roots along with soil mass were put in bucket and washed with running water to separate the soil from the root mass. Suspension was agitated thoroughly to mix the soil and the water and allowed to stand still for 2 minutes to allow heavier soil particles to settle down. The suspension was then passed through a series of sieves with pores of sizes 420 μm at the top and 250 μm at the bottom. Cysts were collected over the sieve with pore size 250 μm. The cysts were then carefully washed with water and collected in a clean beaker with water. To collect eggs, cysts are pulverized thoroughly with glass pestle. Egg masses were collected by passing the pulverized cyst suspension through a 37 μm sieve. Eggs were re-suspended on sterile water, and population density was determined by counting the eggs in a counting dish.

2.3 Seed treatments and planting

Harpin protein was obtained as Messenger (1% a. i. harpin Ea), an industrial formulation from Plant Health Care Inc. NC, US. Seed treatments were applied in two different ways. For dry treatment, soybean and cottonseeds were dusted with harpin proteins at the rate of 0.25 oz per

100 pounds of seed. In separate treatment method, seeds of soybean and cotton were soaked in 10 μg/ml harpin protein solution in sterile water for one hour before planting. Tween-20 (0.02%) was added as surfactant to facilitate proper binding of harpin onto the seed surface. Seeds soaked in Maltrin-M100 + 0.02% Tween 20 served as control. After treating soybean and cotton seeds with harpin Ea, seeds were immediately planted into 10 cm Styrofoam pots that were filled with 1:1 sterilized field soil and sand mixture. Holes were made at base of the pots to allow drainage of excess water. The pots were placed on benches in green houses. Initially, two seeds were planted in each styrofoam pot. After germination, plants were thinned to contain one plant per pot. Plants were maintained in green house conditions of 28-30 °C, constant humidity, 16:8 hours light: dark cycle and regular watering. In the first experiment conducted with treating soybean and cotton seeds with harpin protein at 0.25oz/100 lb seeds the plants were not randomized. For all the subsequent experiments, pots were placed in a completely randomized design.

2.4 Inoculation of nematodes into the soil

Inoculations of all vermiform nematodes and egg masses into soil were done at two-leaf stage where two holes, about 2 cm deep, were created around the base of the soybean and cotton plants. Water suspension containing the inoculums was stirred at regular interval to prevent nematodes and eggs from settling at bottom of container. Nematodes were pipetted out of suspension using a 1 ml pipette and about 3,500 vermiform *R. reniformis* of mixed juvenile and adult stages were carefully inoculated into the 2 cm holes around the plants. Similarly, for soybean cyst nematodes, around 1,500 eggs per plant were inoculated at base of each plant. Holes were covered immediately after inoculation to prevent splashing of nematodes during watering of pots.

2.5 Analyses of plant phenotypes and nematode population

The analyses of plant phenotypes and nematode population were performed six weeks after inoculation of nematodes. Firstly, shoots were cut off from the base of the plants. Roots were extracted from soil by gentle agitation in a bucket containing water. After complete removal of soil particles from the roots, both shoots and roots were padded dry in a paper towel. Fresh weight of shoot and roots were measured and samples were dried in hot air oven at 90 °C overnight. Dry weights of roots and shoots were measured, separately.

To analyze reniform population in soil, roots were removed, and soil suspension in water was passed through a series of screens of pore sizes 150 µm and 38 µm. RN that collected over the sieve with pore size 38 µm were re-suspended in water. Because this suspension also contained very fine soil particles that need to be removed for counting the reniform nematode population, soil and nematode suspension was then centrifuged in 30% sugar solution to separate the reniform nematodes from finer soil particles. Reniform nematodes were collected over a sieve with pore size 25 µm and re-suspended in clean water. The reniform nematodes were visualized and counted under a dissecting microscope.

To collect SCN, soil and roots were removed from pots and washed with running water in a bucked. Soil particles were completely washed off, suspension was mixed thoroughly and allowed to stand still for 2 minutes to ensure that heavier soil particles settled at the bottom. Water was then passed through a series of sieves of sizes 420 µm and 250 µm. SCN are large enough to collect over a lower sieve with pore sizes of 250 µm. Cysts were counted on a plate using a dissecting microscope.

The statistical analyses were performed in the R studio environment. For two treatments, Welch t-test was performed at significance level of $P \leq 0.05$. For analyses of multiple treatments, ANOVA was performed to identify the presence of significant difference between treatments followed by Tukey's HSD test for multiple comparisons at $P \leq 0.05$. Difference in variance was performed by Bartlett test at significance level of $P \leq 0.05$.

2.6 Scanning Electron Microscopy of soybean seeds soaked in harpin

Scanning Electron microscopy of soybean seeds soaked in harpin protein solution was performed to visualize localization of the harpin protein on the seed surface. Briefly, the soybean seeds were soaked in harpin protein solution at 100 µg/ml concentration for one hour. For visual purposes, this concentration used was 10x higher than that used for seed treatments during green house assays. However, concentration of the inert material was increased to the same ratio. Tween-20 (0.02%) was added as a surfactant to facilitate proper binding of harpin protein onto the seed surface. Similarly, a control was set up with 100 µg/ml solution of Maltrin-M100 (inert material without the harpin protein component), with Tween-20 of 0.02 % concentration. Soybean seeds were soaked for one hour at room temperature. After one hour of complete soaking, the seeds were removed and air-dried in sterile conditions at 30 °C over night to completely remove the moisture. The surface of the seeds were mounted onto stubs and coated with 3 nm thin film of gold and then visualized under scanning electron microscope at the Institute of Nanosciences and Engineering, University of Arkansas.

3. Results

3.1 Effect of harpin protein treatment on plant height

In the presence of RN seed coating or dusting with harpin protein did not show consistent increase in plant height compared with untreated soybean seed (Figures 2.1- 2.3). Additionally, experiments conducted with different methods of seed treatments also did not have significant differences in plant height, when compared with the control untreated samples (Figure 2.10).

3.2 Effect of harpin protein treatment on plant biomass

Treating soybean seeds with harpin protein does not have consistent affect on plant biomass (2.4-2.6). Additionally, experiments with different methods of harpin protein treatments showed no significant increase in plant biomass (Figures 2.11-2.14), compared with untreated plants. Under RN infection, soybean plants that were treated with harpin protein had same fresh biomass as that of plants that were untreated. Additionally, dry weights were analyzed and it was observed that harpin protein treated samples had no improvement in plant growth compared with untreated samples.

3.3 Effect of harpin protein treatments on nematode populations

Seed coating or seed dusting resulted in reductions in reniform population compared with untreated control plants (Figure 2.7). Although, there was also a slight reduction in RN population from cotton plants that were treated with harpin protein, the reduction was not significant statistically compared to untreated control plants (Figure 2.8). Experiments conducted with SCN demonstrated that treating soybean seeds with harpin protein prior to planting showed no changes in the soybean cyst nematode population in soil (Figure 2.9). Additionally, the data

from different application methods of harpin protein showed significant reduction in reniform population in soil compared with control (Figure 2.15). However, RN densities did not differ between two different treatment application methods when compared six weeks after inoculation. Coating soybean seeds with harpin protein as recommended by manufacturer (0.25oz/100 lb seeds) before planting or soaking soybean seeds in 10 μg/ml harpin protein solution had similar efficacy on RN population densities. The experiment with different harpin protein treatments was repeated with similar response to RN population densities. While the treatments differed significantly from the control untreated plants, there was no significant difference between different treatment methods (Figure 2.15).

3.4 Harpin protein aggregates on the surface of soybean seeds

To investigate the localization of harpin proteins on soybean seeds after seed treatment, we performed scanning electron microscopy of soybean seeds soaked in harpin protein and control. Based in the observations of SEM of soybean seed surface, the harpin protein appears to form an aggregate on surface seeds soaked for one hour in 100 μg/ml harpin protein solution. However, soybean seeds soaked in a control solution of Maltrin-M100 (inert component of the formulation) of equal concentration did not show any visual aggregation of the inert material on the seed surface (Figure 2.16). The formation of the aggregate in seeds soaked in harpin protein solution is more apparent in higher magnification. The harpin protein aggregates are formed throughout the seed surface and appear as thin layer covering the seed surface.

4. Discussion

When challenged by pathogens, plants mount numerous defense responses including production of toxic compounds, elicitation of hormone signaling pathways, and expression of

pathogenesis related proteins. Harpin proteins are elicitors of bacterial origin and are components of the Type III secretion system (T3SS), which help in delivery of bacterial effectors directly into cytosol (Valls et al., 2006). Harpin proteins were generally thought to elicit just the hypersensitive response; however increasing evidence suggests diverse roles for these proteins, including induction of systemic resistance and improvement of plant vigor (Wang et al., 2007). Previous studies demonstrated that exogenous application of harpin proteins onto plants induced multiple defense pathways mediated by salicylic acid, jasmonic acid, and ethylene (Shao et al., 2008) and resulted in increased resistance against diverse pathogens like bacteria, oomycetes, fungi, virus, and insects, as well as abiotic stresses like drought (Dong et al., 1999; Dong et al., 2004; Jang et al., 2006; Chuang et al., 2010; Zhang et al., 2011a; Zhang et al., 2011b). However, the role of harpin proteins in nematode resistance has been understudied. Findings from these experiments with harpin and reniform nematodes demonstrate that treating soybean and cotton seeds with harpin before planting can effectively reduce *R. reniformis* population in soil under green house conditions. Seeds of soybean and cotton that were either coated with harpin suspension or soaked in harpin solution for one hour before planting showed a significant reduction in *R. reniformis* population in the soil at six weeks after inoculation. The research presented in this chapter is the first evidence of the efficacy of harpin proteins against *R. reniformis*. This suggests the potential of utilizing harpin for management of reniform nematodes. However, molecular mechanisms underlying harpin mediated response of soybean, especially during reniform nematode infection are unclear.

In this study, difference in plant growth parameters between harpin treated and control plants were not consistent. Similar results were observed from previous experiments conducted in tomato treated with harpin protein (Obradovic et al., 2004) and from field experiments

conducted in Arkansas (unpublished). However, other reports have demonstrated the effect of harpin treatment on plant growth phenotypes (Dong et al., 2004; Chuang et al., 2010; Li et al., 2014). One explanation for variability in plant phenotype among experiments could be that inducing resistance against pathogens in plants imposes a fitness cost, resulting in reduced plant growth and reproduction (Cipollini et al., 2003). For example, an ortholog of apple HrpN interacting protein in *Arabidopsis*, AtHIMP, is a negative regulator of growth (Oh and Beer, 2007). Similarly, inhibition of growth was observed in *Nicotiana tabacum* plants after treatment with harpin protein (Dimlioglu et al., 2015).

RN is prevalent in the southern United States, and parasitizes more than 300 plant species, including cotton and soybean (Robinson et al., 1997). Although RN is more problematic in cotton, a shift in acreage from cotton to soybean could pose a serious threat to soybean production due to this pathogen. Although some soybean cultivars contain resistance to reniform nematode (Robbins and Rakes, 1996; Robbins et al., 2002), undesirable agronomic traits associated with these cultivars make them less suitable. With discontinuation of chemical treatments to control plant parasitic nematodes, deployment of elicitors to induce host resistance is advantageous due to simplicity in application, environmentally sustainable, and cost effective (Stetina et al., 2014). Elicitors are molecules that trigger defense response in plants through activation of complex signaling pathways and considered an effective alternate approach to control plant pathogens (Vallad and Goodman, 2004; Bruce, 2010). Previous work demonstrated the effect of elicitors like methyl jasmonate (JAME) and salicylic acid (SA) in reducing the reniform nematode population in soil by reducing fecundity (Soler et al., 2013). These elicitors induced plant defenses by activating hormonal signaling like salicylic and jasmonic acid. Although harpin induces plant resistance against variety of pathogens, its role in activating plant

defenses against plant parasitic nematodes such as the reniform nematode has not been reported. The greenhouse experiments conducted with soybean seeds treated with harpin protein showed significant reductions in RN population compared with untreated control plants but did not consistently influence plant growth. Additionally, treating soybean seeds with harpin proteins developed a coat around soybean seeds, as evident from scanning electron microscopy of soybean seeds, whereas a coating was absent on seeds soaked in Maltrin-100 (the inert material used in Messenger formulation). The formation of a coating on the soybean seed surface by harpin proteins has not been reported previously. Scanning electron micrographs of soybean seeds soaked in harpin could suggest a mode of action on how an elicitor like harpin attaches onto the seed surface and results in activation of defense response.

Plants possess pattern recognition receptors (PRRs), which can recognize specific patterns in microbial molecules called pathogen- or microbe- associated molecular patterns (PAMPs/MAMPs), and provide PAMP triggered immunity (PTI) (Boller and Felix, 2009; Lacombe et al., 2010). However, the role of such receptors on seed surface has been understudied. Studies, however, have shown the involvement of plant lectin receptor-like kinases in plant vigor and immunity during germination (Cheng et al., 2013). Previous research on *Arabidopsis* treated with harpin showed increased root and shoot growth and induced resistance against green peach aphids (Dong et al., 2004). The mechanism underlying the recognition of harpin proteins by seeds and transmission of signals during germination is unknown. Soybean contains proteins that bind to specific elicitors like syringolide, which are highly expressed in seeds (Ji et al., 1998). A similar mechanism of recognition to harpin proteins could exist in soybean seeds responsible for induction of resistance against nematodes in later stages of growth.

Plants do possess different receptors in cell walls to recognize harpin proteins (Lee et al., 2001; Fontanilla et al., 2005), however, their induction and expression in seeds in unknown.

Plants parasitic nematodes are serious threat to crop yield and productivity. With the loss of some efficacious chemical nematicides nematodes, the search for alternative controls has met with limited success. Since the discovery of harpin protein's role in activating plants defense against major plant pathogens, this research analyzes novel function of harpin that seed treatments can activate defense in roots against plant parasitic nematodes. Based on these findings, harpin proteins could provide an additional tactic for nematode management. Additional advantages include ease of application, user and environmental safeties, and cost effectiveness. Although the exact mechanism of harpin proteins against reniform nematodes remains to be seen, the mechanism could be through activation of salicylic acid mediated pathway, a hormone that predominates in systemic acquired resistance (SAR) during pathogens infection. Taken together, harpin protein can act as an important component of nematode management in conjunction with other methods.

References

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.-C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.-N., Schiex, T., Smant, G., Weissenbach, J., and Wincker, P. 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26:909-915.

Boller, T., and Felix, G. 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. Annual Review of Plant Biology 60:379-406.

Bruce, T.J.A. 2010. Tackling the threat to food security caused by crop pests in the new millennium. Food Security 2:133-141.

Che, Y.-Z., Li, Y.-R., Zou, H.-S., Zou, L.-F., Zhang, B., and Chen, G.-Y. 2011. A novel antimicrobial protein for plant protection consisting of a Xanthomonas oryzae harpin and active domains of cecropin A and melittin. Microbial Biotechnology 4:777-793.

Cheng, X.Y., Wu, Y., Guo, J.P., Du, B., Chen, R.Z., Zhu, L.L., and He, G.C. 2013. A rice lectin receptor-like kinase that is involved in innate immune responses also contributes to seed germination. Plant Journal 76:687-698.

Choi, M.-S., Kim, W., Lee, C., and Oh, C.-S. 2013. Harpins, Multifunctional Proteins Secreted by Gram-Negative Plant-Pathogenic Bacteria. Molecular Plant-Microbe Interactions 26:1115-1122.

Chuang, H.-w., Harnrak, A., Chen, Y.-C., and Hsu, C.-M. 2010. A harpin-induced ethylene-responsive factor regulates plant growth and responses to biotic and abiotic stresses. Biochemical and Biophysical Research Communications 402:414-420.

Cipollini, D., Purrington, C.B., and Bergelson, J. 2003. Costs of induced responses in plants. Basic and Applied Ecology 4:79-89.

Decraemer, W., and Hunt, D.J. 2006. Structure and classification. Plant nematology.:3-32.

Dimlioglu, G., Das, Z.A., Bor, M., Ozdemir, F., and Turkan, I. 2015. The impact of GABA in harpin-elicited biotic stress responses in Nicotiana tabaccum. Journal of Plant Physiology 188:51-57.

Dong, H.P., Peng, J.L., Bao, Z.L., Meng, X.D., Bonasera, J.M., Chen, G.Y., Beer, S.V., and Dong, H.S. 2004. Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. Plant Physiology 136:3628-3638.

Dong, H.S., Delaney, T.P., Bauer, D.W., and Beer, S.V. 1999. Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. Plant Journal 20:207-215.

Engelhardt, S., Lee, J., Gaebler, Y., Kemmerling, B., Haapalainen, M.-L., Li, C.-M., Wei, Z., Keller, H., Joosten, M., Taira, S., and Nuernberger, T. 2009. Separable roles of the Pseudomonas syringae pv. phaseolicola accessory protein HrpZ1 in ion-conducting pore formation and activation of plant immunity. Plant Journal 57:706-717.

Faske, T.R., and Starr, J.L. 2006. Sensitivity of Meloidogyne incognita and Rotylenchulus reniformis to abamectin. Journal of Nematology 38:240-244.

Fontanilla, J.M., Montes, M., and De Prado, R. 2005. Effects of the foliar-applied protein 'Harpin(Ea)'. (Messenger (R)) on tomatoes infected with Phytophthora infestans. Communications in Agricultural and Applied Biological Sciences 70:41-45.

Ganji, S., Wubben, M.J., and Jenkins, J.N. 2013. Two Simple Methods for the Collection of Individual Life Stages of Reniform Nematode, Rotylenchulus reniformis. Journal of Nematology 45:87-91.

Gardner, M., Heinz, R., Wang, J.Y., and Mitchum, M.G. 2017. Genetics and Adaptation of Soybean Cyst Nematode to Broad Spectrum Soybean Resistance. G3-Genes Genomes Genetics 7:835-841.

Gheysen, G., and Mitchum, M.G. 2011. How nematodes manipulate plant development pathways for infection. Current Opinion in Plant Biology 14:415-421.

Haapalainen, M., Engelhardt, S., Kuefner, I., Li, C.-M., Nuernberger, T., Lee, J., Romantschuk, M., and Taira, S. 2011. Functional mapping of harpin HrpZ of Pseudomonas syringae

reveals the sites responsible for protein oligomerization, lipid interactions and plant defence induction. Molecular Plant Pathology 12:151-166.

Hammerschmidt, R. 1999. Induced disease resistance: how do induced plants stop pathogens? Physiological and Molecular Plant Pathology 55:77-84.

Jang, Y.S., Sohn, S.I., and Wang, M.H. 2006. The hrpN gene of Erwinia amylovora stimulates tobacco growth and enhances resistance to Botrytis cinerea. Planta 223:449-456.

Jenkins, W.R. 1980. Citation Classic- A rapid centrifugal-flotation technique for separating nematodes from soil. Current Contents/Agriculture Biology & Environmental Sciences:14-14.

Ji, C., Boyd, C., Slaymaker, D., Okinaka, Y., Takeuchi, Y., Midland, S.L., Sims, J.J., Herman, E., and Keen, N. 1998. Characterization of a 34-kDa soybean binding protein for the syringolide elicitors. Proceedings of the National Academy of Sciences of the United States of America 95:3306-3311.

Jones, J.T., Haegeman, A., Danchin, E.G.J., Gaur, H.S., Helder, J., Jones, M.G.K., Kikuchi, T., Manzanilla-Lopez, R., Palomares-Rius, J.E., Wesemael, W.M.L., and Perry, R.N. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14:946-961.

Kariola, T., Palomaki, T.A., Brader, G., and Palva, E.T. 2003. Erwinia carotovora subsp carotovora and Erwinia-derived elicitors HrpN and PehA trigger distinct but interacting defense responses and cell death in Arabidopsis. Molecular Plant-Microbe Interactions 16:179-187.

Kinloch, R.A., and Rich, J.R. 2001. Management of root-knot and reniform nematodes in ultra-narrow row cotton with 1,3-dichloropropene. Journal of Nematology 33:311-313.

Kvitko, B.H., Ramos, A.R., Morello, J.E., Oh, H.-S., and Collmer, A. 2007. Identification of harpins in Pseudomonas syringae pv. tomato DC3000, which are functionally similar to HrpK1 in promoting translocation of type III secretion system effectors. Journal of Bacteriology 189:8059-8072.

Lacombe, S., Rougon-Cardoso, A., Sherwood, E., Peeters, N., Dahlbeck, D., van Esse, H.P., Smoker, M., Rallapalli, G., Thomma, B., Staskawicz, B., Jones, J.D.G., and Zipfel, C. 2010. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nature Biotechnology 28:365-U394.

Lawrence, G.W., and McLean, K.S. 2002. Foliar applications of oxamyl with aldicarb for the management of Meloidogyne incognita on cotton. Nematropica 32:103-112.

Lawrence, K.S., Price, A.J., Lawrence, G.W., Jones, J.R., and Akridge, J.R. 2008. Weed hosts for Rotylenchulus reniformis in cotton fields rotated with corn in the Southeast United States. Nematropica 38:13-22.

Lee, J., Klessig, D.F., and Nurnberger, T. 2001. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. Plant Cell 13:1079-1093.

Li, X., Han, B., Xu, M., Han, L., Zhao, Y., Liu, Z., Dong, H., and Zhang, C. 2014. Plant growth enhancement and associated physiological responses are coregulated by ethylene and gibberellin in response to harpin protein Hpa1. Planta 239:831-846.

McGawley, E.C., Overstreet, C., and Pontif, M.J. 2011. Variation in reproduction and pathogenicity of geographic isolates of Rotylenchulus reniformis on soybean. Nematropica 41:12-22.

Molinari, S. 2011. Natural genetic and induced plant resistance, as a control strategy to plant-parasitic nematodes alternative to pesticides. Plant Cell Reports 30:311-323.

Obradovic, A., Jones, J.B., Momol, M.T., Balogh, B., and Olson, S.M. 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. Plant Disease 88:736-740.

Oh, C.-S., and Beer, S.V. 2007. AtHIPM, an ortholog of the apple HrpN-Interacting protein, is a negative regulator of plant growth and mediates the growth-enhancing effect of HrpN in Arabidopsis. Plant Physiology 145:426-436.

Oh, J., Kim, J.-G., Jeon, E., Yoo, C.-H., Moon, J.S., Rhee, S., and Hwang, I. 2007. Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. Journal of Biological Chemistry 282:13601-13609.

Reboutier, D., and Bouteau, F. 2008. Harpins and ion channels modulations: Many ways to die. Plant signaling & behavior 3:314-316.

Reboutier, D., Frankart, C., Briand, J., Biligui, B., Laroche, S., Rona, J.-P., Barny, M.-A., and Bouteau, F. 2007. The HrpN(ea) harpin from Erwinia amylovora triggers differential responses on the nonhost Arabidopsis thaliana cells and on the host apple cells. Molecular Plant-Microbe Interactions 20:94-100.

Robbins, R.T., and Rakes, L. 1996. Resistance to the reniform nematode in selected soybean cultivars and germplasm lines. Journal of Nematology 28:612-615.

Robbins, R.T., Shipe, E.R., Rakes, L., Jackson, L.E., Gbur, E.E., and Dombek, D.G. 2002. Host suitability of soybean cultivars and breeding lines to reniform nematode in tests conducted in 2001. Journal of Nematology 34:378-383.

Robinson, A.F. 2007. Reniform in US cotton: When, where, why, and some remedies. Annual Review of Phytopathology 45:263-288.

Robinson, A.F., Inserra, R.N., Caswell-Chen, E.P., Vovlas, N., and Troccoli, A. 1997. Rotylenchulus species: Identification, distribution, host ranges, and crop plant resistance. Nematropica 27:127-180.

Sasser, J.N., and Freckman, D.W. 1986. A world perspective on nematology - the role of the society. Journal of Nematology 18:596-596.

Shao, M., Wang, J., Dean, R.A., Lin, Y., Gao, X., and Hu, S. 2008. Expression of a harpin-encoding gene in rice confers durable nonspecific resistance to Magnaporthe grisea. Plant Biotechnology Journal 6:73-81.

Shi, Z., Liu, S.M., Noe, J., Arelli, P., Meksem, K., and Li, Z.L. 2015. SNP identification and marker assay development for high-throughput selection of soybean cyst nematode resistance. Bmc Genomics 16.

Shoresh, M., Harman, G.E., and Mastouri, F. 2010. Induced Systemic Resistance and Plant Responses to Fungal Biocontrol Agents. Annual Review of Phytopathology, Vol 48 48:21-43.

Sohn, S.-I., Kim, Y.-H., Kim, B.-R., Lee, S.-Y., Lim, C.K., Hur, J.H., and Lee, J.-Y. 2007. Transgenic tobacco expressing the hrpN(EP) gene from Erwinia pyrifoliae triggers defense responses against Botrytis cinerea. Molecules and Cells 24:232-239.

Soler, A., Marie-Alphonsine, P.-A., Corbion, C., and Queneherve, P. 2013. Differential response of two pineapple cultivars (Ananas comosus (L.) Merr.) to SAR and ISR inducers against the nematode Rotylenchulus reniformis. Crop Protection 54:48-54.

Stetina, S.R., Smith, J.R., and Ray, J.D. 2014. Identification of Rotylenchulus reniformis Resistant Glycine Lines. Journal of Nematology 46:1-7.

Tampakaki, A.P., Skandalis, N., Gazi, A.D., Bastaki, M.N., Sarris, P.F., Charova, S.N., Kokkinidis, M., and Panopoulos, N.J. 2010. Playing the "Harp": Evolution of Our Understanding of hrp/hrc Genes. Annual Review of Phytopathology, Vol 48 48:347-370.

Vallad, G.E., and Goodman, R.M. 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Science 44:1920-1934.

Valls, M., Genin, S., and Boucher, C. 2006. Integrated regulation of the type III secretion system and other virulence determinants in Ralstonia solanacearum. Plos Pathogens 2:798-807.

van Loon, L.C., Bakker, P., and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36:453-483.

Wang, X., Li, M., Zhang, J., Zhang, Y., Zhang, G., and Wang, J. 2007. Identification of a key functional region in harpins from Xanthomonas that suppresses protein aggregation and mediates harpin expression in E-coli. Molecular Biology Reports 34:189-198.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., and Beer, S.V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.

Zhang, C., Shi, H., Chen, L., Wang, X., Lue, B., Zhang, S., Liang, Y., Liu, R., Qian, J., Sun, W., You, Z., and Dong, H. 2011a. Harpin-induced expression and transgenic overexpression of the phloem protein gene AtPP2-A1 in Arabidopsis repress phloem feeding of the green peach aphid Myzus persicae. Bmc Plant Biology 11.

Zhang, L., Xiao, S., Li, W., Feng, W., Li, J., Wu, Z., Gao, X., Liu, F., and Shao, M. 2011b. Overexpression of a Harpin-encoding gene hrf1 in rice enhances drought tolerance. Journal of Experimental Botany 62:4229-4238.

Zhou, Y.Y., Wang, Y.Y., Zhu, X.F., Liu, R., Xiang, P., Chen, J.S., Liu, X.Y., Duan, Y.X., and Chen, L.J. 2017. Management of the soybean cyst nematode Heterodera glycines with combinations of different rhizobacterial strains on soybean. Plos One 12.

Figure legends

Figure 2.1 Soybean plant height measurement with different harpin protein treatments, and reniform infection. Seeds of soybean were coated with harpin protein @ 0.25 oz/ 100 lb seeds. Reniform nematodes (RN) were inoculated at two-leaf stage. All measurements were taken 6 weeks after inoculation of reniform nematodes.

Figure 2.2 Cotton plant height measurement with different harpin protein treatments, and RN infection. Cottonseeds were coated with harpin proteins @ 0.25oz/ 100 lb seeds. RN were inoculated at two-leaf stage. All measurements were taken 6 weeks after inoculation of reniform nematodes.

Figure 2.3 Soybean plant height with different treatments and soybean cyst nematode (SCN). Seeds of soybean were coated with harpin protein @ 0.25oz/ 100 lb seeds. Eggs of soybean cyst nematode were inoculated at two-leaf stage. All measurements were taken 6 weeks after inoculation of SCN.

Figure 2.4 Fresh soybean biomass with different harpin protein treatments and RN inoculation. Seeds of soybean were coated with harpin protein @ 0.25oz/ 100 lb seeds. RN were inoculated at two-leaf stage. All measurements were taken 6 weeks after inoculation of RN.

Figure 2.5 Fresh cotton biomass under different harpin protein treatments and RN inoculation. Cottonseeds were coated with harpin protein @ 0.25oz/ 100 lb seeds. RN were inoculated at two-leaf stage. All measurements were taken 6 weeks after inoculation with RN.

Figure 2.6 Fresh soybean biomass under different harpin protein treatments and SCN inoculations. Seeds of soybean were coated with harpin protein @ 0.25oz/ 100 lb seeds. Eggs of soybean cyst nematodes were inoculated into the soil at two-leaf stage. All measurements were taken 6 weeks after inoculation of SCN.

Figure 2.7 Effect of harpin protein treatments of soybean seeds on RN population in soil. Soybean seeds were coated with harpin protein @ 0.25oz/ 100 lb seeds. RN were inoculated at two-leaf stage of soybean plants. Populations were quantified 6 weeks after RN inoculation.

Figure 2.8 Effect of harpin protein treatments of cotton seeds on RN populations in soil. Cottonseeds were coated with harpin protein @ 0.25oz/ 100 lb seeds. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after inoculation of RN. Welch two sample t-test was performed at $P \leq 0.05$ for significance.

Figure 2.9 Effect of treatment of soybean seeds on SCN population in soil. Soybean seeds were coated with harpin protein @ 0.25oz/ 100 lb seeds. Eggs of cyst nematodes were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after inoculation of SCN.

Figure 2.10 Effect of different seed treatments on soybean plant height. Soybean seeds were treated with harpin protein in two different methods. Seeds were coated with harpin protein @ 0.25oz/ 100 lb seeds (HA) or soaked with harpin protein @ 10 μg/ml (HB) for one hour

immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after inoculation of RN.

Figure 2.11 Effect of different methods treatment on fresh shoot weight of soybean. Soybean seeds were treated with harpin protein in two different ways. Seeds were coated with harpin @ 0.25oz/ 100 lb seeds (HA) or soybean seeds were soaked with harpin protein @ 10 μg/ml (HB) immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after inoculation of RN.

Figure 2.12 Effect of different treatments dry shoot weight of soybean. Soybean seeds were treated with harpin protein in two different ways. Seeds were coated with harpin protein @ 0.25oz/ 100 lb seeds (HA) or soaked with harpin protein @ 10 μg/ml (HB) for one hour immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after RN inoculation.

Figure 2.13 Effect of different treatments on fresh root weight of soybean. Soybean seeds were treated with harpin protein in two different ways. Seeds were coated with harpin protein @ 0.25oz/ 100 lb seeds (HA) or soaked with harpin protein @ 10 μg/ml (HB) for one hour immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after RN inoculation.

Figure 2.14 Effect of different methods of treatment on dry root weight of soybean. Soybean seeds were treated with harpin protein in two different ways. Seeds were coated with harpin protein @ 0.25oz/100 lb seeds (HA) or soaked with harpin protein @ 10 μg/ml (HB) for one hour immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after RN inoculation.

Figure 2.15 Effect of different methods of treatment of soybean seeds on RN population in soil. Soybean seeds were treated with harpin protein in two different ways. Seeds were coated with harpin protein @ 0.25oz/100 lb seeds (HA) or soaked with harpin protein @ 10 μg/ml (HB) for one hour immediately before planting. RN were inoculated at two-leaf stage of soybean plants. All measurements were taken 6 weeks after RN inoculation.

Figure 2.16 Scanning electron micrographs of seed surface treated with 100 ug/ml Messenger (Panel B) and 100 ug/ml Maltrin-100 (Panel A). Seeds soaked in a solution of harpin protein showed aggregates on the seed surface suggesting interaction between the harpin protein and the seed surface. In brief, seeds were soaked in solution for one hour and were air dried overnight. Seeds were coated with a 3-nm thin layer of gold particles and visualized under Scanning Electron Microscope.

Figures

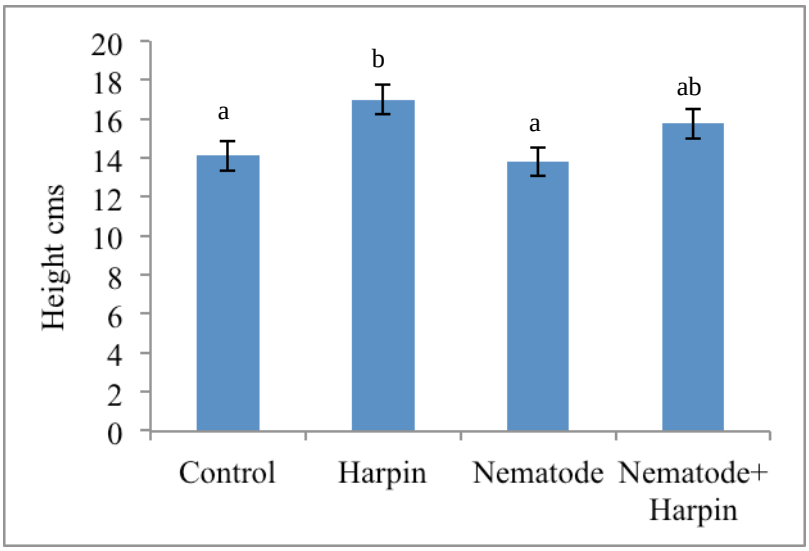


Figure 2.1

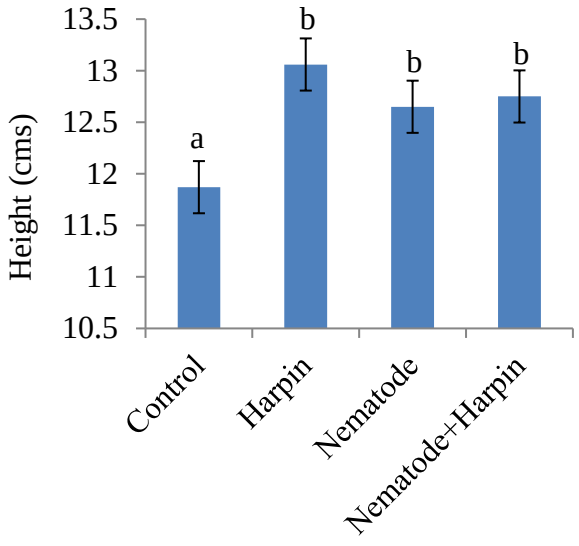


Figure 2.2

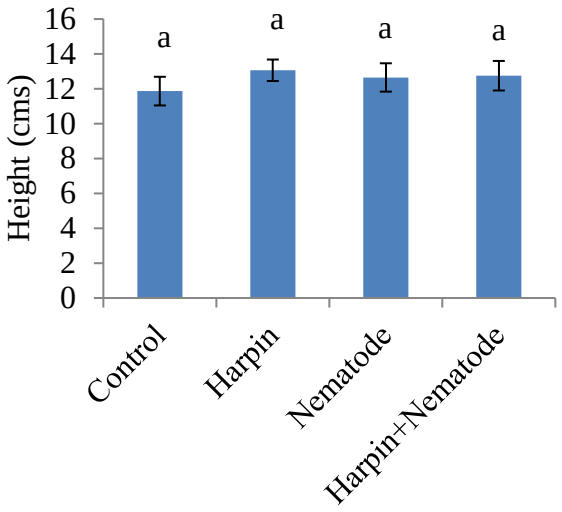


Figure 2.3

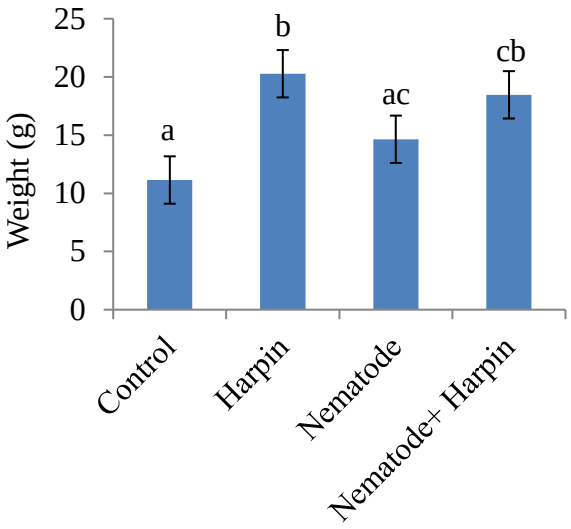


Figure 2.4

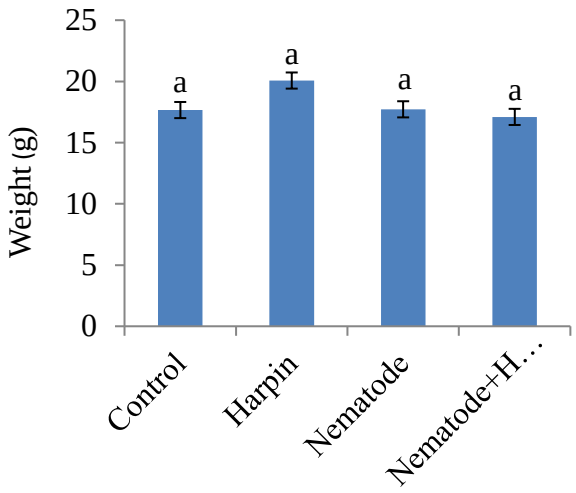


Figure 2.5

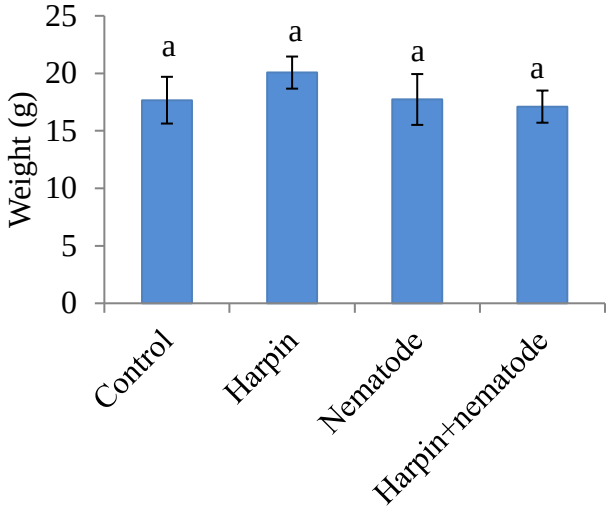


Figure 2.6

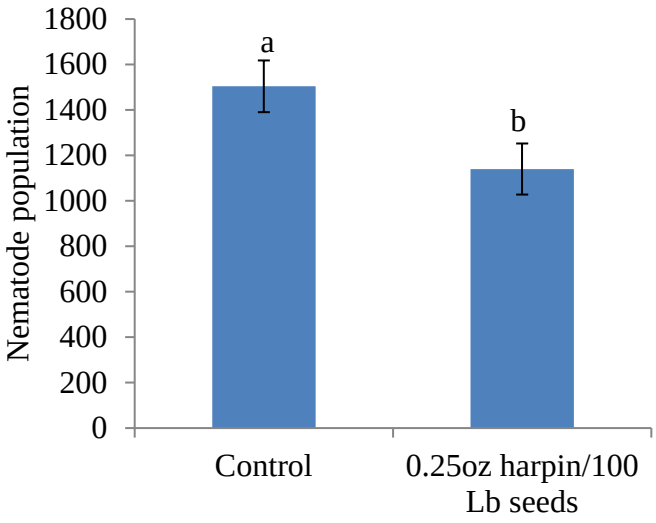


Figure 2.7

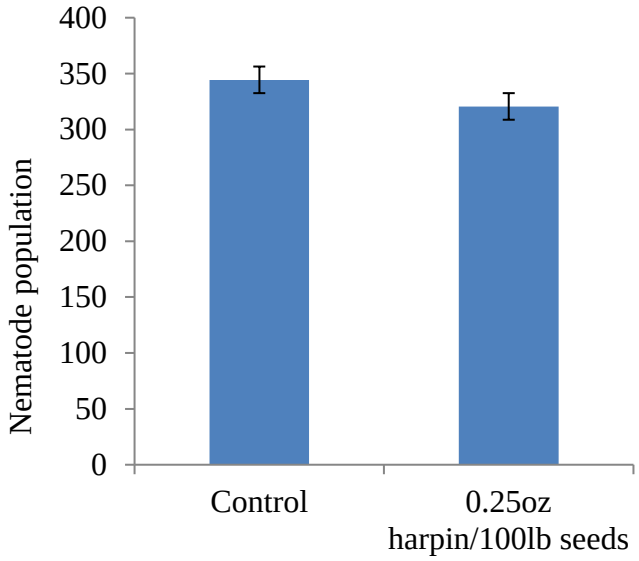


Figure 2.8

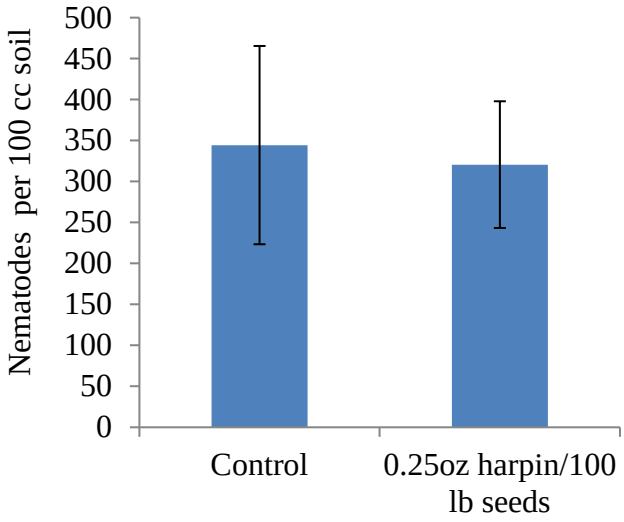


Figure 2.9

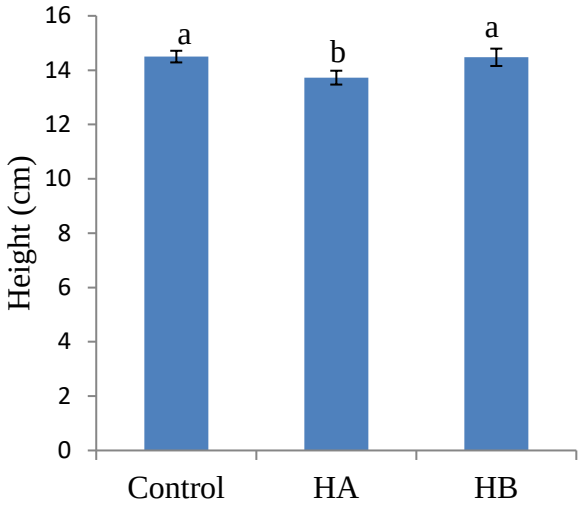


Figure 2.10

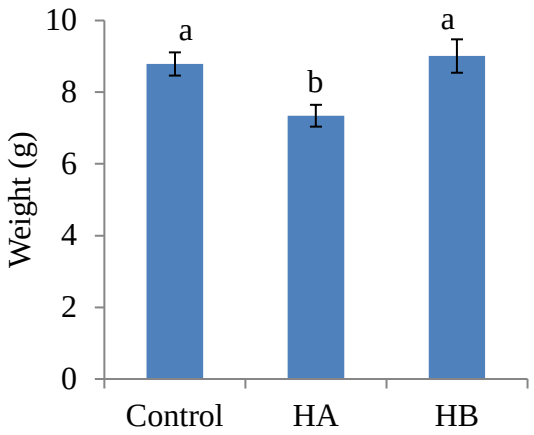


Figure 2.11

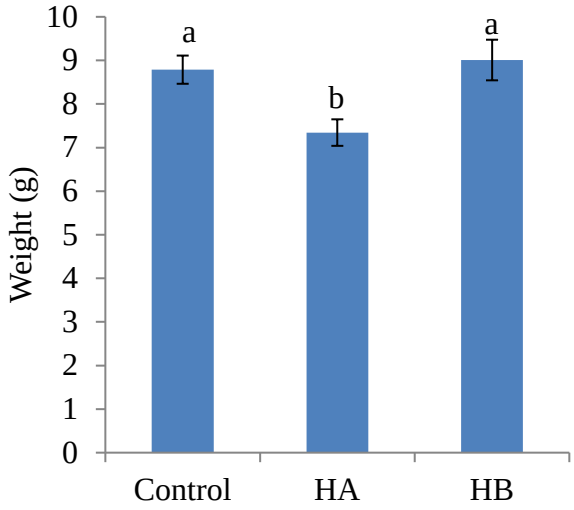


Figure 2.12

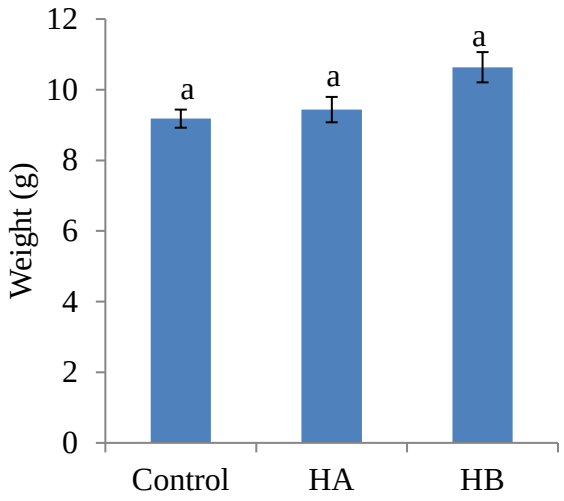


Figure 2.13

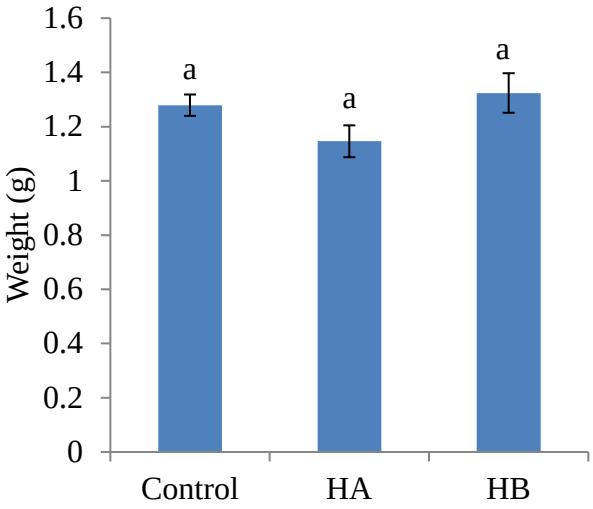


Figure 2.14

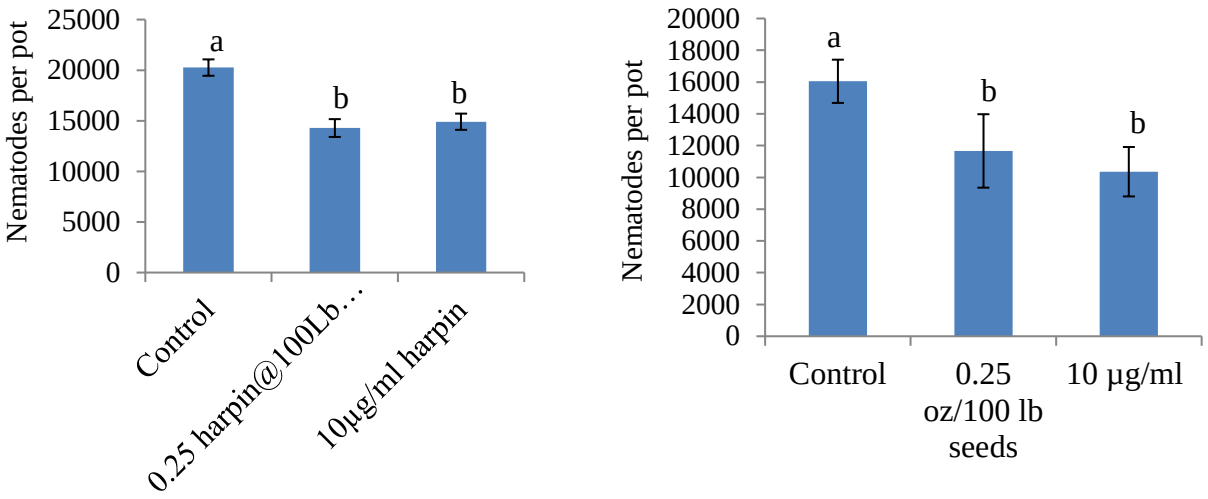


Figure 2.15

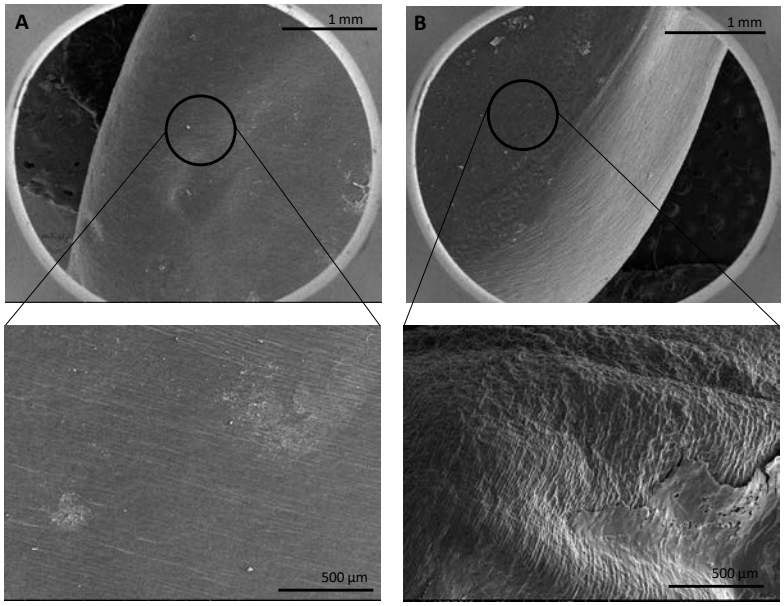


Figure 2.16

CHAPTER III
RNA sequencing of Harpin Protein Treated and Control Soybean Roots Identifies Genes Expressed During Reniform infection

Abstract

Harpin proteins are of bacterial origin. Treatment with harpin proteins improves plant overall fitness and also induces defense responses against a myriad of pathogens including bacteria, fungi, and insects. The defense response in plants is likely due to activation of a general defense response in plants involving hormonal signaling pathways like salicylic acid. Our previous studies under greenhouse conditions shows that soybean seeds treated with harpin show a significant reduction in reniform nematode population in the soil. To investigate the mechanism of this response, we sequenced the transcriptome of soybean roots. Additionally, transgenic soybean plants constitutively expressing salicylate hydroxylase (*nahG*) were created to investigate the role of salicylic acid in the harpin-mediated response. RNA sequencing performed on the Ion Torrent PGM platform obtained more than 5 million reads, of which 67% were mapped to the soybean genome. Mapping of genes revealed expression of several genes in soybean roots including defense-related genes like *PR2* and *NPR1*. Identification of differentially expressed genes among treatments was hindered due to low sequencing depth. Similarly, the *Agrobacterium*-mediated transformation of Williams 82 soybean with the salicylate hydroxylase (*nahG*) gene successfully produced transgenic soybean plants. The expression of the gene was confirmed through semi-quantitative RT-PCR analyses. The growth of the soybean plants in the greenhouse predisposed them to *Fusarium oxysporum*, a common soil inhabiting pathogen. Plants infected with the fungus showed typical wilting symptoms about 10 weeks after germination and failed to mature to the reproductive stage. This experiment describes the first attempt to understand signaling pathway induced by harpin in soybean roots and could provide a valuable resource for the future design of RNA sequencing experiments and generation of transgenic soybean constitutively expressing *nahG*.

1. Introduction

Plants have the ability to respond to a variety of stimuli, including invading pathogens, by activating defense reactions (Killebrew *et al.*, 1993, Zhu *et al.*, 1996). Activation of plant defense works via reprogramming of gene expression leading to a robust response against the pathogen (Buscaill & Rivas, 2014, Tsuda & Somssich, 2015). To combat infection by pathogens, plants have evolved multilayer resistance mechanisms that act in a coordinated fashion to enhance resistance to pathogens. This inherent feature in plants is called innate immunity. The innate immune system in plants is comprised of two layers: pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006). PTI is initiated when special receptors, pattern recognition receptors (PRRs), in the plasma membrane, recognize special pathogen- or microbe-associated molecular patterns (PAMP/MAMP), which activate the MAP Kinase signaling cascade (Macho & Zipfel, 2014). ETI, on the other hand, is activated when effectors from pathogens are recognized by special intercellular proteins containing nucleotide-binding site (NBS) and leucine rich repeats (LRRs), which elicit programmed cell death leading to the hypersensitive response (HR) (Zebell & Dong, 2015).

PTI is a general defense response to a wide range of pathogens, while ETI is a response against a specific pathogen (Li *et al.*, 2016). A main feature of the induction of plant defense responses is that localized responses can be relayed to distal tissues through a hormone signaling pathway leading to the development of systemic resistance. This systemic resistance can protect plants against a subsequent pathogen attack (Spoel & Dong, 2012). Plant defense responses can also be activated using elicitors. Elicitors are compounds derived from a pathogen, or synthetic molecules that are known to induce a defense response in many plant species (Chuang *et al.*, 2014). These compounds can be applied exogenously in plants to trigger defense responses.

Harpins are a group of proteins that are secreted by gram-negative plant pathogenic bacteria and perform diverse functions (Wei *et al.,* 1992). HrpN was the first harpin, and it was isolated and characterized from *Erwinia amylovora*, the causal organism of fire blight in apple. HrpN has been commercialized and is used extensively as a biorational pesticide (Dong *et al.*, 1999, Peng *et al.,* 2003, Dong *et al.,* 2004, Dong *et al.,* 2005, Ren *et al.,* 2008).

Harpins determine susceptibility or resistance of the host to the bacterial pathogens. The purified form of the protein is known to perform multiple functions in different plant species, including activating defense responses against bacteria, viruses, insects, nematodes, and some types of abiotic stress (Dong *et al.*, 2004, Dong *et al.,* 2005, Reboutier *et al.,* 2007, Che *et al.*, 2011). The mechanism that orchestrates the defense response against plant-parasitic nematodes is poorly understood. Soybean seeds treated with harpin proteins show a significant reduction in reniform nematode population in the soil. We hypothesize that the reduction in reniform population is due to transcriptional changes in soybean resulting in the induction of various genes conferring resistance to reniform nematodes in the roots.

RNA sequencing is a powerful tool to identify and measure gene expression (Cloonan *et al.*, 2008, Trapnell *et al.*, 2010). Increased availability and affordability of next generation sequencing has made the technology available, and RNA sequencing has surpassed the use of the microarray method of quantifying gene expression (Marioni *et al.*, 2008). RNA sequencing is a high-throughput method for quantifying gene expression. The method is highly reproducible, with less variation, and can provide sufficient depth to identify less abundant transcripts (Fang & Cui, 2011). Additionally, RNA sequencing allows pooling of different samples and replicates, provided the samples contain specially barcoded adapters. RNA sequencing has been regularly

used to identify differentially expressed transcripts in many plant pathogen interactions (Marioni *et al.,* 2008, Li *et al.,* 2011, Xu *et al.,* 2011, Kawahara *et al.,* 2012).

RNA sequencing and differential expression analyses have allowed identification of several genes regulating development patterns (Severin *et al.,* 2010). Additionally, comparative analyses of different soybean varieties revealed genes that were differentially regulated during drought stress (Fan *et al.,* 2013, Prince *et al.,* 2015) and potassium deficiency (Wang *et al.,* 2012). They also helped identify genes regulating nodulation (Zhu *et al.,* 2013).

RNA sequencing studies on soybean roots during interaction with *Bradyrhizobium,* and the soybean cyst nematode has shed new light on the role of micro RNAs during root colonization (Subramanian *et al.,* 2008, Li *et al.,* 2011, Li *et al.,* 2012). Knowledge of soybean defense responses against reniform nematode infection is limited, however. Most studies of this nature have been with limited to the soybean-cyst nematode pathosystem. The reniform nematode is a common pathogen of cotton, which has encouraged research to understand the cotton-reniform nematode interaction (Li *et al.,* 2015). Previous work has demonstrated the role of hormones including gibberellin and auxin during soybean-root knot interaction (Beneventi *et al.,* 2013). Profiling soybean roots during infection with soybean cyst nematodes show a possible role of cell wall modifying enzymes mediated by jasmonic acid (Ithal *et al.,* 2007), including the role of reactive oxygen species that are generated in response to nematode infection.

Harpin treatment is known to induce defense response through various pathways (Dong *et al.,* 1999, Dong *et al.,* 2005, Dong *et al.,* 2004), as well as having a positive influence on plant growth and development when it is used as a seed treatment (Dong *et al.,* 2004). However, harpin-mediated responses during reniform nematode infection has not been elucidated. To

dissect signaling pathways due to harpin protein treatment, and to identify changes in gene expression during reniform infection, we sequenced the transcriptome of roots from harpin protein-treated and control soybean seeds, with and without reniform infection. Additionally, to investigate the role of salicylic acid-mediated response during harpin protein treatment, we created transgenic soybean lines expressing the salicylate hydroxylase (*nahG*) gene.

2. Materials and methods

2.1. Seed treatments and growth of soybean plants

Soybean plants of the cultivar 'Williams 82' were grown in a greenhouse 24-26 °C and a diurnal cycle of 16 hours of light and eight hours of darkness. Four different seed treatments were used - 1) control seeds without harpin protein treatment, no reniform nematodes (C), 2) soybean seeds treated with harpin protein but without reniform nematodes (H), 3) soybean seeds without harpin protein but inoculated with reniform nematodes (N), and 4) soybean with seeds treated with harpin protein and inoculated with reniform inoculation (HN). For harpin protein treatments, seeds were soaked in 10 μg/ml harpin protein solution for one hour before planting. Seeds were then immediately planted in 12 oz Styrofoam cups filled with 1:1 field soil and sand mixture.

2.2. Extraction and inoculation of reniform nematodes

Reniform nematodes were propagated on cotton cultivar Phytogen. Nematodes were extracted from the soil using decanting-sieving followed by sugar flotation (Jenkins, 1980) Inoculations with the reniform nematodes were performed when the cotyledonary leaves were fully expanded. About 3,500 reniform nematodes were inoculated on each plant using a 1-ml pipette. Two holes about 1 cm deep were made around the base of the plants and the nematodes consisting of a mixed population of juvenile and pre-infective adults were inoculated around the

base of the plants. The holes were immediately covered to prevent splashing of the nematode during watering of soybean plants.

2.3. Collection of samples

Root samples were collected three days after inoculation with the reniform nematodes. Three biological replicates for each treatment were collected for RNA extraction. Roots of the soybean plants from different treatments were gently removed from pots with minimal displacement and disturbance. The roots were washed in a bucket of water to remove all the soil particles. Care was taken to minimize the damage to the roots. After completely removing soil particles from the roots, they were blotted dry with a paper towel. The roots were then flash frozen in liquid nitrogen and stored at -80 °C until further use.

2.4. Extraction of total RNA

Total RNA was extracted from soybean root samples with the Direct-zol RNA Miniprep Kit (Zymo research, Irvine, CA, USA) following manufacturer's instructions. The RNA was quantified with a Nano spectrophotometer (Thermo Fisher Scientific) (Table 4.1), and the quality and integrity of the total RNA extracted were determined on a 1% agarose gel electrophoresis (Figure 4.1).

2.5. Removal of rRNA

Ribosomal RNA was removed with a MagJET mRNA Enrichment Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Twelve μg total RNA from each sample was used for enrichment of mRNA. The rRNA-free mRNA was eluted in 17 μl of nuclease-free water.

2.6. Fragmentation of mRNA

Messenger RNA enriched from the samples was fragmented with the NEBNext RNase III RNA Fragmentation Module (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. The set-up of the reaction mixture was as follows: 17 µl of purified mRNA was mixed with 0.75 µl of RNase III (1u/ µl), RNase III 10× reaction buffer and 0.25 µl nuclease free water. The reaction mixture was incubated at 37 °C for 5 minutes. Following incubation, 80 µl cold water was immediately added to the reaction mixture which as then transferred to ice. The fragmented RNA was cleaned using the Zymo RNA Clean & Concentrator kit (Zymo Research). The fragmented mRNA was eluted in 15 µl nuclease free water.

2.7. First and second strand cDNA synthesis

First strand cDNA synthesis was performed on the fragmented mRNA using random hexamers, and M-MLV reverse transcriptase (Promega Corp., Madison, WI, USA) using 500 nanograms of random hexamers. Second strand synthesis was performed with the NEBNext Second Strand Synthesis Module kit (New England Biolabs) following the manufacturer's protocol. The reaction mixture was cleaned up with the GeneJET PCR Purification Kit (Thermo Fisher Scientific) to remove the enzymes. The second strand cDNA was eluted in 17 µl nuclease free water.

2.8. End repair and ligation of sequencing adapters

Following preparation of double strand cDNA from the mRNA, the end repair was performed with the NEB End Repair Module kit (New England Biolabs) following manufacturer's instructions. End repair of the fragments was followed by ligation of the sequencing adapters. Each sample was ligated with a uniquely barcoded adapter to differentiate

the samples during sequencing. The ligation reaction was set up with 20 µl end repaired cDNA, 4 µl of T4 DNA ligase buffer, 2 µl each of adapters A1 and P, 4 µl of T4 DNA ligase, 1 µl of Bst DNA polymerase and 7 µl sterile water to a total volume of 40 µl. The reaction mixture was incubated in a thermal cycler for 15 minutes and 25 °C followed by 5 minutes at 65 °C.

2.9. Size selection and PCR amplification of the libraries

Sterile water was added to the above reaction mixture to bring the volume to 100 µl. Size selection was performed with the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Size selection was performed to obtain 480 bp libraries, including the adapters. The DNA was eluted using 46 µl sterile water. A final PCR reaction to amplify the library was performed according to the PCR step mentioned in NEBNext Fast DNA Fragmentation and Library Preparation for Ion Torrent kit (New England Biolabs). The PCR products were cleaned with the 100 µl Agencourt AMPure XP beads (Beckman Coulter). The libraries were eluted in 25 µl 0.1× TE buffer and quantified with an Agilent 2200 Tapestation D1K (Agilent Technologies, Santa Clara, USA) at the Department of Biological Sciences, University of Arkansas. Size distribution and the molar concentrations of the individual cDNA libraries are given in figure 4.2 and table 4.2.

2.10. Pooling and sequencing libraries

The libraries were pooled in equimolar concentration. Six libraries were pooled for sequencing in one chip. Template preparation was performed on the Ion OT2 with the Ion OT2 Template Preparation Kit (Thermo Fisher Scientific). Following template preparation, the enrichment of the DNA-bound Ion Sphere Particles was performed with the Ion PGM Enrichment Kit (Thermo Fisher Scientific). Sequencing was performed on the Ion Personal Genome Machine sequencer with a 318 Chip Kit V2. The sequencing was performed on two 318

chips with 6 samples on each chip, and the samples were randomly assigned on each chip to minimize variability arising out of sequencing.

2.11. Mapping reads to the soybean genome

Reads obtained from the sequencing were checked for quality with FASTQ with Q20 was set as the threshold. Reads that did not meet the criteria were discarded. A reference index for the reference genome assembly was built with GMAP v2014-08-04 (Wu and Watanabe, 2005) with default settings. The reads were mapped with GSNAP v2014-10-09 (Wu and Nacu, 2010) with the options to enable spliced alignments and to exclude failed mapped reads. SAMtools v0.1.19 (Li et al., 2009) was used to sort, exclude secondary alignments, and to create a binary version of the alignment file which served as input to BEDtools v2.26.0 (Quinlan and Hall, 2010) to calculate the number of mapped reads to each gene. The reads that mapped consisted of raw counts, unlike FPKM values that work well with other sequencing platforms.

2.12. Differential expression analyses

Analyses of differential expression were performed with the R-studio computing environment. For differential expression analyses, the reads from different treatments were separated into 4 groups for statistical analyses. Expression analyses were performed between different groups of treatments - control and harpin protein, nematode and harpin protein + nematode, harpin protein and harpin protein + nematode, and control and nematode. Differential expression between treatments was determined by DESeq package in R studio. Differential expression analyses were performed based on FDR cutoff of 0.05, and the fold change expression was quantified on the log2 scale with the LFC threshold of 1, and at 95% confidence interval.

2.13. Cloning and sequencing of the *nahG* gene

For the generation of constructs for transformation of soybeans, the *nahG* gene was first amplified from the plasmid pCAMBIA1300-NahG (Ying *et al.*, 2010) using the primer pair NahG5' /NahG 3'. PCR conditions for amplification of the gene were: initial denaturation cycle at 94 °C for 5 minutes, 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 65 °C for 1.5 minutes. A final amplification cycle at 65 °C for 10 minutes was performed. The amplification was performed with the Long Amp Crismon *Taq* polymerase (New England Biolabs) to minimize the chance of error during PCR. The fragment was then purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA). The fragment obtained from the PCR was sequenced at the University of Arkansas DNA Sequencing Facility in the Don Tyson Center for Excellence for Poultry Science. The sequence of the fragment was compared with the *nahG* gene in the GenBank to check for accuracy of the PCR. After sequence validation, the 1.5 kb *nahG* fragment was cloned into the vector pXcmI to generate the plasmid pSB005.

2.14. Construction of the plant transformation cassette

CAM35S promoter was amplified from the plasmid pCAMBIA-1300 using the primer pair P35SF/ P35SR (table 4.7). Similarly, the NosA terminator was also amplified from the pCAMBIA-1300 using the primer pair NosAF/NosAR. The promoter and the terminator were then fused together via fusion PCR with primer pair P35SFN /NosARN. The fusion product of the promoter and the terminator was then digested with the enzymes *PstI* and *EcoRI* and ligated into plasmid pTF101.1, also digested with *PstI* and *EcoRI* to generate the plasmid pSB006.12. The ligation of the promoter-terminator construct was confirmed using restriction digestion with *PstI* and *EcoRI* and also via Sanger sequencing at the DNA sequencing facility of the

Department of Microbiology and Immunology, University of Arkansas for Medical Science at Little Rock. The *nahG* gene was digested from the plasmid pSB005 with *XbaI* and *SacI* and ligated into *XbaI*/*SacI* site of the plasmid pSB006.12 to generate a new plasmid pSB007.17. Prior to sending the plasmid for transformation at the transformation facility, a diagnostic was performed by digesting 100 ng of plasmid pSB007.17 with the restriction enzymes *EcoRV* and *XcmI*. The digested plasmid was run on a 1% agarose gel to confirm the size of the fragments.

2.15. Soybean transformation

Transformation of soybean Williams 82 was performed at Plant Transformation Facility at the Iowa State University, Iowa, USA. Five independent transformation events were conducted for the generation of *nahG* expression plants. For transformation of soybean, a previously described cotyledonary node method using matured soybean seeds was used (Paz *et al.*, 2006). Following transformation, the soybean plants were tested for expression of bar gene using Basta herbicide. The T0 plants were grown in a greenhouse to maturity to collect seeds.

3. Results

3.1. Extraction of total RNA from soybean roots

The agarose gel image of the total RNA indicating the quantity and quality of total RNA extracted from the roots of soybean plants from different treatments is shown in figure 4.1. The agarose gel image showed the integrity of the total RNA, which is depicted by the intactness of the ribosomal RNA. The bands of ribosomal RNA were intact with minimal degradation. Similarly, the yield of the total RNA ranged from 252 ng/µl to 643.5 ng/µl. The amount of total RNA used in library preparation was 15 µg for each sample.

3.2. rRNA removal and cDNA library preparation

The size distribution and the amount of each library are shown in figure 4.2 and table 4.2 respectively. The libraries showed normal distribution of fragments with the mean peak close to 400 bp. Some libraries contained adapter dimers, which were evident by sharp bands below the smear. The concentration of the libraries ranged from a minimum of 12.9 ng/ul to 61.4 ng/ul with peak molarity from 59.3 to 247 mol/l. The peak molarity of the samples is important during pooling of the samples where the samples are pooled in equal molar amounts. The formula for calculation of the amount of sample needed during pooling is $V_i = (C_f \times V_f)/(\text{number of samples} \times C_i)$, where V_i is the volume of indexing of the library, C_f is the final concentration of the pooled libraries, V_f is the final volume, and C_i is the initial concentration of the library.

3.3. Sequencing data

The RNA libraries were run on the Ion Torrent Personal Genome Machine sequencer on 318 chip kit V2. Total sequencing reads obtained from sequencing runs for each sample are shown in table 4.3. A total of 5.827 million reads was obtained from sequencing of 12 mRNA samples, with an average of 485,584 reads per sample. Treatment Harpin protein Nematode3 had the lowest number of reads with 358,052, while Harpin proteinNematode1 had the maximum read with 621480 reads. Additionally, the number of bases that passed the quality threshold (>=Q20) were 923,086, 836 bases averaging 76,923,603 bases per sample.

3.4. Mapping of sequencing reads to the soybean genome

The genome of soybean was obtained from the Soybase browser and the version used was Glyma 1.01. The size of the soybean genome is estimated to be 1,115 Megabases (Schmutz *et al.*, 2010). Surprisingly there are 46430 loci that are predicted to encode proteins and additional 20,000 loci that are predicted to encode proteins with low confidence. The reads from

the sequencing were mapped onto the annotated soybean genome with GSNAP (Wu & Nacu, 2010). The mapping and the percentage of the reads obtained from the sequencing run for all the samples are shown in Table 4.4. total of 3,920,664 reads mapped onto the soybean genome across all samples. The number of reads that mapped for each sample ranged from 276,986, which represented 55.8% of the sequenced reads, to about 440,769 reads, or about 77% of the sequenced reads per sample.

3.5. Differential expression analyses

The differential sequencing analyses were performed in the R-studio environment. The results from the differential sequencing analyses between different treatment groups are shown in Table 4.5. All the comparisons were performed at 95% confidence interval. Comparison between control and harpin protein show that one gene is significantly up-regulated in the control while it is down-regulated in roots from soybean treated with harpin protein. However, the difference is insufficient for $\log_2$ fold change analyses. Similarly, comparing expression from nematode and control plants, the results show that 58 genes are significantly up-regulated while eight genes are down-regulated. However, the $\log_2$ fold change significance is also lacking between these treatments. Interestingly, the differential expression analyses between harpin protein-harpin protein nematode and nematode-harpin protein nematode did not yield significant differences in gene expression (Table 4.5).

3.6. Expression of genes involved in defense

The reads from RNA sequencing data that mapped to *PR1*, *PR2* and *NPR1* were analyzed to determine their expression in the dataset. The mapping of the reads to the soybean genome is shown in Table 4.6. *PR1* was represented by transcript 16292368, while *PR2* by transcript 16252661 and *NPR1* by transcript 16298647. Our results show that on an average no reads

mapped to *PR1*, while 231 reads mapped to *PR2* and 162 reads mapped to *NPR1*. Analyses of the *PR2* transcript showed mapping of 64 reads for control (average 21), 95 reads for harpin protein (average 32), 33 reads (average 11) for harpin protein nematode and 39 reads (average 13) for nematode treatment. Similarly, for NPR1 transcript, reads that mapped were 51 for control (average 27), 60 for harpin protein (average of 20), 21 for harpin protein nematode (average 7), and 30 for harpin protein nematode (average 10). The DE seq analyses using R platform, however, did not show any significant difference in the expression levels for either of these genes.

3.7. Transformation of soybean Williams-82

The results for transformation events of soybean Williams-82 is shown in Table 4.6. Although five transformation events were expected, only two events were obtained from Iowa State University Plant Transformation Facility. From one event 229 seeds were obtained while from the second event only 3 seeds were obtained. A majority of the seeds appeared shriveled and were smaller in size compared to normal Williams-82 seeds. All three seeds in the second event were shriveled in appearance.

4. Discussion

RNA sequencing is one of the most powerful techniques to identify and quantify gene expression. It is a high-throughput analytical method, and simultaneously allows quantification and identification of transcripts across different treatments (Cloonan *et al.*, 2008, Mortazavi *et al.*, 2008, Trapnell *et al.*, 2010, Li *et al.*, 2014). Previously the most popular method to quantify gene expression in different samples was microarray analyses. However, the microarray method is limited to quantifying expression of known genes and does not permit identification of novel transcripts in different samples. RNA sequencing of soybean roots during cyst nematode

infection has identified several genes and micro RNAs that could be potential targets for nematode control (Li *et al.*, 2011, Li *et al.*, 2012). Similarly, transcriptome analyses from roots of different cotton genotypes showing different responses to reniform nematodes shed light on genes that were important for resistance to the reniform nematode (Li *et al.*, 2015). Genome-wide expression profiling of soybean has helped identify regulation of numerous genes, particularly pathogenesis-related proteins, auxin transport proteins, ethylene responsive factors, and expansin family proteins at different stages of infection (Ithal *et al.*, 2007).

Transcriptome analyses of soybean roots during infection with *Meloidogyne javanica* identified several genes related to stress response (de Sa *et al.*, 2012). Additionally, time course analyses of soybean roots have been performed to compare compatible and incompatible reactions during cyst nematode infection (Klink *et al.*, 2007). Due to popularity of RNA sequencing to identify differentially expressed genes in other host-nematode interactions, the approach was utilized to dissect soybean-reniform interaction during harpin protein treatment. However, the analyses to identify differentially expressed genes in either sample were inconclusive. The genome of soybean is estimated to be about 1115 Mb containing around 46,430 putative transcripts or genes (Schmutz *et al.*, 2010). Although, not all the genes are expected to express in the root environment, the depth of sequencing limited our differential expression analyses using the DEseq method. Differential expression analyses was also performed using alternate program Cuffdiff (Trapnell *et al.*, 2013), but the analyses to identify differentially expressed genes was inconclusive.

Mapping pattern of reads to specific genes like pathogenesis related protein 1 (*PR1*) *PR2*, and *NPR1* were analyzed. These genes are a hallmark of the salicylic acid induction pathway, and their expression is also correlated with resistance to nematode infection (Molinari *et al.*,

2014). The analyses showed that no reads mapped to the putative *PR1* gene. Surprisingly a total of only about 0.005% of the total transcripts mapped to the locus. Additionally, the locus for *NPR1* in the soybean genome was identified and the total reads the mapped to the transcript was calculated. The total reads that mapped to this locus represented only 0.004% of the total reads mapped. The fraction of the transcripts mapped to the locus of interest seems really low to perform any statistical analyses. However, not all transcripts will be expected to express in soybean roots at a given condition. Analyses of the RNA sequencing data suggest that we did not attain sufficient coverage of soybean genes to derive a conclusion on differential expression analyses. Sequencing in an alternative platform such as Illumina Hi-seq could provide improved depth of sequencing and results for statistical analyses. Although the RNA sequencing data from the roots cannot be utilized to perform differential expression analyses, the data could provide a resource to design RNA sequencing experiments for soybean nematode interaction, and identify the genes that are expressed in soybean for future experiments.

Transgenic soybean plants expressing *nahG* (salicylic hydroxylase) is not available in public resources. Previous researches have expressed the gene exclusively in soybean roots through hairy root transformation using *Agrobacterium rhizogenes* (Mitchum, 2016, Youssef *et al.*, 2013). Search for soybean plants expressing the gene are not available in seed banks either. The transgenic soybean created in this project will provide a valuable resource to study immunity in soybean against various soybean pathogens and uncover the defense pathways involved in resistance.

References

Beneventi, M.A., da Silva, O.B., de Sa, M.E.L., Firmino, A.A.P., de Amorim, R.M.S., Albuquerque, E.V.S., da Silva, M.C.M., da Silva, J.P., Campos, M.D., Lopes, M.J.C., Togawa, R.C., Pappas, G.J., and Grossi-de-Sa, M.F. (2013) Transcription profile of soybean-root-knot nematode interaction reveals a key role of phythormones in the resistance reaction. *Bmc Genomics* **14**.

Buscaill, P., and Rivas, S. (2014) Transcriptional control of plant defence responses. *Current Opinion in Plant Biology* **20**: 35-46.

Che, Y.-Z., Li, Y.-R., Zou, H.-S., Zou, L.-F., Zhang, B., and Chen, G.-Y. (2011) A novel antimicrobial protein for plant protection consisting of a Xanthomonas oryzae harpin and active domains of cecropin A and melittin. *Microbial Biotechnology* **4**: 777-793.

Chuang, H.W., Chang, P.Y., and Syu, Y.Y. (2014) Harpin Protein, an Elicitor of Disease Resistance, Acts as a Growth Promoter in Phalaenopsis Orchids. *Journal of Plant Growth Regulation* **33**: 788-797.

Cloonan, N., Forrest, A.R.R., Kolle, G., Gardiner, B.B.A., Faulkner, G.J., Brown, M.K., Taylor, D.F., Steptoe, A.L., Wani, S., Bethel, G., Robertson, A.J., Perkins, A.C., Bruce, S.J., Lee, C.C., Ranade, S.S., Peckham, H.E., Manning, J.M., McKernan, K.J., and Grimmond, S.M. (2008) Stem cell transcriptome profiling via massive-scale mRNA sequencing. *Nature Methods* **5**: 613-619.

de Sa, M.E.L., Lopes, M.J.C., Campos, M.D., Paiva, L.V., dos Santos, R.M.A., Beneventi, M.A., Firmino, A.A.P., and de Sa, M.F.G. (2012) Transcriptome analysis of resistant soybean roots infected by Meloidogyne javanica. *Genetics and Molecular Biology* **35**: 272-282.

Dong, H.P., Peng, J.L., Bao, Z.L., Meng, X.D., Bonasera, J.M., Chen, G.Y., Beer, S.V., and Dong, H.S. (2004) Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiology* **136**: 3628-3638.

Dong, H.P., Yu, H.Q., Bao, Z.L., Guo, X.J., Peng, J.L., Yao, Z., Chen, G.Y., Qu, S.P., and Dong, H.S. (2005) The ABI2-dependent abscisic acid signalling controls HrpN-induced drought tolerance in Arabidopsis. *Planta* **221**: 313-327.

Dong, H.S., Delaney, T.P., Bauer, D.W., and Beer, S.V. (1999) Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. *Plant Journal* **20**: 207-215.

Fan, X.D., Wang, J.Q., Yang, N., Dong, Y.Y., Liu, L., Wang, F.W., Wang, N., Chen, H., Liu, W.C., Sun, Y.P., Wu, J.Y., and Li, H.Y. (2013) Gene expression profiling of soybean leaves and roots under salt, saline-alkali and drought stress by high-throughput Illumina sequencing. *Gene* **512**: 392-402.

Fang, Z.D., and Cui, X.Q. (2011) Design and validation issues in RNA-seq experiments. *Briefings in Bioinformatics* **12**: 280-287.

Ithal, N., Recknor, J., Nettleton, D., Hearne, L., Maier, T., Baum, T.J., and Mitchum, M.G. (2007) Parallel genome-wide expression profiling of host and pathogen during soybean cyst nematode infection of soybean. *Molecular Plant-Microbe Interactions* **20**: 293-305.

Jenkins, W.R. (1980) Citation Classic- A rapid centrifugal-flotation technique for separating nematodes from soil. *Current Contents/Agriculture Biology & Environmental Sciences*: 14-14.

Jones, J.D.G., and Dangl, J.L. (2006) The plant immune system. *Nature* **444**: 323-329.

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., and Minami, E. (2012) Simultaneous RNA-Seq Analysis of a Mixed Transcriptome of Rice and Blast Fungus Interaction. *Plos One* **7**.

Killebrew, J.F., Roy, K.W., and Abney, T.S. (1993) Fusaria and other fungi on soybean seedlings and roots of older plants and interrelationships among fungi, symptoms, and soil characteristics. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **15**: 139-146.

Klink, V.P., Overall, C.C., Alkharouf, N.W., MacDonald, M.H., and Matthews, B.F. (2007) A time-course comparative microarray analysis of an incompatible and compatible response by Glycine max (soybean) to Heterodera glycines (soybean cyst nematode) infection. *Planta* **226**: 1423-1447.

Li, B., Meng, X.Z., Shan, L.B., and He, P. (2016) Transcriptional Regulation of Pattern-Triggered Immunity in Plants. *Cell Host & Microbe* **19**: 641-650.

Li, R.J., Rashotte, A.M., Singh, N.K., Lawrence, K.S., Weaver, D.B., and Locy, R.D. (2015) Transcriptome Analysis of Cotton (Gossypium hirsutum L.) Genotypes That Are Susceptible, Resistant, and Hypersensitive to Reniform Nematode (Rotylenchulus reniformis). *Plos One* **10**.

Li, S., Tighe, S.W., Nicolet, C.M., Grove, D., Levy, S., Farmerie, W., Viale, A., Wright, C., Schweitzer, P.A., Gao, Y., Kim, D., Boland, J., Hicks, B., Kim, R., Chhangawala, S., Jafari, N., Raghavachari, N., Gandara, J., Garcia-Reyero, N., Hendrickson, C., Roberson, D., Rosenfeldr, J., Smith, T., Underwood, J.G., Wang, M., Zumbo, P., Baldwin, D.A., Grills, G.S., and Mason, C.E. (2014) Multi-platform assessment of transcriptome profiling using RNA-seq in the ABRF next-generation sequencing study. *Nature Biotechnology* **32**: 915-925.

Li, X., Wang, X., Zhang, S., Liu, D., Duan, Y., and Dong, W. (2011) Comparative profiling of the transcriptional response to soybean cyst nematode infection of soybean roots by deep sequencing. *Chinese Science Bulletin* **56**: 1904-1911.

Li, X.Y., Wang, X., Zhang, S.P., Liu, D.W., Duan, Y.X., and Dong, W. (2012) Identification of Soybean MicroRNAs Involved in Soybean Cyst Nematode Infection by Deep Sequencing. *Plos One* **7**.

Macho, A.P., and Zipfel, C. (2014) Plant PRRs and the Activation of Innate Immune Signaling. *Molecular Cell* **54**: 263-272.

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M., and Gilad, Y. (2008) RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. *Genome Research* **18**: 1509-1517.

Mitchum, M.G. (2016) Soybean Resistance to the Soybean Cyst Nematode Heterodera glycines: An Update. *Phytopathology* **106**: 1444-1450.

Molinari, S., Fanelli, E., and Leonetti, P. (2014) Expression of tomato salicylic acid (SA)-responsive pathogenesis-related genes in Mi-1-mediated and SA-induced resistance to root-knot nematodes. *Molecular Plant Pathology* **15**: 255-264.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods* **5**: 621-628.

Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M., and Wang, K. (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient Agrobacterium-mediated soybean transformation. *Plant Cell Reports* **25**: 206-213.

Peng, J.L., Dong, H.S., Dong, H.P., Delaney, T.P., Bonasera, J.M., and Beer, S.V. (2003) Harpin-elicited hypersensitive cell death and pathogen resistance require the NDR1 and EDS1 genes. *Physiological and Molecular Plant Pathology* **62**: 317-326.

Prince, S.J., Joshi, T., Mutava, R.N., Syed, N., Vitor, M.D.J., Patil, G., Song, L., Wang, J.J., Lin, L., Chen, W., Shannon, J.G., Valliyodan, B., Xu, D., and Nguyen, H.T. (2015) Comparative analysis of the drought-responsive transcriptome in soybean lines contrasting for canopy wilting. *Plant Science* **240**: 65-78.

Reboutier, D., Frankart, C., Briand, J., Biligui, B., Laroche, S., Rona, J.-P., Barny, M.-A., and Bouteau, F. (2007) The HrpN(ea) harpin from Erwinia amylovora triggers differential responses on the nonhost Arabidopsis thaliana cells and on the host apple cells. *Molecular Plant-Microbe Interactions* **20**: 94-100.

Ren, X.Y., Liu, F., Bao, Z.L., Zhang, C.L., Wu, X.J., Chen, L., Liu, R.X., and Dong, H.S. (2008) Root Growth of Arabidopsis thaliana Is Regulated by Ethylene and Abscisic Acid Signaling Interaction in Response to HrpN(Ea), a Bacterial Protein of Harpin Group. *Plant Molecular Biology Reporter* **26**: 225-240.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J.X., Mitros, T., Nelson, W., Hyten, D.L., Song, Q.J., Thelen, J.J., Cheng, J.L., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S.Q., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J.C., Tian, Z.X., Zhu, L.C., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., and Jackson, S.A. (2010) Genome sequence of the palaeopolyploid soybean. *Nature* **463**: 178-183.

Severin, A.J., Woody, J.L., Bolon, Y.T., Joseph, B., Diers, B.W., Farmer, A.D., Muehlbauer, G.J., Nelson, R.T., Grant, D., Specht, J.E., Graham, M.A., Cannon, S.B., May, G.D., Vance, C.P., and Shoemaker, R.C. (2010) RNA-Seq Atlas of Glycine max: A guide to the soybean transcriptome. *Bmc Plant Biology* **10**.

Spoel, S.H., and Dong, X.N. (2012) How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology* **12**: 89-100.

Subramanian, S., Fu, Y., Sunkar, R., Barbazuk, W.B., Zhu, J.K., and Yu, O. (2008) Novel and nodulation-regulated microRNAs in soybean roots. *Bmc Genomics* **9**.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology* **31**: 46-+.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28**: 511-U174.

Tsuda, K., and Somssich, I.E. (2015) Transcriptional networks in plant immunity. *New Phytologist* **206**: 932-947.

Wang, C., Chen, H.F., Hao, Q.N., Sha, A.H., Shan, Z.H., Chen, L.M., Zhou, R., Zhi, H.J., and Zhou, X.A. (2012) Transcript Profile of the Response of Two Soybean Genotypes to Potassium Deficiency. *Plos One* **7**.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., and Beer, S.V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**: 85-88.

Wu, T.D., and Nacu, S. (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics* **26**: 873-881.

Xu, L., Zhu, L.F., Tu, L.L., Liu, L.L., Yuan, D.J., Jin, L., Long, L., and Zhang, X.L. (2011) Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *Journal of Experimental Botany* **62**: 5607-5621.

Ying, X.-B., Dong, L., Zhu, H., Duan, C.-G., Du, Q.-S., Lv, D.-Q., Fang, Y.-Y., Antonio Garcia, J., Fang, R.-X., and Guo, H.-S. (2010) RNA-Dependent RNA Polymerase 1 from Nicotiana tabacum Suppresses RNA Silencing and Enhances Viral Infection in Nicotiana benthamiana. *Plant Cell* **22**: 1358-1372.

Youssef, R.M., MacDonald, M.H., Brewer, E.P., Bauchan, G.R., Kim, K.H., and Matthews, B.F. (2013) Ectopic expression of AtPAD4 broadens resistance of soybean to soybean cyst and root-knot nematodes. *Bmc Plant Biology* **13**.

Zebell, S.G., and Dong, X.N. (2015) Cell-Cycle Regulators and Cell Death in Immunity. *Cell Host & Microbe* **18**: 402-407.

Zhu, M.Z., Dahmen, J.L., Stacey, G., and Cheng, J.L. (2013) Predicting gene regulatory networks of soybean nodulation from RNA-Seq transcriptome data. *Bmc Bioinformatics* **14**.

Zhu, Q., DrogeLaser, W., Dixon, R.A., and Lamb, C. (1996) Transcriptional activation of plant defense genes. *Current Opinion in Genetics & Development* **6**: 624-630.

Figures and tables legends

Figures and Tables

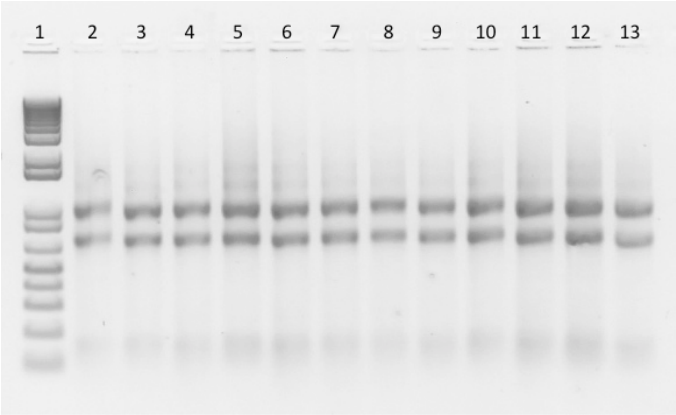


Figure 4.1

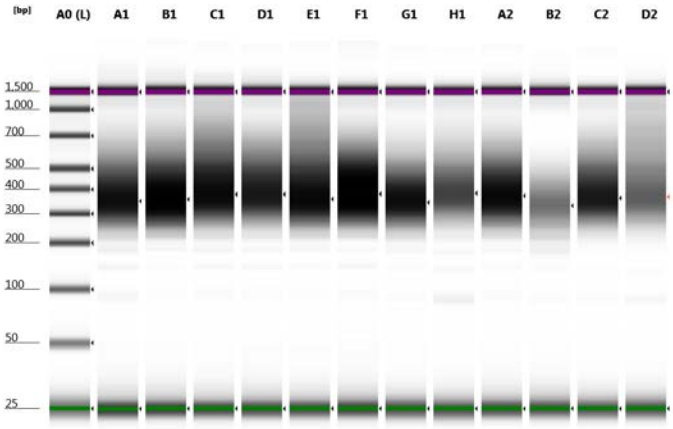


Figure 4.2

Table 4.1

Sample	Quantity ng/µl	260/230
Control 1 (C1)	252.0	1.85
Control 2 (C2)	329.2	1.60
Control 3 (C3)	345.8	1.55
Harpin 1 (H1)	563.9	1.83
Harpin 2 (H2)	516.2	1.76
Harpin 3 (H3)	380.5	1.69
Nematode 1 (N1)	428.7	1.75
Nematode 2 (N2)	377.4	1.64
Nematode 3 (N3)	439.6	1.25
Harpin+ nematode 1 (HN1)	603.6	1.84
Harpin+ nematode 2 (HN2)	643.5	1.88
Harpin+ nematode 3 (HN3)	524.1	1.52

Table 4.2

Sample	Size [bp]	Concentration	Peak Molarity [nmol/l]
C1	354	36.1	157
C2	361	54.2	231
C3	381	48.6	196
H1	362	48.9	208
H2	381	36.2	146
H2	348	36.0	159
H3	382	61.4	247
N1	348	36.0	159
N2	386	23.2	92.6
N3	375	41.7	171
HN1	334	12.9	59.3
HN2	366	36.1	152
HN3	372	22.1	91.3

Table 4.3

Sample	Total reads from Ion Torrent	>= Q20 Bases
Control 1	571,741	103,810,551
Control 2	475,027	81,567,830
Control 3	382,647	63,949,922
Harpin 1	374,214	63,835,831
Harpin 2	589,999	95,835,050
Harpin 3	569,421	96,727,946
Nematode 1	500,182	74,242,886
Nematode 2	496,132	69,948,034
Nematode 3	432,821	65,399,163
Harpin+Nematode 1	621,480	87,519,081
Harpin+Nematode 2	455,284	68,557,065
Harpin+Nematode 3	358,052	51,693,477
Total	5,827,000	

Table 4.4

Sample	Reads mapped	% Mapping
Control 1	440769	77.0
Control 2	344791	72.6
Control 3	274337	71.7
Harpin 1	259892	69.5
Harpin 2	416701	70.6
Harpin 3	412737	72.5
Nematode 1	319120	63.8
Nematode 2	276986	55.8
Nematode 3	282835	65.3
Harpin+ nematode	371291	59.7
Harpin+ nematode	297098	65.3
Harpin+ nematode	224107	62.6
Total	3,92,0664	67.28

Table 4.5

	DDS (adjusted p-value <0.05)				LFC (adjusted p-value <0.05)			
	CH	CN	HHN	NHN	CH	CN	HHN	NHN
LFC > 0 (up)	1	58	0	0	0	1	0	0
LFC < 0 (Down)	1	8	0	0	0	0	0	0
Outliers	1	8	0	0	1	8	0	0
Low counts	0	0	0	0	0	0	0	0

Table 4.6

Event ID	R1 seeds		R1 analysis						
	#Pod	#Seeds	Date of planting	# Seeds planted	# Seeds germinated	# Resistant plants	# Sensitive plants	Seed weight	#Seeds
ST170-45	106	229	9/30/13	4	2	2	0	30.35	229
ST170-57	4	3						0.55	3
							Total	30.90	232

Table 4.7

Primer name	Sequence
NahG5’	TCTAGATGAAAAACAATAAACTTGGC
NahG3’	GAGCTCACCCTTGACGTAGCGCACCC
P35SF	TGCGGGCCTCTTCGCTATTA
P35SR	GGGCCCGAGCTCTACGTAAAGCTTTCTAGACGAGAGAGATAG ATTTGTAGAGAGAGACTG
NosAF	TCTAGAAAGCTTTACGTAGAGCTCGGGCCCCCGATCGTTCAAA CATTTGGCAATA
NosAR	AGCCTGTCGCGTAACTTAGGACTT
P35SFN	CTGCAGGCGTATTGGCTAGAGCAGCTT
NosARN	GAATTCCCCGATCTAGTAACATAGATGACAC
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
nahGF1	TCGATGTCGAGGTATTCGAACAGG
nahGR1	ATTCGGTAGCGTCGATCAGCTTCT
GmUBl3F	GTGTAATGTTGGATGTGTTCCC
GmUBL3R	ACACAATTGAGTTCAACACAAACCG

CHAPTER IV
Genome Sequencing and Analyses of ARF18 Identifies Genes Related to Nematophagy and Provides Clues on its Potential Use as Biological Control Against Plant-Parasitic Nematodes

Abstract

Plant-parasitic nematodes are one of the most destructive pathogens of crop plants worldwide. Discontinued use of harmful chemicals has prompted the search for feasible and effective alternative control strategies. Nematophagous fungi are widely distributed in the environment and have evolved different strategies to parasitize and derive nutrition from nematodes. In this study, a previously characterized, but unnamed nematophagous fungus isolate (designated as ARF18) was taxonomically classified, and its ability to infect cyst nematode was examined. Additionally, growth of ARF18 in different nutritional conditions was analyzed to uncover its ability to produce spores. To identify nematophagy in ARF18 and to establish it as a potential biological control its genome was sequenced using the Pacific Biosciences long read technology and assembled. Based on the initial taxonomic analyses using ITS1-5.8S-ITS2, ARF18 was predicted to form a distinct monophylogenetic clade with the genus *Brachyphoris*, a genus of nematophagous fungi closely related to *Dactylella* and *Vermispora*. The draft genome assembly showed a genome size of 46.3 Mb. There were 14461 predicted proteins in the genome, which enabled us to identify many genes potentially involved in nematode parasitism. Taken together, our study from the infection biology will propel ARF18 as a biological control for controlling plant parasitic nematodes and its genome will provide resource to study nematode parasitism and will also assist in developing ARF18 as a commercial product for application in agricultural settings.

1. Introduction

Plant-parasitic nematodes are destructive pathogens of crop plants and cause estimated losses in excess of $150 million annually (Abad *et al.*, 2008). More than 4100 species of nematode parasitize crop plants (Decraemer & Hunt, 2006) out of which only about 100 species are economically important . Previously, control of plant-parasitic nematodes relied solely on chemical nematicides. However, key chemical nematicides are being discontinued due to human health risks and environmental hazards. Additionally, chemical control only provide short term solution to control of plant pathogens (Jatala, 1986). Thus, alternative measures to control plant-parasitic nematodes are urgently needed (Oka *et al.*, 2000, Tian *et al.*, 2007). Alternative approaches to control plant-parasitic nematodes include cultural control, crop rotation, non-host resistance, deployment of resistance genes, and RNAi. While some methods like RNAi and host resistance are still in the development phase, others like crop rotation and non-host resistance are limited by economic constraints and cannot be adapted to all cropping systems due to the wide host range of some species like reniform nematodes and root-knot nematodes. Additionally, the genetic diversity of some of plant-parasitic nematode species undermines the effectiveness of resistance in crops (Castagnone-Sereno, 2002). All the above-mentioned factors demonstrate an urgent need to devise alternate effective management strategies for these important pests.

Biological control is defined as the use of living organisms either as parasites, predators or pathogens to prevent the growth of another organism especially pests that have a harmful impact on the human health and agriculture (Eilenberg *et al.*, 2001). Biological control has shown tremendous promise to control important agricultural pests in a sustainable way (Hashem & Abo-Elyousr, 2011, Perez-Garcia *et al.*, 2011). Additionally, control of plant pathogens, especially plant-parasitic nematodes through antagonistic microbes is one of the most effective

and ecologically friendly methods, and can be easily adapted to replace harmful chemicals (Li *et al.*, 2015). Numerous microorganisms, including bacteria and fungi inhabit the soil and have developed several specialized strategies to attack and parasitize plant-parasitic nematodes.

Nematophagous fungi are a special class of fungi that trap and parasitize nematodes in soil. More than 700 species of nematophagous fungi have been described till date, and belong to different groups such as *Ascomycota*, *Zygomycota*, *Basidiomycota*, and *Chytridomycota* (Li *et al.*, 2015). Based on the mechanism by which they parasitize nematodes, nematophagous fungi have been classified into four broad groups: nematode-trapping fungi, egg-parasitizing fungi, endoparasitic fungi, and toxin producing fungi (Hyde *et al.*, 2014). Nematode-trapping fungi have developed mechanisms enabling them to capture nematodes using specialized structures. The nematophagous fungi within this group belong to the order *Orbiliaceae* within Ascomycota. These fungi generally exist as saprophytes in the soil, however, in the presence of nematodes, they switch to parasitic mode and trap nematodes. Some of the well knows structures these nematodes form to capture nematodes are constricting rings (*Drechslerella stenobrocha*), non-constricting rings (*Dactylellina copepodii*), sticky knobs (*Monachrosporium haptotylum*), adhesive networks (*Arthrobotrys oligospora*) (Yang *et al.*, 2007b). These structures capture nematodes through the presence of an adhesive layer around the site of trapping device (Su *et al.*, 2017). Currently, more than 100 species of nematode-trapping fungi, and their unique structures have been identified and described (Yang *et al.*, 2007b). Endoparasitic fungi, on the other hand, do not form specialized structures but infect nematodes through their zoospores or conidia. In the presence of nematodes, their conidia germinate and penetrate into the nematode through assimilative hyphae (Lopez-Llorca *et al.*, 2008). Unlike the nematode-trapping fungi, a saprophytic phase is absent or limited in this group, and the fungi fail to produce mycelium in

soil (Moosavi & Zare, 2012). This feature makes the fungi in this group undesirable for use as a biological control. The most studied species in this group is *Drechmeria coniosporia*. *D. coniosporia* is known to produce a large number of adhesive conidia, which adhere and infect nematodes. Infection is followed by growth of hypha and digestion of nematode body, and the emergence of new conidia from conidiophores from the nematode body (Moosavi & Zare, 2012). Egg-parasitizing nematodes infect nematodes through specialized structures like appressoria, special penetration peg, or lateral mycelium branches (Lopez-Llorca *et al.,* 2008). The fungi in this group belong to clavicitipaceous fungi in Ascomycota, and infect nematode through secretion of chitinases and proteases degrading chitin and proteins that are an integral part of the nematode body and nematode eggs (Yang *et al.*, 2007a). Species that belong to this group are *Pochonia chlamydosporia*, *Paecilomyces lilacunus*, *Clonostachys rosea*, and *Lecanicillium psalliotae*. Final group of fungi is the toxin producing nematophagous fungi. The fungi produce toxins to immobilize nematodes before penetrating the cuticle using hyphae (Lopez-Llorca *et al.*, 2008). Fungi in this group belong to Ascomycota and Basidiomycota (Li *et al.*, 2007, Li & Zhang, 2014), and approximately 280 species have been identified. Numerous compounds with nematicidal activities have been identified from this group and belong to a diverse chemical group, including alkaloids, peptides, terpenoids, macrolides, quinones, aliphatic compounds, aromatic compounds and sterols (Li *et al.*, 2007). Presence of such compounds in this group makes them a promising tool as biological control agents. Additionally, two Basidiomycete species, *Coprinus comatus*, and *Stropharia rugosoannulata*, produce special nematode-attacking devices to parasitize nematodes (Luo *et al.*, 2006, Luo *et al.*, 2007).

Additional fungal species, not present in above-mentioned groups, also have the ability to parasitize and kill nematodes. For example, a *Trichoderma* species has been described as a biological control agent against numerous plant-parasitic nematode, although the mechanism of parasitism has not been elucidated (Affokpon *et al.*, 2011). It was demonstrated that the species can kill nematodes through secretion of various extracellular proteolytic enzymes like trypsin-like protease PRA1 (Suarez *et al.*, 2004), serine proteases (Chen *et al.*, 2009), and chitinolytic enzymes (Szabo *et al.*, 2012). Additional nematicidal compounds have been isolated from other *Trichoderma* species, which include trichodermin (Yang *et al.*, 2010), β-vinylcyclopentane-1α, 3α-diol, 6-pentyl-2H-pyran-2-one, and 4-(2-hydroxyethyl) phenol (Yang *et al.*, 2012, Degenkolb & Vilcinskas, 2016). Some additional endophytic fungi and AVM fungi have also been reported to reduce nematode population in soil and enhance plant growth (Veresoglou & Rillig, 2012, Vos *et al.*, 2012).

ARF18, a taxonomically uncharacterized nematode parasitizing fungal isolate, was first isolated from infected cysts of *Heterodera glycines* nearly 30 years ago (Kim & Riggs, 1991) in the Department of Plant Pathology, the University of Arkansas. The fungus parasitizes all stages of the cyst nematode, including eggs, sedentary juveniles, and adults in both soil and culture media (Timper *et al.*, 1999), and is a promising tool as a biological control of plant-parasitic nematodes. Greenhouse experiments have demonstrated that the fungus can suppress the population of *H. glycines* by 86-99% (Kim & Riggs, 1995). The fungus can infect all the sedentary stages of cyst nematodes (Timper & Riggs, 1998). Additionally, controlled experiments in the greenhouse conditions also demonstrated the potential of ARF18 in suppressing the population of the reniform nematode in cotton (Wang *et al.*, 2004). ARF18 has the ability to infect the cyst and reniform nematodes and is characterized by sterile white

mycelium. In corn meal agar medium, the fungus produces sclerotium-like structures, which are masses of highly melanized hyphae clustered together, which have also been associated with penetration site during cyst infection (Kim *et al.*, 1992). Several isolates of ARF (Arkansas Fungus) were collected, and based on the hyphal morphology and sclerotium-like structures produced, they were grouped as either compact (ARF-C), or loose (ARF-L) (Kim *et al.*, 1998). Of the two groups, isolates from ARF-L consistently demonstrated the ability to suppress nematode population in the soil. One of the isolates not only effectively reduced nematode population but also showed excellent growth in the rhizosphere, and was designated as TN14. Although the isolate showed promising results in controlling cyst and reniform nematodes, currently culture conditions that induce conidiation have not yet been identified, and little is known about the phylogeny of the fungal isolate.

Next-generation sequencing has made huge advances in recent years and has allowed the access to information on genomes of numerous organisms. Furthermore, the reduced cost and depth of sequencing have revolutionized sequencing projects (Nowrousian *et al.*, 2010). Although the number of fungal genomes sequenced has increased dramatically, genomes of just a few nematophagous fungi have been sequenced and deposited in the public database (Yang *et al.*, 2011, Meerupati *et al.*, 2013, Liu *et al.*, 2014, Lai *et al.*, 2014, Larriba *et al.*, 2014, Prasad *et al.*, 2015, Lebrigand *et al.*, 2016). Although the potential application of nematophagous fungi in agriculture is essential, the rate of sequencing of the genomes of other economically important filamentous fungi has overtaken those of nematophagous fungi. Not only the biology of infection in these fungi is interesting, their potential use as biological control makes them tractable organisms for further functional genomic studies. Additionally, no genome resources are currently available within the genus *Brachyphoris*. This chapter deals with growth habit of

ARF18 in different nutritional conditions, its phylogeny, infection biology on cyst nematodes, and the genome of the fungal isolate.

2. Materials and methods

2.1. Phylogenetic analyses of ITS region

For phylogenetic analyses, ITS1-5.8s-ITS2 region of the ribosomal DNA was amplified using primer pairs ITS1 and ITS4. The amplified PCR product was sequenced using Sanger sequencing at the DNA sequencing center, Poultry Science building, University of Arkansas. For taxonomic placement, an initial BLAST analysis (blastn) (Altschul *et al.*, 1990) against the GenBank nucleotide was performed. Subsequently, DNA sequences of ITS regions from *Orbiliaceae* were obtained from GenBank. Sequences were initially aligned with ClustalX (Larkin *et al.*, 2007). Alignments were manually curated, and ambiguously aligned regions were removed using Gblocks (Castresana, 2000). The assembled data set was subjected to phylogenetic analyses using neighbor-joining methods implemented in Phylip 3.66 and maximum likelihood methods implemented in RAxML v 7.0.0 (Stamatakis, 2006). Both programs were accessed via the CIPRES web-portal (Miller *et al.*, 2010). Internal branch support was evaluated in both Phylip and RAxML using 1,000 replicates. The ITS1-5.8S-ITS2 rDNA dataset consisted of 41 taxa, including *Neurospora crassa*, *Sordaria fimicola*, and *Apodus oryzae* as outgroups (Figure 3.1).

2.2. Growth in different nutritional conditions

To characterize the growth and identify conditions for spore production in ARF18, analyses of growth were performed in different nutritional conditions. A plug containing actively growing hypha from 0.2× strength potato dextrose agar (PDA) plates were transferred to different nutrient conditions and incubated at room temperature in dark. The nutritional

conditions used in the study were complete medium, minimal medium, yeast glucose agar (YGA) medium, potato dextrose agar (PDA), corn meal agar (CMA) medium, malt extract agar (MEA) at pH 7, and MEA at pH 4. The colony diameter was recorded at 5,7, 14, and 21 days after inoculation (3.2) and hyphal growth was visually recorded. Colony growth was quantified by measuring the diameter from the center inoculation of the plug. The colony diameter was quantified at different directions from the center and an average of the readings were recorded. Formation of sclerotia-like structures in corn meal agar medium were visualized and photographed under a light microscope (Figure 3.3).

2.3. Scanning electron microscopy

To identify the mechanism of cyst infection in ARF18, we performed a scanning electron microscopy on cyst nematodes that were infected with the fungus. For infection of the cysts, a plug of the hyphal mass of actively growing fungus in 0.2× PDA was transferred to a plate of minimal medium overlaid with cellophane, and allowed to grow for few days. Cysts of freshly harvested *Heterodera glycines* were surface sterilized with 10% bleach, carefully placed on the growing hyphae of ARF18, and incubated for 7 days (Figure 3.4). After 7 days, cellophane around the infected cysts was cut with razor-sharp blade and the cut portion was fixed overnight at 4 °C in 2.5% gluteraldehyde in 0.05 M sodium phosphate buffer. After overnight fixation, the samples were washed with 0.05 M sodium phosphate buffer for 30 minutes. The fixed samples were treated with 1% osmium tetroxide (in 0.05 M sodium phosphate buffer) for 2 hours. Following post-fixation treatment with osmium tetroxide, samples were rinsed with sterile water three times for 30 minutes each. A serial dehydration was set up with subsequent washes in 25%, 50%, 75% and 100% ethanol for 30 minutes each. Dehydration was followed by critical point drying using ethanol-hexamethyldisilizane gradient mixtures, 3:1 mixture of ethanol:

hexamethyldisilizane, followed by 2:1, 1:1 and 1:3 and finally 100 percent hexamethyldisilizane. Following 30 minutes incubation in each solution, the samples were air dried at room temperature for 30 minutes. The samples were then coated with thin film of gold particles (2 nanometers) and visualized with a scanning electron microscope at the Nanotech building in the University of Arkansas.

2.4. Identification of nematode parasitism genes

To identify genes involved in nematode parasitism, a BLAST search was performed against the genome of ARF18. Genes that have been previously characterized as involved in nematode parasitism were identified and their nucleotide or amino acid sequences were retrieved from GenBank (NCBI). Using the sequences retrieved from the GenBank as the query, BLASTp or BLASTn was performed on the ARF18 genome.

3. Results

3.1. Taxonomic placement of ARF18

An initial BLAST of the ITS1-5.8s-ITS2 region in NCBI database was performed to identify the taxonomic placement of ARF18. BLAST analyses (Altschul *et al.*, 1990) querying the ITS region of ARF18 against the GenBank nucleotide collection indicated high sequence similarity with species from the Ascomycete family *Orbiliaceae* (i.e. *Dactylella* spp.). Subsequently, the ITS1-5.8S-ITS2 rDNA dataset consisting of 41 taxa, including *Neurospora crassa*, *Sordaria fimicola*, and *Apodus oryzae* as outgroups were obtained from the GenBank database. The resulting phylogenies indicated that ARF18 is an Ascomycete in the family *Orbiliaceae* (Orbiliomycetes, Figure 3.1). Additionally, ARF18 formed a well-supported monophylogenetic clade within the genus *Brachyphoris* (Figure 3.1), a genus of nematophagous fungi closely related to the genera *Dactylella* and *Vermispora* (Chen *et al.*, 2007).

3.2. Growth in different nutritional conditions

ARF18 showed morphologically distinct growth characteristics in different nutritional conditions. However, the fungal isolate was unable to produce spores in different conditions tested. The diameter of the hyphal growth was highest in MEA medium (8 cm after 21 days) while the growth was lowest in PDA medium, with 6.6 cm diameter of hyphal growth (Figure 3.2). The pH of the medium did not significantly affect the colony diameter of the fungal isolate. Additionally, hyphal mass observed in PDA, YGA, and complete medium was substantially high while sparse hyphal growth was observed in minimal medium. ARF18 also produced sclerotia-like structures in CMA medium (Figure 3.3). Sclerotia-like structures have been previously reported to form during the infection of the cyst nematode, and appear as a compact mass of highly melanized hyphae.

3.3. Infection biology

Scanning electron micrographs of ARF18 infecting cysts of *H. glycines* were obtained using scanning electron microscope. ARF18 appears to completely grow over the cysts and infect them through direct penetration. The figures provide evidence of ARF18 hyphae penetrating the cysts at different positions. Interestingly, at the site of penetration, the hyphae appeared swollen to produce an appresorium-like structure (Figure 3.4). A report of the fungal isolate producing such structure during cyst infection has not been reported previously.

3.4. Identification of putative nematode parasitism genes

A BLAST analysis was performed to identify genes encoding putative nematode parasitism genes. Genes or protein sequences were downloaded from the GenBank database. The genes were identified from previously published research and selected based on their antagonizing effects on plant-parasitic nematodes. The genome of ARF18 contained a single

copy of cuticle degrading serine protease (TN14_07867A), which was similar to *A. musiformis* serine protease, with an e value of $2e^{-24}$, and shared 41.6% similarity. Similarly, ARF18 also contained orthologs of alkaline serine protease (TN14_07831A, and TN14_08784A) from *Hirsutella rhossiliensis*, with 47 and 34 percent similarity respectively. Search for cysteine rich secretory protein family, previously described in *Nectria haematococca*, identified 4 proteins: TN14_04654A, TN14_05792A, TN14_12017A, and TN14_01827A with a similarity of 33, 30, 36 and 33 percent respectively.

4. Discussion

Plant-parasitic nematodes cause enormous losses to agricultural yield annually (Siddiqui & Mahmood, 1996, Degenkolb & Vilcinskas, 2016). With the discontinued use of harmful chemical pesticides, an effective and environmentally friendly management strategy is urgently needed (Chitwood, 2003). Current management options of plant-parasitic nematodes are limited to crop resistance and cultivation of non-host crops. These options, however, are constrained by economic feasibility and cultivation practices. Alternative management options such as use of plant resistance genes, induced resistance and RNAi are still in the research phase and may take few years for their deployment in agricultural setting. Biological control, defined as the use of living organisms, and their metabolites to suppress the growth of pests, has shown tremendous promise in controlling plant pathogens (Li *et al.*, 2015). Nematophagous fungi are ubiquitous in soil, usually inhabit top 20 cm of the soil, and play an important role in maintaining the natural population of nematodes (Persmark & NordbringHertz, 1997). Most species of nematophagous fungi exist as saprophytes in the soil. However, in the presence of nematodes, they convert to parasitic mode and parasitize nematodes, which makes them ideal biocontrol agents (Nordbring-Hertz *et al.*, 2006, Yang *et al.*, 2007b). The ability to exist in dual lifestyle gives them a

nutritional advantage over other saprophytes in the soil, and potential as a tool for sustainable agriculture. Furthermore, their lifestyle supports mode of application either through the addition of large amounts of inoculum to the soil, or stimulating fungal species preexisting in the soil environment. Increase in knowledge of their different modes of infection biology and improvement in formulation development and application have increased the interest in using nematophagous fungi as a biological control against plant-parasitic nematodes (Nordbring-Hertz *et al.*, 2006).

The cost of genome sequencing has reduced significantly in recent years and is affordable to smaller groups. Genomes of a handful of nematophagous fungi have been sequenced recently. Analyses of their genomes have provided useful insight into their infection biology and their mode of action on plant-parasitic nematodes. Additionally, sequencing and analyzing their genomes are accelerating research on their ability to be used as biocontrol agents (Ahren *et al.*, 2005, Meerupati *et al.*, 2013, Liu *et al.*, 2014, Larriba *et al.*, 2014, Lebrigand *et al.*, 2016).

ARF18 is an understudied nematophagous fungal isolate, which was first identified in the Department of Plant Pathology at the University of Arkansas (Kim & Riggs, 1991). The fungal isolate has the ability to parasitize and kill cyst and reniform nematodes (Kim & Riggs, 1995, Wang *et al.*, 2004). Additionally, the taxonomic placement of this important fungal species is unresolved. Phylogenetic analyses based on the ITS region suggests, however, that ARF18 groups within *Brachyphoris*, a genus similar to *Vermispora* and *Dactylelllina* (Chen *et al.*, 2007). The genus *Brachyphoris* (synonym *Dactylella*) was recently introduced and constitutes a group of saprophytic fungi with conidiophores short, simple or branched, hyaline. Conidia are borne either single or sometimes double, are spindle-shaped, hyaline smooth walled, and rarely longer than conidiophores (Chen *et al.*, 2007). Multiple species of *Dactylella* have been previously

described (Stirling & Mankau, 1978b, Liu *et al.*, 1996). The species have been mainly studied in China, and isolated from decaying twigs of broad-leaf tree or rotten bamboo (Chen *et al.*, 2007).

The ability of ARF18 to infect multiples species of plant-parasitic nematodes, and exist as a saprophyte, makes it ideal to be used in different cropping systems. However, the fungus was unable to sporulate in a variety of nutritional conditions tested in current research. Similarly, in previous studies, the fungal isolate failed to sporulate even in different carbon and nitrogen sources for nutrition (Liu & Chen, 2003). Additionally, the fungus failed to produce spores in 20 different media conditions previously tested (Kim & Riggs, 1991). A previously described species, *Dactylella oviparasitica* required a "complex" media for sporulation, and only in the presence of light the fungus was able to produce spores (Stirling & Mankau, 1978a). Additionally, enriched Emerson's yeast extract soluble starch (YpSs) was also sufficient to induce spore production in another isolate. However, attempt to induce spore production in ARF18 using YpSs was not successful (Kim & Riggs, 1991). The fungal isolate has been characterized as a sterile hyphomycete due to its inability to produce spores in tested conditions.

A BLAST analysis was performed to identify putative nematode parasitism genes in ARF18 genome. The analyses identified several important genes that have been previously identified and characterized in other nematophagous and entomopathogenic fungi. Initial analyses showed the genome contained putative cuticle degrading serine protease (TN14_07867A). The enzyme has been well characterized in *Arthrobotrys musiformis* and is important for digesting nematode cuticle. Another enzyme alkaline serine protease, similar to *Hirsutella rhossiliensis* serine protease was identified with two different copies (TN14_7831A, and TN14_8784A). In *H. rhossiliensis* the enzyme is secreted in liquid culture and is highly effective against juveniles of cyst nematodes (Wang *et al.*, 2009). The enzyme helps to degrade

cuticle during pathogenicity. Similarly, another group of enzymes for nematode pathogenicity belongs to cysteine-rich secretory protein family. ARF18 genome contained 4 putative genes (TN14_4654A, TN14_5792A, TN14_12017A, and TN14_01827A) belonging to cysteine-rich secretory protein family. Although the enzymes are also present in fungi that parasitize plants (Rep *et al.*, 2004, Dean *et al.*, 2005), evidence shows that it is essential for nematode parasitism in *Drechmeria coniosporia* (Lebrigand et al., 2016).

Compared to the genomes of important plant pathogenic fungi, the availability of genomes of nematophagous fungi is very limited. We sequenced and assembled the genome of a previously unnamed nematophagous fungal isolate ARF18 and is presented in the next section of the chapter. The information from genome assembly and analyses will provide the first step towards its taxonomic placement and ultimately naming the fungus. Additionally, the genome contains several genes related to nematode parasitism, which identifies its potential as a strong biological control agent of plant-parasitic nematodes. Although the functional assignment of the genes is purely speculative at this point, use of molecular genetics on this fungus can provide additional evidence towards gene function. Additionally, the genome also provides resources for ARF18 for further studies on nematophagy and also aid in developing the fungal isolate as a biocontrol agent, and patent developments. The draft genome will advance functional genomics research within the genus *Brachyphoris*, and nematophagous fungi in general, to dissect nematode parasitism. The information gathered could further provide tools to increase the virulence of the fungus on several other species of nematodes and steer the development of ARF18 as an ideal biological control for controlling plant-parasitic nematodes.

References

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.-C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.-N., Schiex, T., Smant, G., Weissenbach, J., and Wincker, P. (2008) Genome sequence of the metazoan plant-parasitic nematode Meloidogyne incognita. *Nature Biotechnology* **26**: 909-915.

Affokpon, A., Coyne, D.L., Htay, C.C., Agbede, R.D., Lawouin, L., and Coosemans, J. (2011) Biocontrol potential of native Trichoderma isolates against root-knot nematodes in West African vegetable production systems. *Soil Biology & Biochemistry* **43**: 600-608.

Ahren, D., Tholander, M., Fekete, C., Rajashekar, B., Friman, E., Johansson, T., and Tunlid, A. (2005) Comparison of gene expression in trap cells and vegetative hyphae of the nematophagous fungus Monacrosporium haptotylum. *Microbiology-Sgm* **151**: 789-803.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403-410.

Castagnone-Sereno, P. (2002) Genetic variability in parthenogenetic root-knot nematodes, Meloidogyne spp., and their ability to overcome plant resistance genes. *Nematology* **4**: 605-608.

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540-552.

Chen, J., Xu, L.-L., Liu, B., and Liu, X.-Z. (2007) Taxonomy of *Dactylella* complex and *Vermispora*. I. Generic concepts based on morphology and ITS sequences data. *Fungal Diversity* **26**: 73-83.

Chen, L.L., Liu, L.J., Shi, M., Song, X.Y., Zheng, C.Y., Chen, X.L., and Zhang, Y.Z. (2009) Characterization and gene cloning of a novel serine protease with nematicidal activity from Trichoderma pseudokoningii SMF2. *Fems Microbiology Letters* **299**: 135-142.

Chitwood, D.J. (2003) Nematicides. *Encyclopedia of agrochemicals*.

Dean, R.A., Talbot, N.J., Ebbole, D.J., Farman, M.L., Mitchell, T.K., Orbach, M.J., Thon, M., Kulkarni, R., Xu, J.R., Pan, H.Q., Read, N.D., Lee, Y.H., Carbone, I., Brown, D., Oh, Y.Y., Donofrio, N., Jeong, J.S., Soanes, D.M., Djonovic, S., Kolomiets, E., Rehmeyer, C., Li, W.X., Harding, M., Kim, S., Lebrun, M.H., Bohnert, H., Coughlan, S., Butler, J., Calvo, S., Ma, L.J., Nicol, R., Purcell, S., Nusbaum, C., Galagan, J.E., and Birren, B.W.

(2005) The genome sequence of the rice blast fungus Magnaporthe grisea. *Nature* **434**: 980-986.

Decraemer, W., and Hunt, D.J. (2006) Structure and classification. *Plant nematology*.: 3-32.

Degenkolb, T., and Vilcinskas, A. (2016) Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes. *Applied Microbiology and Biotechnology* **100**: 3799-3812.

Eilenberg, J., Hajek, A., and Lomer, C. (2001) Suggestions for unifying the terminology in biological control. *Biocontrol* **46**: 387-400.

Hashem, M., and Abo-Elyousr, K.A. (2011) Management of the root-knot nematode Meloidogyne incognita on tomato with combinations of different biocontrol organisms. *Crop Protection* **30**: 285-292.

Hyde, K., Swe, A., and Zhang, K.-Q., (2014) Nematode-Trapping Fungi. In: Nematode-Trapping Fungi. Springer, pp. 1-12.

Jatala, P. (1986) Biological-control of plant parasitic nematodes. *Annual Review of Phytopathology* **24**: 453-489.

Kim, D.G., and Riggs, R.D. (1991) Characteristics and efficacy of a sterile hyphomycete (ARF18), a new biocontrol agent for *Heterodera glycines* and other nematodes. *Journal of Nematology* **23**: 275-282.

Kim, D.G., and Riggs, R.D. (1995) Efficacy of the nematophagous fungus ARF18 in alginate-clay pellet formulations against Heterodera glycines. *Journal of Nematology* **27**: 602-608.

Kim, D.G., Riggs, R.D., and Correll, J.C. (1998) Isolation, characterization, and distribution of a biocontrol fungus from cysts of *Heterodera glycines*. *Phytopathology* **88**: 465-471.

Kim, D.G., Riggs, R.D., and Kim, K.S. (1992) Ultrastructure of *Heterodera glycines* parasitized by Arkansas Fungus-18. *Phytopathology* **82**: 429-433.

Lai, Y.L., Liu, K.K., Zhang, X.Y., Zhang, X.L., Li, K., Wang, N.N., Shu, C., Wu, Y.P., Wang, C.S., Bushley, K.E., Xiang, M.C., and Liu, X.Z. (2014) Comparative Genomics and Transcriptomics Analyses Reveal Divergent Lifestyle Features of Nematode Endoparasitic Fungus Hirsutella minnesotensis. *Genome Biology and Evolution* **6**: 3077-3093.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007) Clustal W and clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Larriba, E., Jaime, M., Carbonell-Caballero, J., Conesa, A., Dopazo, J., Nislow, C., Martin-Nieto, J., and Lopez-Llorca, L. (2014) Sequencing and functional analysis of the genome

of a nematode egg-parasitic fungus, Pochonia chlamydosporia. *Fungal Genetics and Biology* **65**: 69-80.

Lebrigand, K., He, L.D., Thakur, N., Arguel, M.J., Polanowska, J., Henrissat, B., Record, E., Magdelenat, G., Barbe, V., Raffaele, S., Barbry, P., and Ewbank, J.J. (2016) Comparative Genomic Analysis of Drechmeria coniospora Reveals Core and Specific Genetic Requirements for Fungal Endoparasitism of Nematodes. *Plos Genetics* **12**.

Li, G., Zhang, K., Xu, J., Dong, J., and Liu, Y. (2007) Nematicidal substances from fungi. *Recent Patents on Biotechnology* **1**: 212-233.

Li, G.-H., and Zhang, K.-Q., (2014) Nematode-toxic fungi and their nematicidal metabolites. In: Nematode-Trapping Fungi. Springer, pp. 313-375.

Li, J., Zou, C.G., Xu, J.P., Ji, X.L., Niu, X.M., Yang, J.K., Huang, X.W., and Zhang, K.Q. (2015) Molecular Mechanisms of Nematode-Nematophagous Microbe Interactions: Basis for Biological Control of Plant-Parasitic Nematodes. *Annual Review of Phytopathology, Vol 53* **53**: 67-95.

Liu, K.K., Zhang, W.W., Lai, Y.L., Xiang, M.C., Wang, X.N., Zhang, X.Y., and Liu, X.Z. (2014) Drechslerella stenobrocha genome illustrates the mechanism of constricting rings and the origin of nematode predation in fungi. *Bmc Genomics* **15**.

Liu, X.Z., Gao, R.H., Zhang, K.Q., and Cao, L. (1996) Dactylella tenuifusaria sp nov, a rhizopod-capturing hyphomycete. *Mycological Research* **100**: 236-238.

Liu, X.Z.Z., and Chen, S.Y.Y. (2003) Nutritional requirements of Pochonia chlamydospora and ARF18, fungal parasites of nematode eggs. *Journal of Invertebrate Pathology* **83**: 10-15.

Lopez-Llorca, L., Maciá-Vicente, J., and Jansson, H.-B., (2008) Mode of action and interactions of nematophagous fungi. In: Integrated management and biocontrol of vegetable and grain crops nematodes. Springer, pp. 51-76.

Luo, H., Li, X., Li, G.H., Pan, Y.B., and Zhang, K.Q. (2006) Acanthocytes of Stropharia rugosoannulata function as a nematode-attacking device. *Applied and Environmental Microbiology* **72**: 2982-2987.

Luo, H., Liu, Y.J., Fang, L., Li, X., Tang, N.H., and Zhang, K.Q. (2007) Coprinus comatus damages nematode cuticles mechanically with spiny balls and produces potent toxins to immobilize nematodes. *Applied and Environmental Microbiology* **73**: 3916-3923.

Meerupati, T., Andersson, K.M., Friman, E., Kumar, D., Tunlid, A., and Ahren, D. (2013) Genomic Mechanisms Accounting for the Adaptation to Parasitism in Nematode-Trapping Fungi. *Plos Genetics* **9**.

Miller, M.A., Pfeiffer, W., and Schwartz, T., (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE), 2010. Ieee, pp. 1-8.

Moosavi, M.R., and Zare, R., (2012) Fungi as biological control agents of plant-parasitic nematodes. In: Plant defence: biological control. Springer, pp. 67-107.

Nordbring-Hertz, B., Jansson, H.B., and Tunlid, A. (2006) Nematophagous fungi. *Els*.

Nowrousian, M., Stajich, J.E., Chu, M.L., Engh, I., Espagne, E., Halliday, K., Kamerewerd, J., Kempken, F., Knab, B., Kuo, H.C., Osiewacz, H.D., Poggeler, S., Read, N.D., Seiler, S., Smith, K.M., Zickler, D., Kuck, U., and Freitag, M. (2010) De novo Assembly of a 40 Mb Eukaryotic Genome from Short Sequence Reads: Sordaria macrospora, a Model Organism for Fungal Morphogenesis. *Plos Genetics* **6**.

Oka, Y., Koltai, H., Bar-Eyal, M., Mor, M., Sharon, E., Chet, I., and Spiegel, Y. (2000) New strategies for the control of plant-parasitic nematodes. *Pest Management Science* **56**: 983-988.

Perez-Garcia, A., Romero, D., and de Vicente, A. (2011) Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Current Opinion in Biotechnology* **22**: 187-193.

Persmark, L., and NordbringHertz, B. (1997) Conidial trap formation of nematode-trapping fungi in soil and soil extracts. *Fems Microbiology Ecology* **22**: 313-323.

Prasad, P., Varshney, D., and Adholeya, A. (2015) Whole genome annotation and comparative genomic analyses of bio-control fungus Purpureocillium lilacinum. *Bmc Genomics* **16**.

Rep, M., van der Does, H.C., Meijer, M., van Wijk, R., Houterman, P.M., Dekker, H.L., de Koster, C.G., and Cornelissen, B.J.C. (2004) A small, cysteine-rich protein secreted by Fusarium oxysporum during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Molecular Microbiology* **53**: 1373-1383.

Siddiqui, Z.A., and Mahmood, I. (1996) Biological control of plant parasitic nematodes by fungi: A review. *Bioresource Technology* **58**: 229-239.

Stamatakis, A. (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688-2690.

Stirling, G.R., and Mankau, R. (1978a) Dactylella oviparasitica, a new fungal parasite of Meloidogyne eggs. *Mycologia* **70**: 774-783.

Stirling, G.R., and Mankau, R. (1978b) Dactylella oviparasitica, a new fungal parasite of *Meloidogyne* eggsDactylella oviparasitica, a new fungal parasite of Meloidogyne eggs. *Mycologia* **70**: 774-783.

Su, H., Zhao, Y., Zhou, J., Feng, H.H., Jiang, D.W., Zhang, K.Q., and Yang, J.K. (2017) Trapping devices of nematode-trapping fungi: formation, evolution, and genomic perspectives. *Biological Reviews* **92**: 357-368.

Suarez, B., Rey, M., Castillo, P., Monte, E., and Llobell, A. (2004) Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent Trichoderma harzianum CECT 2413 displaying nematicidal activity. *Applied Microbiology and Biotechnology* **65**: 46-55.

Szabo, M., Csepregi, K., Galber, M., Viranyi, F., and Fekete, C. (2012) Control plant-parasitic nematodes with Trichoderma species and nematode-trapping fungi: The role of chi18-5 and chi18-12 genes in nematode egg-parasitism. *Biological Control* **63**: 121-128.

Tian, B., Yang, J., and Zhang, K.-Q. (2007) Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. *Fems Microbiology Ecology* **61**: 197-213.

Timper, P., and Riggs, R.D. (1998) Variation in efficacy of isolates of the fungus ARF against the soybean cyst nematode *Heterodera glycines*. *Journal of Nematology* **30**: 461-467.

Timper, P., Riggs, R.D., and Crippen, D.L. (1999) Parasitism of sedentary stages of Heterodera glycines by isolates of a sterile nematophagous fungus. *Phytopathology* **89**: 1193-1199.

Veresoglou, S.D., and Rillig, M.C. (2012) Suppression of fungal and nematode plant pathogens through arbuscular mycorrhizal fungi. *Biology Letters* **8**: 214-217.

Vos, C., Claerhout, S., Mkandawire, R., Panis, B., De Waele, D., and Elsen, A. (2012) Arbuscular mycorrhizal fungi reduce root-knot nematode penetration through altered root exudation of their host. *Plant and Soil* **354**: 335-345.

Wang, B., Liu, X.Y., Wu, W.P., Liu, X.Z., and Li, S.D. (2009) Purification, characterization. and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus Hirsutella rhossiliensis. *Microbiological Research* **164**: 665-673.

Wang, K.N., Riggs, R.D., and Crippen, D. (2004) Suppression of *Rotylenchulus reniformis* on cotton by the nematophagous fungus ARF. *Journal of Nematology* **36**: 186-191.

Yang, J., Tian, B., Liang, L., and Zhang, K.-Q. (2007a) Extracellular enzymes and the pathogenesis of nematophagous fungi. *Applied Microbiology and Biotechnology* **75**: 21-31.

Yang, J., Wang, L., Ji, X., Feng, Y., Li, X., Zou, C., Xu, J., Ren, Y., Mi, Q., Wu, J., Liu, S., Liu, Y., Huang, X., Wang, H., Niu, X., Li, J., Liang, L., Luo, Y., Ji, K., Zhou, W., Yu, Z., Li, G., Liu, Y., Li, L., Qiao, M., Feng, L., and Zhang, K.-Q. (2011) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. *Plos Pathogens* **7**.

Yang, Y., Yang, E., An, Z.Q., and Liu, X.Z. (2007b) Evolution of nematode-trapping cells of predatory fungi of the Orbiliaceae based on evidence from rRNA-encoding DNA and multiprotein sequences. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 8379-8384.

Yang, Z.S., Li, G.H., Zhao, P.J., Zheng, X., Luo, S.L., Li, L., Niu, X.M., and Zhang, K.Q. (2010) Nematicidal activity of Trichoderma spp. and isolation of an active compound. *World Journal of Microbiology & Biotechnology* **26**: 2297-2302.

Yang, Z.S., Yu, Z.F., Lei, L.P., Xia, Z.Y., Shao, L., Zhang, K.Q., and Li, G.H. (2012) Nematicidal effect of volatiles produced by Trichoderma sp. *Journal of Asia-Pacific Entomology* **15**: 647-650.

Figures and Table legends

Figure 3.1 Taxonomic placement of ARF18/TN14. Phylogenetic tree was made using the ITS1-5.8S-ITS2 region. DNA sequences of ITS regions from *Orbiliaceae* were obtained from GenBank. Sequences were initially aligned with ClustalX. Alignments were manually curated, and ambiguously aligned regions were removed using Gblocks. The assembled data set was subjected to phylogenetic analyses using neighbor-joining methods implemented in Phylip 3.66 and maximum likelihood methods implemented in RAxML v 7.0.0. Internal branch support was evaluated in both Phylip and RAxML using 1,000 replicates.

Figure 3.2 Quantification of growth of ARF18 in different nutritional conditions- malt extract agar (MEA) in pH 4.2 and pH 7, complete medium (CM), minimal medium (MM), yeast-extract glucose agar (YGA), potato dextrose agar (PDA), and cornmeal agar (CMA). Plugs of equal diameter from actively growing fungus were inoculated in the center of the petri dishes with different media and growth was measured at 5, 7, 14, and 21 days after inoculation. Growth was quantified as diameter of hyphal mass from the center of the petri dish.

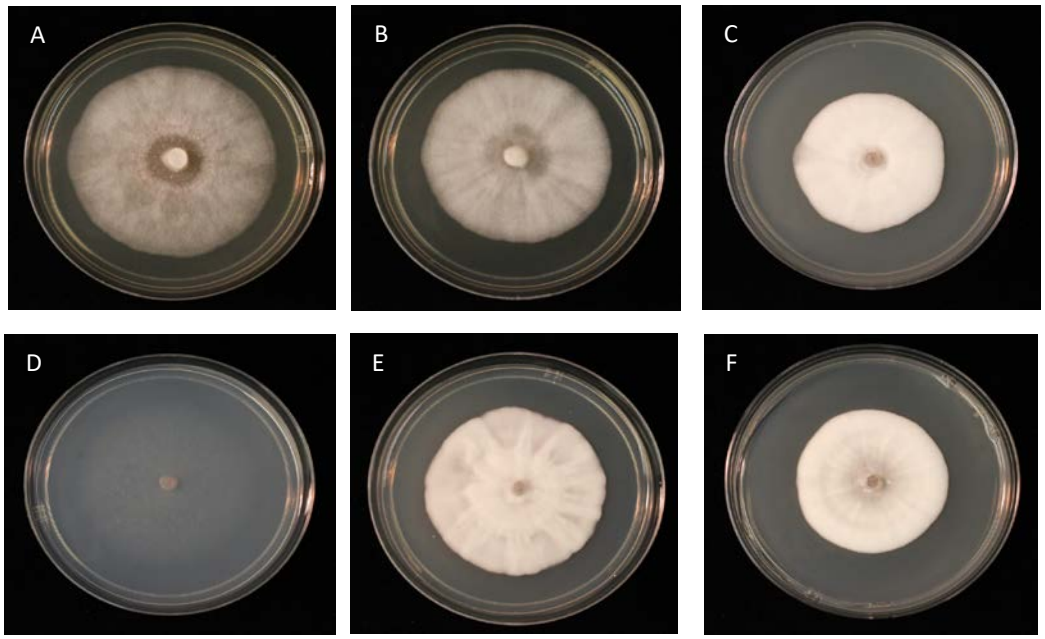
Figure 3.3 Light microscope figures of sclerotium-like structure produced by ARF18 in corn meal agar (CMA) medium. The fungus was unable to produce such structures in other medium.

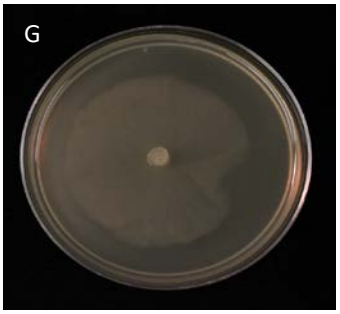
Figure 3.4 Scanning electron micrograph of ARF18 parasitizing cysts of *Heterodera glycines in-vitro.* Panel A shows the growth of fungal hyphae over the cyst, while panel B demonstrates the penetration of the cyst by the hypha of ARF18. Panel C is magnified 14991× at one of the penetration sites to demonstrate appresorium-like structures formed at the site of infection.

Table 3.1 Genes identified in ARF18 that are known to be important for nematode parasitism from other fungal species.

Figures and tables

Figure 3.1





A. Malt Extract Agar pH 7
B. Malt Extract Agar pH 4.2
C. PDA
D. Minimal medium
E. Complete medium
F. YGA
G. CMA

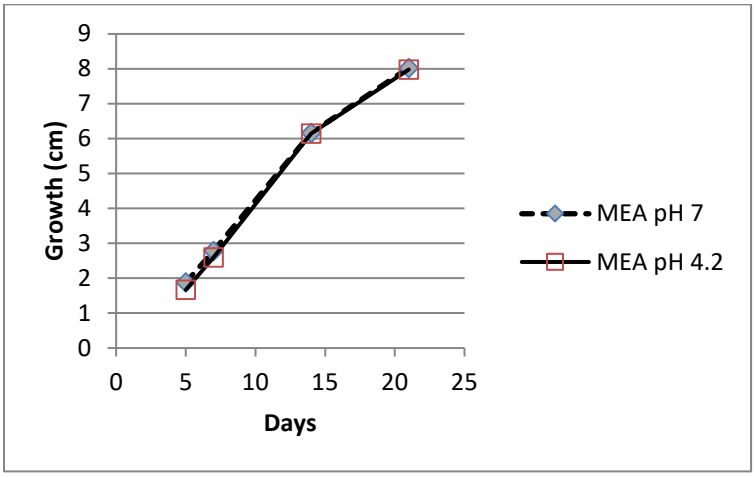
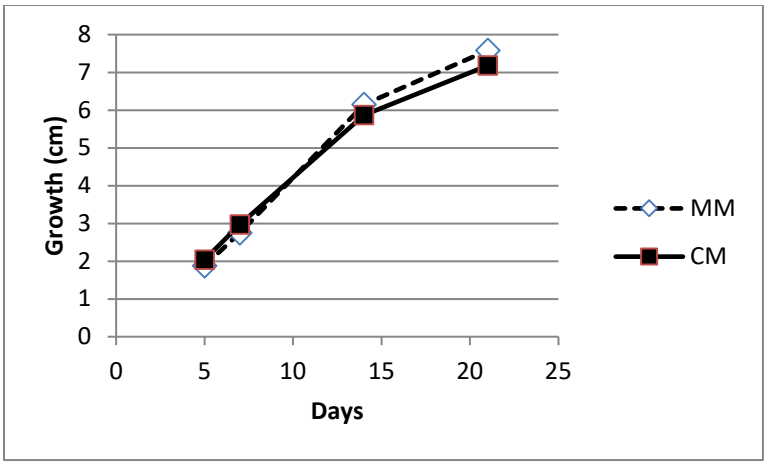


Figure 3.2



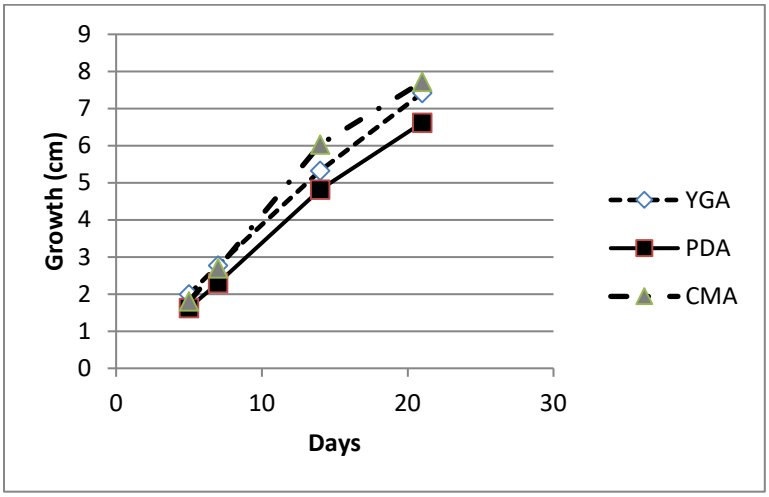


Figure 3.2

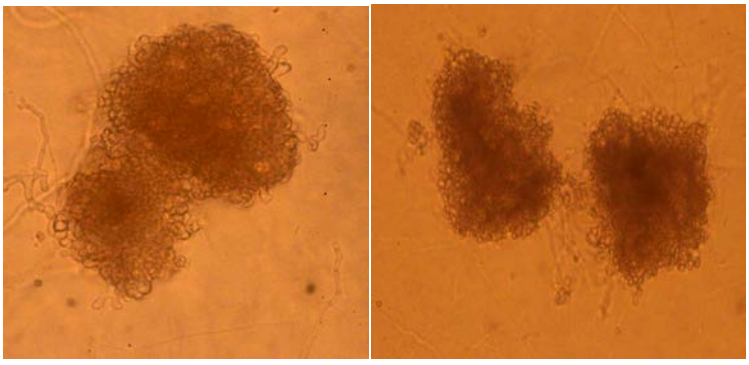


Figure 3.3

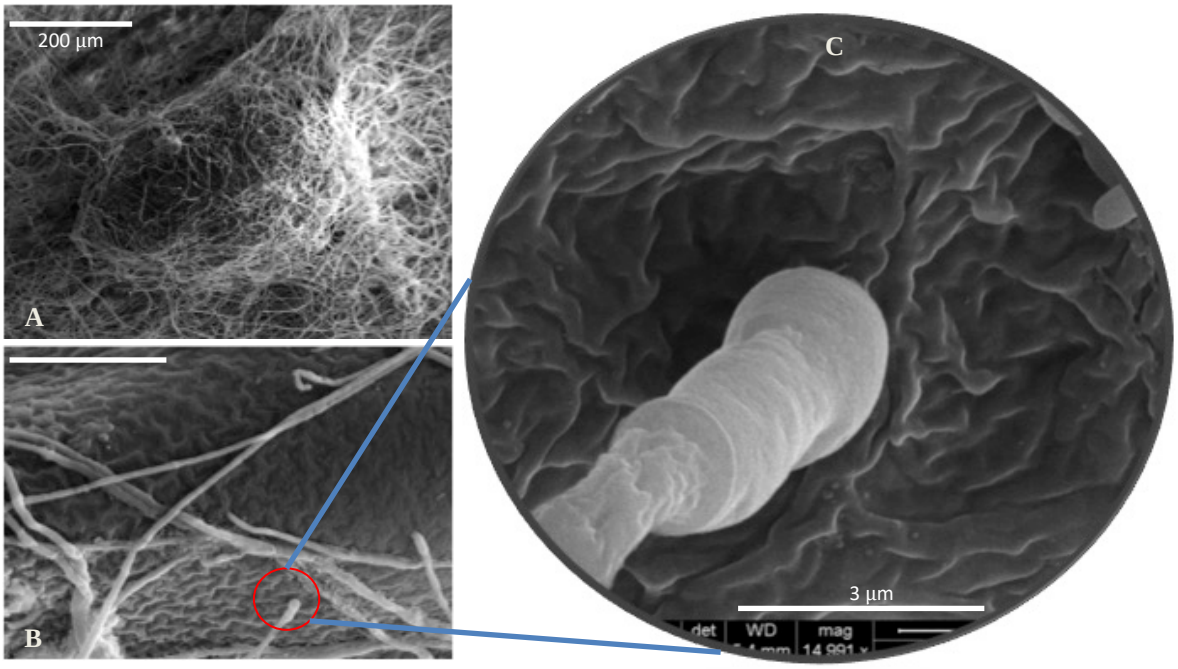


Figure 3.4

Table 3.1

Function	ARF18 gene Id	Organism	GenBank Id	Similarity (%)
Cuticle degrading serine protease	TN14_07867A	*Arthrobotrys musiformis*	EF113088.1	41.6
Alkaline serine protease	TN14_07831A TN14_08784A	*Hirsutella rhossiliensis*	GI:799240667	47 34
Cysteine rich secretory protein family	TN14_04654A TN14_05792A TN14_12017A TN14_01827A	*Nectria haematococca*	GI: 302895657	33 30 36 33

Appendix

Title: Taxonomic resolution of the nematophagous fungal isolate ARF18 via genome sequencing

Authors: Sandeep Sharma[a], Alex Z, Zaccaron[a], John B. Ridenour[a], Amy Bradshaw[b], Terry L. Kirkpatrick[a], Kelly Cartwright [a,c], Burton H. Bluhm[a #]

[a] Department of Plant Pathology, University of Arkansas, Division of Agriculture, Fayetteville, AR 72701, USA.

[b] Southern Arkansas University, Magnolia, AR 71753

[c] Agricultural Research Initiatives, Inc. Fayetteville, AR 72701, USA.

[#]Address correspondence to Burton H. Bluhm, bbluhm@uark.edu

ABSTRACT

A taxonomically uncharacterized, nematophagous fungus ARF18 that parasitizes cysts, juveniles, and adults of the soybean cyst nematode (*Heterodera glycines*) was proposed as a nematode biological control agent in 1991. A 46.3 Mb draft genome sequence of this fungus is presented, and a tentative taxonomic identification as a novel species of *Brachyphoris* is proposed.

Plant-parasitic nematodes are destructive pathogens of crop plants worldwide and cause estimated losses in excess of $150 billion annually (1). Control of plant-parasitic nematodes relies on chemical nematicides and cultural practices including crop rotation and resistant cultivars. Manufacture and use of several key chemical nematicides have been discontinued due to human health risks and environmental concerns. Resistant cultivars do not currently exist for all crops, and effective crop rotation schemes are lacking for many cropping systems due to economic concerns. Alternative nematode control tactics are urgently needed for many major economic crops (2, 3). The hyphomycete fungus ARF18 was first isolated from infected cysts of *Heterodera glycines* nearly 30 years ago (4). Because the fungus parasitizes all stages of the nematode, including eggs, juveniles, and adults in both soil and culture media (5), it was suggested as a potential biological control organism. Culture conditions have not yet been identified that induce conidiation or other morphological features that are required for classical taxonomic identification. Additionally, nothing is yet known about nematophagy in ARF18 at the molecular level.

The genome of ARF18 was sequenced with Pacific Biosciences (PacBio) technology, which generated 142,598 reads. Lengths varied from 35 bp to 43,743 bp with an average length of 7686 bp. A draft genome assembly of the fungus was obtained with Canu v1.1 (6), following the program instructions for low coverage datasets. The ARF18 draft genome assembly was

improved by merging contigs into scaffolds with AHA from the smrtanalysis suite v2.3.0 (http://www.pacb.com/products-and-services/analytical-software/devnet/devnet-analysis-tools/). The resulting genome assembly had 46639970 bp organized into 412 scaffolds with N50 of 177 kb, L50 of 76, and GC content of 44.6%. Compared to the genome of *Arthrobotrys oligospora* and many other ascomycetes, ARF18 had a slightly larger genome (7,8).

Gene prediction was performed with the Maker pipeline v2.31.6 (9) with homology evidence proteins from *Arthrobotrys oligospora* ATCC 24927, *Monacrosporium haptotylum* CBS 200.50, and *Drechslerella stenobrocha* 248. A total of 14,461 protein-encoding genes with average length of 1028 bp were predicted in the ARF18 genome assembly. Through BLAST analyses, several genes were identified that could play roles in nematode pathogenesis, including cuticle-degrading serine proteases, alkaline serine proteases, and chitinases (10, 11, 12). Further examination of the ITS1-5.8s-ITS2 rDNA region suggested that ARF18 belongs to a distinct monophylogenetic clade within *Brachyphoris*, a genus of nematophagous fungi that belongs to the Ascomycete family Orbiliaceae (13, 14, 15). Based on BLAST analyses, most of the genes analyzed showed high identity to *A. oligospora*, and *Dactylellina haptotyla*, both nematophagous fungi within the Orbiliaceae family, supporting the taxonomic placement of ARF18 within the Orbiliaceae family.

Currently, only a few nematophagous fungal genomes are publicly available. Thus, the genome sequence of this fungus will provide a useful resource to study the biology of nematophagous fungi, especially within the *Brachyphoris* genus. Further analyses of the genome of ARF18 will also provide important information regarding the molecular basis of fungal nematophagy and guide the potential development of this nematode pathogen as a biological control agent.

Nucleotide sequence accession numbers: This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession AZLU00000000. The version described in this paper is version AZLU01000000.

ACKNOWLEDGEMENTS

This work was supported by funding from the U.S. National Science Foundation (STTR: 1009585) and The Arkansas Soybean Promotion Board. Additionally, this work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1008895.

REFERENCES

1. **Abad P, Gouzy J, Aury J-M, Castagnone-Sereno P, Danchin EGJ, Deleury E, Perfus-Barbeoch L, Anthouard V, Artiguenave F, Blok VC, Caillaud M-C, Coutinho PM, Dasilva C, De Luca F, Deau F, Esquibet M, Flutre T, Goldstone JV, Hamamouch N, Hewezi T, Jaillon O, Jubin C, Leonetti P, Magliano M, Maier TR, Markov GV, McVeigh P, Pesole G, Poulain J, Robinson-Rechavi M, Sallet E, Segurens B, Steinbach D, Tytgat T, Ugarte E, van Ghelder C, Veronico P, Baum TJ, Blaxter M, Bleve-Zacheo T, Davis EL, Ewbank JJ, Favery B, Grenier E, Henrissat B, Jones JT, Laudet V, Maule AG, Quesneville H, Rosso M-N, Schiex T, Smant G, Weissenbach J, Wincker P.** 2008. Genome sequence of the metazoan plant-parasitic nematode Meloidogyne incognita. Nature Biotechnology **26:**909-915.
2. **Oka Y, Koltai H, Bar-Eyal M, Mor M, Sharon E, Chet I, Spiegel Y.** 2000. New strategies for the control of plant-parasitic nematodes. Pest Management Science **56:**983-988.
3. **Tian B, Yang J, Zhang K-Q.** 2007. Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. Fems Microbiology Ecology **61:**197-213.
4. **Kim DG, Riggs RD.** 1991. Characteristics and efficacy of a sterile hyphomycete (arf18), a new biocontrol agent for Heterodera glycines and other nematodes. Journal of Nematology **23:**275-282.
5. **Timper P, Riggs RD, Crippen DL.** 1999. Parasitism of sedentary stages of Heterodera glycines by isolates of a sterile nematophagous fungus. Phytopathology **89:**1193-1199.
6. **Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM**. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighing and repeat separation. Genome Research **27**: 722-736.
7. **Yang J, Wang L, Ji X, Feng Y, Li X, Zou C, Xu J, Ren Y, Mi Q, Wu J, Liu S, Liu Y, Huang X, Wang H, Niu X, Li J, Liang L, Luo Y, Ji K, Zhou W, Yu Z, Li G, Liu Y, Li L, Qiao M, Feng L, Zhang K-Q.** 2011. Genomic and Proteomic Analyses of the Fungus Arthrobotrys oligospora Provide Insights into Nematode-Trap Formation. Plos Pathogens **7**.
8. **Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma LJ, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang SG, Nielsen CB, Butler J,**

Endrizzi M, Qui DY, Ianakiev P, Pedersen DB, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Stabge-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishman D, Krystofova S, Rasmussen C, Metzenberg RL, Perkins DD, Kroken S, Cogoni C, Macino G, Catcheside D, Li WX, Pratt RJ, Osmani SA, DeSouza CPC, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiler S, Dunlap J, Radford A, Aramayo R, Natvig DO, Alex LA, Mannhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B. 2003. The genome sequence of the filamentous fungus Neurospora crassa. Nature **422:**859-868.

9. **Huang XW, Zhao NH, Zhang KQ.** 2004. Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. Research in Microbiology **155:**811-816.
10. **Lopez-Llorca LV, Gomez-Vidal S, Monfort E, Larriba E, Casado-Vela J, Elortza F, Jansson H-B, Salinas J, Martin-Nieto J.** 2010. Expression of serine proteases in egg-parasitic nematophagous fungi during barley root colonization. Fungal Genetics and Biology **47:**342-351.
11. **Yang J, Tian B, Liang L, Zhang K-Q.** 2007. Extracellular enzymes and the pathogenesis of nematophagous fungi. Applied Microbiology and Biotechnology **75:**21-31.
12. **Yang J, Yu Y, Li J, Zhu W, Geng Z, Jiang D, Wang Y, Zhang K-Q.** 2013. Characterization and functional analyses of the chitinase-encoding genes in the nematode-trapping fungus Arthrobotrys oligospora. Archives of Microbiology **195:**453-462.
13. **Chen J, Xu L-L, Liu B, Liu X-Z.** 2007. Taxonomy of Dactylella complex and Vermispora. I. Generic concepts based on morphology and ITS sequences data. Fungal Diversity **26:**73-83.
14. **Chen J, Xu L-L, Liu B, Liu X-Z.** 2007. Taxonomy of Dactylella complex and Vermispora. II. The genus Dactylella. Fungal Diversity **26:**85-126.
15. **Chen J, Xu L-L, Liu B, Liu X-Z.** 2007. Taxonomy of Dactylella complex and Vermispora. III. A new genus brachyphoris and revision of vermispora. Fungal Diversity **26:**127-142.

CHAPTER V

A Forward genetic screen coupled with novel target-enrichment sequencing approach identifies novel genes regulating cercosporin in *Cercospora zeae-maydis*, a foliar pathogen of corn

Abstract

Molecular genetics studies have helped identify genes regulating numerous biological processes in filamentous fungi. For example, the improvement and deployment of biological control organisms could be accelerated rapidly by a more complete understanding of the underlying genetic mechanisms. Polyethylene glycol (PEG)- and *Agrobacterium*-mediated transformation are effective ways to create populations of mutants in many fungal species. However, identifying where mutagenesis cassettes integrated into genomes can be laborious and challenging. In this study, a novel target-enrichment sequencing method was developed to efficiently characterize cassette insertions in *Cercospora zeae-maydis*, a model plant pathogenic fungus. A collection of 3500 tagged mutants of *C. zeae-maydis* was created via PEG- and *Agrobacterium*-mediated transformation and screened for production of cercosporin, a phytotoxin that can be scored visually in defined growth media. Eighty mutants produced substantially higher or lower levels of cercosporin than the wild-type strain. Biotinylated oligonucleotides were designed to hybridize with border regions of the insertion cassette and enrich cassette-genome break junctions from whole-genome DNA sequencing libraries. For target-enrichment sequencing, up to 16 uniquely barcoded libraries were pooled per capture reaction, and a maximum of two such pools were sequenced simultaneously on the Ion Torrent Personal Genome Machine to identify cassette insertion sites. Target-enrichment sequencing identified 49 mutants with single T-DNA insertions, and 26 mutants with multiple insertions. Novel genes potentially regulating cercosporin biosynthesis were identified, including genes involved in signal transduction, primary and secondary metabolism, growth and development, and stress responses. Additionally, RNAi lines created for selected genes of interest confirmed associations between cassette insertions and cercosporin-related phenotypes. This approach provided expansive, unique insight into the regulation of cercosporin biosynthesis, and could

easily be adapted to dissect the genetic basis of parasitism in nematophagous fungi such as ARF18 to enhance its efficacy in controlling plant-parasitic nematodes.

1. Introduction

Molecular genetics is broadly defined as the application of tools and strategies to understand the function, expression, and regulation of a gene at the molecular level. Forward genetic screens can be a valuable tool of molecular genetic studies and have been widely used to identify novel genes, such as those encoding enzymess (Reilly *et al.*, 2018) and genes involved in virulence (O'Meara *et al.*, 2015). All forward genetic screens follow a similar principle wherein an organism undergoes mutagenesis to create a collection of random mutants. The population is then screened for a phenotype of interest and the gene(s) underlying the phenotype is identified. Validating that the candidate gene is responsible for the phenotype is the next step and involves mutating the wild type strain at the same locus and confirming the phenotype. Although forward genetic screens are widely applicable in many organisms, they are largely limited to model organisms (Patton & Zon, 2001, Forsburg, 2001, Casselton & Zolan, 2002, Kile & Hilton, 2005). Forward genetic studies in filamentous fungi and oomycetes have identified genes underlying several developmental processes such as pathogenesis and secondary metabolism (Kamoun, 2003, Yu & Keller, 2005, Jeon *et al.*, 2008, Pfannenstiel *et al.*, 2017). With improvement in techniques for genetic manipulation and availability of genome resources, forward genetic screens are being widely used in non-model organisms. Although genetic manipulation of tools are available for nematophagous fungi (Ahman *et al.*, 2002, Atkins *et al.*, 2004, Shen *et al.*, 2015), the use of forward genetic screens to identify genes regulating pathogenesis and secondary metabolism production in nematophagous fungi has been underutilized (Xu *et al.*, 2005)

However, the ability to efficiently identify genomic lesions in random mutants represents a bottleneck in forward genetic screens. Several approaches have been developed to define

insertion sites of mutagenesis DNA insertion cassettes, including plasmid rescue (Tam & Lefebvre, 1993), thermal asymmetric interlaced PCR (Dent *et al.*, 2005), restriction enzyme site-directed amplification PCR (Gonzalez-Ballester *et al.*, 2005), and site finding PCR (Li *et al.*, 2012). These methods, however, are limited by throughput, labor intensive, and expensive. Recently, whole-genome re-sequencing at shallow coverage has been utilized successfully to identify mutations in some species of fungi (Esher *et al.*, 2015). However, current costs associated with whole-genome re-sequencing limit the number of mutants that can be analyzed. Additional methods based on restriction enzyme associated DNA sequencing (RAD-seq) approaches were developed to identify the insertion sites of the mutagenesis cassette (Zhang *et al.*, 2014, Zaccaron *et al.*, 2018). These methods have limitations due selection of restriction enzymes, truncation of the mutagenesis cassette and aberrant insertion of the mutagenesis cassette.

Target capture sequencing or target-enrichment sequencing enriches selected regions of the genome and has been widely utilized in both genome sequencing (Mertes *et al.*, 2011) and RNA-sequencing experiments (Mercer *et al.*, 2014). Previous methods used for target-enrichment included PCR and molecular inversion probes, which are error prone (Schmitt *et al.*, 2015). In solution hybridization is another method of target capture and uses biotinylated oligos to enrich selected regions of the genome (Penalba *et al.*, 2014). Although target enrichment sequencing have been applied to identify the site of insertion of the mutagenesis cassettes in plant species like corn (Williams-Carrier *et al.*, 2010) and *Arabidopsis* (Lepage *et al.*, 2013, Inagaki *et al.*, 2015), the method has not been applied as a tool in filamentous fungi, especially in the context of forward genetic screens.

Cercospora zeae-maydis Tehon & Daniels, the causal organism of the gray leaf spot in corn, is the most common and one of the most destructive foliar pathogens of corn in the United States (Shim & Dunkle, 2002) and causes significant damage to both yield and quality (Ward *et al.*, 1999, Dunkle & Levy, 2000, Goodwin *et al.*, 2001, Crous *et al.*, 2004). *Cercospora zeae-maydis* and other species of *Cercospora* produce a phytotoxin, cercosporin (Daub, 1982, Shim & Dunkle, 2002). Cercosporin belongs to a pyrelenequinone class of toxin, which is activated by light (Bluhm *et al.*, 2008, Kim *et al.*, 2011a) and causes cell death through lipid peroxidation, membranes leakage, and cytoplasmic leakage (Lousberg *et al.*, 1971). Cercosporin can be induced in an artificial medium like 0.2× strength potato dextrose agar (PDA) (Shim & Dunkle, 2002). This ability to produce cercosporin makes the phenotype easy to identify and quantify. Despite the importance of cercosporin in pathogenesis, its regulation is understudied at the molecular level. Therefore, as a proof of concept, we utilized *C. zeae-maydis* as a model system and utilized a forward genetic screen coupled with a novel target-enrichment sequencing technology to identify putative genes regulating cercosporin production. *C. zeae-maydis* was used as a system because 1) it is routine to transform and is amenable to both protoplast- and *Agrobacterium*-mediated transformation, 2) it has an easy visual screen for cercosporin production, and 3) has a near-complete genome that is completely annotated. The method described in this chapter utilizing *C. zeae-maydis* as a model system can be successfully applied to diverse filamentous fungi, including nematophagous fungus like ARF18, to accelerate molecular genetic studies of different metabolic pathways including pathogenesis and secondary metabolism.

2. Materials and Methods

2.1. Generation of mutants

Random insertional mutants of *Cercospora zeae-maydis* strain SCOH1-5 were created using polyethyleneglycol (PEG)-mediated and *Agrobacterium*-mediated transformation. For PEG-mediated transformation of protoplasts, a previously described protocol was followed (Ridenour *et al.*, 2012). Briefly, the mutagenesis cassette, GFP-Gen was amplified from the plasmid pBR0073 using primer pairs ATKpnI1F and ATKpn1R and purified by precipitating with ethanol and dissolved in dH_2O. Protoplasts of *C. zeae-maydis* were made as described by Ridenour et al. (2012) with slight modifications. Conidia were harvested from three-day-old actively growing cultures on V8 juice agar medium and inoculated into YEPD liquid medium at room temperature and constant shaking at 200 rpm. Germinated conidia were separated from the liquid YEPD medium after 36-40 hours of incubation using nylon spectra mesh filter. After collecting the germinated conidia on the nylon spectra mesh filter, they were transferred to an Erlenmeyer flask with 20-25 ml of 1.2 M potassium chloride (KCl) solution containing 20% lysing enzymes from *Trichoderma harzianum* (Glucanex; Sigma Aldrich, St Louis, MO, USA) and 0.4% (v/v) β-glucuronidase (Sigma Aldrich) and incubated at room temperature for 8 hours with constant shaking at 50 rpm. Protoplasts were harvested by centrifugation of the lysing solution in 50 ml centrifuge tubes at 4000 rpm in 4 °C. Protoplasts were suspended in 1.2 M KCl solution, diluted to a concentration of 1×10^6 protoplasts per milliliter. Prepared protoplasts were either used immediately for transformation or stored in -80 °C until further use.

For transformation, 10 µg of the amplified mutagenesis cassette was added to the protoplasts suspension, mixed with gentle tapping and incubated in ice for 30 minutes. 200 µl of 60% PEG-2000 in STC (1.2 M sorbitol, 50mM CaCl2 and 10mM Tris pH 8.0), was added and

mixed gently by swirling the tube. After incubating the mixture at room temperature for 30 minutes, 1 ml of regeneration medium (1.0 M sucrose, 0.02% yeast extract) was added to the tube and incubated at room temperature for 48 hours in a rocking shaker. After 48 hours, the transformation mixture was diluted to 10 ml with regeneration medium and 1 ml of the diluted mixture was plated with regeneration agar medium (1.0 M sucrose, 0.02% yeast extract and 1.0% agar) amended with 200 µg/ml G-418 disulphate (geneticin, Research Products Inter. Corp., Mt. Prospect, IL) and incubated at room temperature in dark until colonies were visible for transfer to a selection medium.

For *Agrobacterium*-mediated transformation, the plasmid pBHt2-sGFP (Mullins *et al.*, 2001) transformed into the vector *Agrobacterium tumefaciens*, strain AGL1 (Lazo *et al.*, 1991) was used. A single colony of the *Agrobacterium*, containing the pBHt2-sGFP plasmid (Mullins *et al.*, 2001), from a 3 day-old culture in LB-agar plate, amended with appropriate antibiotics, was inoculated in Luria broth with 100 $\mu g ml^{-1}$ carbenicillin and 100 $\mu g ml^{-1}$ kanamycin for selection. Following 2-3 days of incubation at 28 ºC, the OD of the culture was quantified using a spectrophotometer. The culture was spun down and re-suspended in *Agrobacterium* inducing medium (IMM), with 100 $\mu g ml^{-1}$ carbenicillin and 100 $\mu g ml^{-1}$ kanamycin for selection, to an OD_{600} of 0.2. After 24 hours, the OD_{600} of the bacterial culture in the IMM was quantified and diluted with IMM to set the final OD_{600} value to 0.2. This was the induced *Agrobacterium* culture used for transformation.

For transformation of *C. zeae-maydis*, conidia from a three-day-old culture on V8 agar were harvested with IMM. Approximately 10^6 conidia ml^{-1} were mixed with the induced *Agrobacterium* cultures in equal volumes. Two hundred microliters of the agro-spore suspension were plated onto IMM agar plates overlaid with cellophane and incubated for 4 days at room

temperature. The cellophane membranes were removed and flipped onto 0.2× strength PDA plates amended with 100 μgml^{-1} hygromycin and 200 μgml^{-1} cephotaxime, 100 μgml^{-1} carbenicillin and 100 μgml^{-1} kanamycin as selection antibiotics. The cellophane membranes were removed four days after the initial transfer. Transformed colonies were visible approximately 10 days after the removal of the cellophane membranes. GFP expressing colonies were identified through visual examination using GFP light. Fluorescing colonies were transferred onto V8 agar medium amended with the 100 μgml^{-1} hygromycin. Colonies were screened for cercosporin production once sufficient growth was observed on V8 agar medium.

2.2. Identifying mutants with altered cercosporin production

The mutants were screened for cercosporin production on 0.2× strength PDA (Difco Microbiology, Lawrence, KS) in constant light (Shim & Dunkle, 2002). Cercosporin causes dark red pigmentation on the PDA medium (Figure 2). To screen mutants for cercosporin, 1mm x 1mm plugs from actively growing cultures in V8 agar were transferred onto 0.2× PDA medium and kept in constant light for 7-10 days. Similarly, a plug from actively growing SCOH1-5 (the wild-type parent strain) was inoculated as a control. The mutants were categorized into three groups: non-producers - which did not show visible pigmentation in 0.2× PDA medium, very less producers- which showed comparatively less pigmentation compared to the wild-type, and over producers- which showed increased pigmentation compared to wild-type parent strain (Figure 2). The mutants that grouped under these three categories were selected for further screening. After two rounds of screening for cercosporin production, the cultures were single sporulated by streaking spores onto 0.2× PDA medium and culturing colonies growing from one spore. Colonies from single spores were then transferred onto V8 agar medium amended with 100 μgml^{-1} hygromycin or 200 μgml^{-1} geneticin as selection antibiotic. After sufficient growth of

the cultures, they were re-screened for cercosporin production with the same method as described above.

2.3. Extraction of genomic DNA and library preparation

Modified CTAB method was followed for DNA extraction. In brief, the strains were grown on liquid yeast extract media at room temperature with constant shaking. Fungal tissue was collected by centrifugation of the culture in 50 ml Falcon tubes. The tissue was extracted and ground with liquid nitrogen and used for genomic DNA extraction. DNA extracted from the tissues was quantified with a spectrophotometer. 400 bp libraries from the mutants were prepared using 200 nanograms DNA with the NEBNext Fast DNA Library Preparation kit for Ion Torrent (New England Biolabs, Ipswich, MA) with minor modifications. In the ligation step, the adapters from the kit were replaced with a different set of barcoded A and P1 adapters (Biooscientific, Austin, TX). The libraries were the quantified with an Agilent Tapestation 2200 D1K (Agilent Technologies, Santa Clara, CA). Up to 16 different barcoded libraries were pooled in equal amounts for a total of 500 nanograms simultaneous target capture reaction.

2.4. Target-enrichment sequencing to identify T-DNA insertion sites

For identification of the site/s of insertion, we followed probe capture followed by sequencing in Ion Torrent PGM machine. For the capture of the target regions the protocol described by Schmitt et al., (2015) was used. Following the final set of amplification for 14 cycles, libraries were pooled in equal amounts. Biotinylated probes for the cassette were designed and ordered from IDT (Integrated DNA Technologies, Coralville, IA). Probes were designed to capture about 240-360 bp from left and the right border of the cassette. Briefly, libraries from the mutants were combined in equal amounts giving a total of 500 ng DNA a pool. 500 ng of the pooled libraries were combined with 1 µl each of xGen Universal Blocking Oligo 1

(IT-P1) and xGen Universal Blocking Oligo 2 (IT-A) (Integrated DNA Technologies). The contents were completely lyophilized with a Savant SpeedVac Concentrator SVC100H (Thermo Fisher Scientific, Waltham, MA, USA). The following reagents were added to each of the lyophilized samples- 8.5 µl of xGen 2× Hybridization Buffer (Integrated DNA Technologies), 2.7 µl of xGen Hybridization Buffer Enhancer (Integrated DNA Technologies), 1.8 µl nuclease free water. The components were thoroughly mixed and were incubated at 95 °C for 10 minutes. Following incubation at 95 °C for 10 minutes, 3 picomoles of the biotinylated probes were immediately added to each reaction and mixed by vortexing. The reaction mixtures were incubated at 65 °C for 4 hours. After 4 hours of incubation, 75 µl of Dynabeads M-270 Streptavidin beads (Thermo Fisher Scientific) were added to each sample, and the incubation and washes were performed according to the protocol described in Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents (Integrated DNA Technologies). The probed libraries were amplified for 14 cycles according to the manufacturer's protocol described in the NEBNext Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs) and purified with 1× volume of Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The purified libraries were combined with 0.5 µl of the blocking oligos and another round of capture was performed with 1.5 picomoles of the biotinylated probes. Additional round of clean up with 1× volume of Agencourt AMPure XP beads was performed followed by a PCR reaction for 14 cycles and the final libraries was analyzed with an Agilent Tapestation 2200 D1K (Agilent Technologies). The libraries were sequenced on an Ion Torrent Personal Genome Machine with an Ion 314 chip kit V2 (Thermo Fisher Scientific).

2.5. Mapping reads to the reference genome

Sequenced reads were processed with FASTQC to filter poor quality reads. The reads above threshold Q20 were selected for further analyses. Additionally, poor quality bases at the 3' end of the reads were trimmed off. The reads were initially mapped to the mutagenesis cassette with Burrows Wheeler Aligner (BWA) (Li & Durbin, 2010). The mapped reads were assembled *de novo* with MAKER pipeline version 2.31.6 (Holt & Yandell, 2011). The mapped and assembled reads were then aligned to the reference genome (SCOH1-5) with BWA to identify the site/s of insertion/s. The site/s of insertion/s were visualized with IGV (Robinson *et al.*, 2011) to identify the number, site, and types of insertions in the mutants. The site or sites of the T-DNA insertion was determined by the percent of reads that mapped to a locus or a set of loci in the genome compared to overall mapping in the genome.

2.6. Validation of cercosporin production phenotype

To validate the robustness of our screen and the link between the mutations and the phenotype, we created individual knock-down mutants for six of the genes identified in the screen. RNA silencing (RNAi) constructs to silence the six genes, including the putative *CTB1* gene, were created. For generating the RNAi vectors, we amplified sense and anti-sense strands for each of the six genes with primers mentioned in Table 3. Intron of cutinase from *Magnaporthe oryzae* was amplified from pSILENT vector (Nakayashiki *et al.*, 2005). Six individual vectors- pBW4, pBW5, pBW7, pBW8, pBW9 and pBW10 were created by incorporating the strands and the intron onto the plasmid pBHT2 following the Gibson Assembly protocol (Gibson *et al.*, 2009). The orientation of the sense and antisense strands and the intron in each of the plasmids were confirmed via polymerase chain reaction. The plasmids were transformed into *Agrobacterium* cells, *AGL1*, via electroporation mentioned above. *C. zeae-*

maydis, strain SCOH1-5, was transformed with the RNAi constructs following the *Agrobacterium*-mediated transformation protocol described above to create individual RNAi lines. The colonies were picked about 10 days after transformation and transferred to V8 media amended with 100 $\mu g ml^{-1}$ hygromycin as the selection antibiotic. The transformed colonies were screened for cercosporin production by inoculating a small plug from an actively growing colony in 0.2× PDA plates and incubating in constant light for 7-10 days. The colonies with the expected cercosporin production phenotype were selected for further analyses.

For quantification of cercosporin production in the RNAi lines generated, 10^5 spores from each of the lines were plated on to 0.2× strength PDA plates and incubated at constant light at room temperature. After 7 days of incubation, cercosporin was quantified with 5N KOH previously described (Kim *et al.*, 2011b). Briefly, the plate was flooded with 10 ml of 5N KOH, mixed by swirling and incubated in dark for 30 minutes. The absorbance of KOH was quantified at 480 nm. Cercosporin concentration was measured using the formula $A = \varepsilon m Cl$, where εm is the molar extinction coefficient 23,300 mol^{-1} cm^{-1}, C is the concentration and l is the path length of the cuvette, which is 1 cm.

3. Results

3.1. Development of target-enrichment sequencing method

A unique protocol was developed using the target-enrichment method to successfully enrich for a selected region of the genome. Using this method, we were able to successfully enrich a desired region for sequencing which improved the depth of coverage of the region several-fold. The method was optimized for Ion Torrent Personal Genome Machine, which is quick to operate, easy to handle. However, the method can be easily adapted to other sequencing platforms such as Illumina, Oxford Nanopore, or Pacbio technologies. Our method enabled us to

successfully sequence multiple *C. zeae-maydis* strains in one run on a 314 chip with a maximum capacity of just 100 million bases. Using the 314 chip, we were able to pool and sequence the desired region for up to 31 mutants in one reaction. Figure 1 depicts a flowchart of the steps that were used for target-enrichment and sequencing. In our protocol, we successfully targeted the end of the T-DNA (Transfer DNA) to identify the insertions (Table 1), although insignificant non-specific enrichment was also obtained. The percentage of mapping reads to a single locus would not be possible with conventional sequencing. Although our method is developed for a single region, we can apply this method to numerous loci simultaneously to increase mapping.

3.2. Determining the site/s of insertion using target-enrichment sequencing

A forward genetic screen was performed to identify genes regulating cercosporin biosynthesis in *C. zeae-maydis.* Mutants were created via PEG-mediated transformation of protoplasts and *Agrobacterium*-mediated transformation. A collection of nearly 3500 mutants was created from the above methods with 350 mutants from PEG-mediated protoplast transformations, and 3150 mutants were created via *Agrobacterium*-mediated transformation. Previously very few studies have been conducted on *C. zeae-maydis* utilizing *Agrobacterium*-mediated transformation (Lu et al., 2016). However, a large-scale genetic screen on *C. zeae-maydi*s to identify genes regulating cercosporin biosynthesis has not been reported.

In many species of *Cercospora*, cercosporin has been shown to be required for pathogenicity. Although genes regulating cercosporin biosynthesis in *C. zeae-maydis* had been identified through subtractive hybridization (Shim & Dunkle, 2002), a genetic screen to identify genes regulating the production of cercosporin biosynthesis has not been reported. For phenotyping cercosporin production in the mutant collection, 1 mm x 1 mm plugs from the mutants and the wild-type parent strain was inoculated onto 0.2× strength PDA plates in

incubated in constant light for 7-10 days (Figure 2). We limited ourselves to the identification of only the cercosporin phenotype, although the method is also applicable to phenotypes such as pathogenicity, sporulation etc. We identified around 81 mutants with altered levels of cercosporin compared to the wild-type parent strain. From the 81 mutants, 29 mutants were created from the protoplasts, while 52 mutants were created from *Agrobacterium*-mediated transformation. The forward genetic screen helped us identify three groups of mutants, based on visual analyses. Mutants that did not produce cercosporin- no color production was observed on 0.2× strength PDA plates, mutants that were severely reduced in cercosporin production- the pigmentation was reduced compared to the wild-type parent strain, and mutants that were increased in cercosporin production- pigmentation on the 0.2× PDA plated were more than the wild-type parent strain. Of the total of 81 mutants we identified 59 mutants that did not show any production of cercosporin, 18 mutants had very reduced production of cercosporin, while 3 mutants were over-producers (Table 4).

Eighty-one mutants with altered levels of cercosporin were identified and we developed a target-enrichment sequencing approach to precisely identify the site and the copy number of insertion of the mutagenesis cassette. Our method also identified multiple insertions in several mutants, which suggests that the method could be optimized to replace Southern hybridization, which is employed to determine the copy number of insertions in mutant strains. In our method, a double capture method previously described (Schmitt *et al.*, 2015) was utilized and optimized for filamentous fungi. The sequencing was performed in the Ion PGM platform, which is cheap and fast compared to other next-generation sequencing platforms like Illumina and Pacific Biosciences long read technology. Additionally, with our approach, we were able to sequence up to 31 mutants in a single run in a 314 chip, which has a sequencing capacity of just 100 Mb. We

obtained a very high coverage for the targeted site to confidently predict the location of the T-DNA/mutagenesis cassette insertion. Such methods to enrich regions for the genome to improve the depth of sequencing in filamentous fungi have not been developed to date.

Using target-enrichment sequencing approach for 81 independent random mutants, we identified a total of 109 putative insertional locations. Fifty-five from the 109 insertional sites show a typical insertional characteristic with mapping to both sides of the insertional cassette (Figure 4). Such insertions are termed as symmetric insertions. From these insertional sites, we identified small and large deletions in the genome during the integration of the mutagenesis cassette. *Agrobacterium*-mediated transformation identified 40 symmetric insertions, while from PEG-mediated transformation we identified 15 symmetric insertions. We also observed 54 asymmetric insertions, wherein only one side of the mutagenesis cassette was mapped onto an insertional site (Figure 4). Of the total insertions, we identified 49 single insertions and 26 multiple insertions. Interestingly, 22 of 29 insertions from PEG-mediated transformation were single and 4 were multiple, whereas *Agrobacterium*-mediated transformation produced a higher percentage of multiple insertions wherein 27 out of 52 were single insertions and 22 were multiple.

3.3. Identifying genes regulating cercosporin production

A forward genetic screen approach to identify genes regulating cercosporin biosynthesis in *C. zeae-maydis* identified novel genes, which were previously unknown regulators of cercosporin biosynthesis in the genus *Cercospora*, including *C. zeae-maydis*. Out of a total of 81 mutants, 51 mutants had single insertions (Table 4). The insertions were present in genes, in between genes, upstream promoter regions, and repetitive elements. Many of the genes identified include novel and previously uncharacterized genes in filamentous fungi. Interestingly, one of

the mutants identified had the cassette integrated into the open reading frame of *CTB1* (Cercosporin Toxin Biosynthesis-1), the polyketide synthase gene catalyzing the first step of cercosporin biosynthesis in *Cercospora* species. The genes identified through the screen could be grouped into three broad categories: 1) Chromatin modifiers such as *SNF5*, *SET3*, bromodomain-containing protein, WD40 repeat-containing protein, SANT domain-containing protein, 2) genes involved in growth and development such as putative scytalone dehydratase *(SDH)*, *ERG5*, *VPS35*, actin depolymerizing factor, and 3) genes involved in signal transduction such as MFS1 transporter family, cytochrome P450, Calcium ion channel protein, Phenol hydroxylase, conserved hypothetical proteins (Figure 6). Interestingly, the sites that were disrupted in chromatin modifying genes were all either non-producers of cercosporin or were severely reduced in cercosporin production. The gene scytalone dehydratase (*SDH1*) has been previously characterized in *Magnaporthe oryzae*, and is required for melanin biosynthesis and pathogenicity (Motoyama *et al.*, 1998). Interestingly the mutation within this gene produced a white-colored fungal colony with red pigmentation on V8 agar medium even in complete darkness (Figure 3). Similarly, one of the insertions identified is present 287 bp upstream of putative glucose-inactivated glycerol proton symporter *STL1*, a gene previously characterized in *Saccharomyces cerevisiae*, expressed during hyperosmotic stress, thus required for homeostasis (Sauday, 2010). One insertion identified is located in an open reading frame highly similar to salicylate hydroxylase, present in plant pathogenic bacteria. Salicylate hydroxylase is required for degrading salicylic acid- a hormone produced during systemic resistance in plants. The disruption caused the mutant to be defective in cercosporin production. In addition, many of the insertions identified were present in genes that do not share any homology to previously known genes and hence could potentially be novel genes regulating secondary metabolism in fungi. All

the insertions were mapped to different locations in the genome. The insertions of the T-DNA or mutagenesis cassette appeared randomly distributed throughout the genome (Figure 5).

3.4. Phenotypic validation of target-enrichment sequencing results

To further validate the rigidity of our screening procedure and to ensure that the altered cercosporin phenotype was linked to the mutation identified through target-enrichment sequencing, we generated six independent mutant lines using RNAi approach. Six different genes were targeted to silence through RNAi (Table 3). Of the 6 genes, 2 were linked to no production of cercosporin, 1 linked to the reduced production of cercosporin and 3 with an increased production of cercosporin. RNAi lines created to target six genes showed similar results when compared to the forward genetic screen (Figure 8). All the RNAi lines targeting *CTB1* (CzmATMT2.277) showed a significant reduction in cercosporin production compared to the wild-type strain. Similarly, silencing of the *SNF5* (CzmRI6.333) gene showed no cercosporin production *in-vitro* confirming our phenotype from the forward genetic screen. Two RNAi strains each for overproducers CzmRI6.274 (mutation in aromatic ring hydroxylase) and CzmRI6.251 (*ERG5*) were analyzed for cercosporin production. One strain from each gene showed increased cercosporin production compared to the wild-type strain SCOH1-5. Strains created to knock down conserved hypothetical protein (CzmRI6.197) and scytalone dehydratase (CzmRI6.285) demonstrated inconsistent results compared to those observed in our forward genetic screen (Figure 7).

4. Discussion

Functional genomics is a powerful approach to identify gene function and regulation in filamentous fungi and oomycetes. Functional genomics studies have been made possible due to improved techniques available for genetic manipulation of fungi, development of efficient

transformation systems, and high-throughput methods for phenotyping (Michielse *et al.*, 2005). Additionally, reduced cost of genome sequencing has increased the availability of assembled genomes in the last few years, which has enhanced functional genomics studies in different fungal species. Although several approaches are available for studying gene function and regulation in filamentous fungi, they are only limited to model organisms. Nematophagous fungi are an important component of biological control of plant-parasitic nematodes. Their use in nematode management could reduce the use of harmful chemicals and provide a sustainable solution towards nematode management. Functional genomics research in nematophagous fungi, however, is hampered due to scarcity of genomic resources and availability of a tractable system. Application of functional genomics studies in nematophagous fungi could potentially augment the acceleration of nematode management strategies through identification of nematode parasitism genes, secondary metabolites or novel compounds toxic to plant-parasitic nematodes. However, development of functional genetics tools needs to be initially validated and verified in a well-established system prior to its potential use in nematophagous fungi. Hence, with the goal of eventually expanding our approach to ARF18, we used *Cercospora zeae-maydis* as a model system develop a high-throughput method to generate a large number of random mutants, screen for a phenotype and precisely identify the underlying mutations associated with the phenotype. Our approach can be easily adapted to other filamentous fungi like ARF18 which also has draft genome sequenced and assembled (Sharma *et al.*, 2017). High throughput nature of our method has widespread applications to discover novel genes regulating nematode parasitism in ARF18, enhance nematophagy, and develop ARF18 as an ideal and effective biological control of plant-parasitic nematodes. In our approach, we successfully identified the location of T-DNA insertions a few mutants. However, the method can be easily expanded to larger mutant

collections, which will enable the construction of genetic networks underlying the regulation of secondary metabolism or other biological processes such as nematode parasitism.

In our method, we were able to identify not only single insertions but also multiple insertions in the mutant strains. Target-enrichment sequencing identified up to four sites of insertions in the samples. Although the insertion numbers needs to be validated through PCR, our method is advantageous over currently used Southern hybridization in which one can only identify the number of copies of insertions but not the location. Additionally, multiple copy insertions are very difficult to resolve using the commonly used genome-walker PCR. Using the target-enrichment sequencing approach we identified both symmetric and non-symmetric insertions. Non-symmetric insertions are cases where only a single end of the cassette is mapped to the genome. This situation could arise due to several possibilities, including insertion-induced chromosomal rearrangements (Esher *et al.*, 2015). Additionally, edges of the T-DNA cassette can be truncated during integration events. Since our biotinylated probes only hybridize and capture 240-360 bases from the left and right border of the mutagenesis cassette, a truncation beyond 360 bases from either border will not be captured. Our method will also fail to detect any aberrant integration events arising out of insertion of a small piece of the mutagenesis cassette or insertion of the vector backbone in addition to T-DNA region, which is a rare but possible phenomenon.

Using the target–enrichment sequencing approach to a population of random mutants in *C. zeae-maydis*, we identified numerous genes potentially regulating cercosporin biosynthesis *in-vitro*. The results from the present work will provide useful insights into the regulation of cercosporin during pathogenicity. Currently, the production and regulation of cercosporin in *C. zeae-maydis* during the pathogenesis of corn is poorly understood. *C. zeae-maydis* is a

hemibiotrophic pathogen and its infection strategy involves a short biotrophic phase of 2-3 days followed by a switch to a necrotrophic stage (Kim *et al.*, 2011a). It has been previously demonstrated that *CTB1* is up-regulated during initial biotrophic phase and declines at the onset of lesions in corn (Bluhm *et al.*, 2008). The different genetic factors that control the expression of the CTB cluster and regulate cercosporin production at different stages of infection remain unknown. The genes identified from our genetic screen can provide valuable clues on the regulation of cercosporin especially during pathogenicity. The differential expression during different stages of pathogenesis suggests that the regulation is tightly under the control of chromatin regulation. Some of the most interesting candidate genes involved in chromatin modifications such as *SNF5*, *SET3*, genes containing WD40 repeats, bromodomain transcription factors, *SANT* domain-containing gene and Velvet domain-containing gene. Interestingly in our forward screen, disruption of the genes involved in chromatin modifications appears to have a similar effect on cercosporin production: either severely reduced in production, or completely abolished which demonstrates the tight regulation of cercosporin through chromatin modification. In *Neurospora crassa, SNF5* is recruited by *WC1* to regulate *FRQ* (frequency), which is involved in the circadian response. In *C. zea-maydis, CRP1,* the homolog of *WCI,* is required for cercosporin production and pathogenicity (Kim *et al.*, 2011b) and also regulates *FRQ* (Bluhm lab, data not shown). *C. zeae-maydis* penetrates the host through the stomata, which is the only known method in entry into the host. The infection through the stomata is regulated by light and is also linked to a circadian clock (Kim *et al.*, 2011b). *SNF5* could provide a link between *CRP1* and *FRQ* to regulate pathogenicity in *C. zeae-maydis. SANT* domain occurs in many ATP dependent chromatin-remodeling enzymes, histone acetyltransferase and deacetylases, and binds to histone tails (Boyer *et al.*, 2002, Boyer *et al.*, 2004) thereby regulating

chromatin accessibility. *SET3* is another chromatin modifying protein that is responsible for histone deacetylation (Kim & Buratowski, 2009), and performs numerous roles in gene regulation through chromatin modification. It is recruited by *SET1* to suppress nucleosome acetylation and remodeling (Kim & Buratowski, 2009). In *Magnaporthe oryzae, SET3,* along with *TIG1, SNT1, HOS2,* form a part of a Tig core complex, involved in infectious growth during rice pathogenicity (Ding *et al.*, 2010). Other genes of interests include *ERG5* and *SDH1,* which are both involved in cell wall development. *ERG5* is required for ergosterol synthesis, while *SDH1* is required for melanin biosynthesis. Interestingly mutations in both the genes lead to an overproduction in cercosporin. It would be interesting to see if as a result of overproduction in cercosporin also increases the virulence in these mutants. Since cercosporin is required to induce cell death in plants, we can hypothesize that these mutants would readily induce necrosis in plants bypassing the biotrophic phase. Additional genes that have not been previously characterized in filamentous fungi can provide novel insights into cercosporin regulation.

With slight modifications and optimizations, our approach could be easily adapted to other filamentous fungi and different mutagenesis cassette. The approach is an improvement over previously used PCR based methods (Gawronski *et al.*, 2009) or whole genome sequencing approach (Esher *et al.*, 2015) to identify the site of cassette integration. For the target-enrichment sequencing approach, we designed probes to span just the borders of the cassette. However, the method can be easily optimized to hybridize and enrich the cassette by synthesizing probes spanning the entire T-DNA/REMI cassette or the entire plasmid. The method could be adapted to accelerate the discovery of novel genes regulating different biological processes in filamentous fungi, including nematode parasitism in nematophagous fungi.

Nematophagous fungi are known to produce a plethora of metabolites. Among many these, linoleic acid is the most prevalent (Anke *et al.*, 1995). Important secondary metabolites produced by nematode-trapping ascomycetes include oligosporon, oligosporol A and oligosporol B, and arthrobotrisins in *Arthrobotrys oligospora* (Yang et al., 2011), flagranones in *A. flagrans*, and paganins from *Dactylellina entomopaga.* Metabolites secreted by cysts and eggs parasitizing nematophagous fungi include pochonins, monocillins, chlamydocin, paecilocin (Degenkolb & Vilcinskas, 2016). Many of these have been implicated in pathogenicity and toxicity of plant-parasitic nematodes (Li *et al.*, 2007). Given the diverse nature of secondary metabolites produced by different nematophagous fungi, exploring their regulation could open new avenues for efficient management of plant-parasitic nematodes. New compounds could potentially be isolated and screened for their efficacy against nematodes or novel strains of fungi created to control diverse species of plant-parasitic nematodes. However, functional genomics research in nematophagous fungi is very limited currently. Application of a forward genetic screen to identify mutants for increased pathogenicity and secondary metabolism combined with target-enrichment sequencing could augment existing nematode management strategies and accelerate their control. High-throughput methods and techniques need to be developed and tested in already established systems to replicate the research in nematophagous fungi. If successfully applied to fungal species like ARF18, our method could have a positive impact on nematode management.

References

Ahman, J., Johansson, T., Olsson, M., Punt, P.J., van den Hondel, C., and Tunlid, A. (2002) Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. *Applied and Environmental Microbiology* **68**: 3408-3415.

Anke, H., Stadler, M., Mayer, A., and Sterner, O. (1995) Secondary metabolites with nematocidal and antimicrobial activity from nematophagous fungi and ascomycetes. *Canadian Journal of Botany-Revue Canadienne De Botanique* **73**: S932-S939.

Atkins, S.D., Mauchline, T.H., Kerry, B.R., and Hirsch, P.R. (2004) Development of a transformation system for the nematophagous fungus Pochonia chlamydosporia. *Mycological Research* **108**: 654-661.

Bluhm, B.H., Dhillon, B., Lindquist, E.A., Kema, G.H.J., Goodwin, S.B., and Dunkle, L.D. (2008) Analyses of expressed sequence tags from the maize foliar pathogen Cercospora zeae-maydis identify novel genes expressed during vegetative, infectious, and reproductive growth. *Bmc Genomics* **9**.

Boyer, L.A., Langer, M.R., Crowley, K.A., Tan, S., Denu, J.M., and Peterson, C.L. (2002) Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Molecular Cell* **10**: 935-942.

Boyer, L.A., Latek, R.R., and Peterson, C.L. (2004) The SANT domain: a unique histone-tail-binding module? *Nature Reviews Molecular Cell Biology* **5**: 158-163.

Casselton, L., and Zolan, M. (2002) The art and design of genetic screens: Filamentous fungi. *Nature Reviews Genetics* **3**: 683-697.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. (2004) Cryptic speciation and host specificity among Mycosphaerella spp. occurring on Australian Acacia species grown as exotics in the tropics. *Studies in Mycology*: 457-469.

Daub, M.E. (1982) Cercosporin, a photosensitizing toxin from Cercospora species. *Phytopathology* **72**: 370-374.

Degenkolb, T., and Vilcinskas, A. (2016) Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes. *Applied Microbiology and Biotechnology* **100**: 3799-3812.

Dent, R.M., Haglund, C.M., Chin, B.L., Kobayashi, M.C., and Niyogi, K.K. (2005) Functional genomics of eukaryotic photosynthesis using insertional mutagenesis of Chlamydomonas reinhardtii. *Plant Physiology* **137**: 545-556.

Ding, S.L., Liu, W.D., Iliuk, A., Ribot, C., Vallet, J., Tao, A., Wang, Y., Lebrun, M.H., and Xu, J.R. (2010) The Tig1 Histone Deacetylase Complex Regulates Infectious Growth in the Rice Blast Fungus Magnaporthe oryzae. *Plant Cell* **22**: 2495-2508.

Dunkle, L.D., and Levy, M. (2000) Genetic relatedness of African and United States populations of Cercospora zeae-maydis. *Phytopathology* **90**: 486-490.

Esher, S.K., Granek, J.A., and Alspaugh, J.A. (2015) Rapid mapping of insertional mutations to probe cell wall regulation in Cryptococcus neoformans. *Fungal Genetics and Biology* **82**: 9-21.

Forsburg, S.L. (2001) The art and design of genetic screens: Yeast. *Nature Reviews Genetics* **2**: 659-668.

Gawronski, J.D., Wong, S.M.S., Giannoukos, G., Ward, D.V., and Akerley, B.J. (2009) Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 16422-16427.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**: 343-U341.

Gonzalez-Ballester, D., de Montaigu, A., Galvan, A., and Fernandez, E. (2005) Restriction enzyme site-directed amplification PCR: A tool to identify regions flanking a marker DNA. *Analytical Biochemistry* **340**: 330-335.

Goodwin, S.B., Dunkle, L.D., and Zismann, V.L. (2001) Phylogenetic analysis of Cercospora and Mycosphaerella based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology* **91**: 648-658.

Holt, C., and Yandell, M. (2011) MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects. *Bmc Bioinformatics* **12**.

Inagaki, S., Henry, I.M., Lieberman, M.C., and Comai, L. (2015) High-Throughput Analysis of T-DNA Location and Structure Using Sequence Capture. *Plos One* **10**.

Jeon, J., Choi, J., Park, J., and Lee, Y.H. (2008) Functional genomics in the rice blast fungus to unravel the fungal pathogenicity. *Journal of Zhejiang University-Science B* **9**: 747-752.

Kamoun, S. (2003) Molecular genetics of pathogenic Oomycetes. *Eukaryotic Cell* **2**: 191-199.

Kile, B.T., and Hilton, D.J. (2005) The art and design of genetic screens: Mouse. *Nature Reviews Genetics* **6**: 557-567.

Kim, H., Ridenour, J.B., Dunkle, L.D., and Bluhm, B.H. (2011a) Regulation of Pathogenesis by Light in Cercospora zeae-maydis: An Updated Perspective. *Plant Pathology Journal* **27**: 103-109.

Kim, H., Ridenour, J.B., Dunkle, L.D., and Bluhm, B.H. (2011b) Regulation of Stomatal Tropism and Infection by Light in Cercospora zeae-maydis: Evidence for Coordinated Host/Pathogen Responses to Photoperiod? *Plos Pathogens* **7**.

Kim, T., and Buratowski, S. (2009) Dimethylation of H3K4 by Set1 Recruits the Set3 Histone Deacetylase Complex to 5 ' Transcribed Regions. *Cell* **137**: 259-272.

Lazo, G.R., Stein, P.A., and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio-Technology* **9**: 963-967.

Lepage, E., Zampini, E., Boyle, B., and Brisson, N. (2013) Time- and Cost-Efficient Identification of T-DNA Insertion Sites through Targeted Genomic Sequencing. *Plos One* **8**.

Li, G., Zhang, K., Xu, J., Dong, J., and Liu, Y. (2007) Nematicidal substances from fungi. *Recent Patents on Biotechnology* **1**: 212-233.

Li, H., and Durbin, R. (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**: 589-595.

Li, X.B., Moellering, E.R., Liu, B.S., Johnny, C., Fedewa, M., Sears, B.B., Kuo, M.H., and Benning, C. (2012) A Galactoglycerolipid Lipase Is Required for Triacylglycerol Accumulation and Survival Following Nitrogen Deprivation in Chlamydomonas reinhardtii. *Plant Cell* **24**: 4670-4686.

Lousberg, R.J., Weiss, U., Salemink, C.A., Arnone, A., Merlini, L., and Nasini, G. (1971) Structure of cercosporin, a naturally occurring quinone. *Journal of the Chemical Society D-Chemical Communications*: 1463-1464.

Mercer, T.R., Clark, M.B., Crawford, J., Brunck, M.E., Gerhardt, D.J., Taft, R.J., Nielsen, L.K., Dinger, M.E., and Mattick, J.S. (2014) Targeted sequencing for gene discovery and quantification using RNA CaptureSeq. *Nature Protocols* **9**: 989-1009.

Mertes, F., ElSharawy, A., Sauer, S., van Helvoort, J.M.L.M., van der Zaag, P.J., Franke, A., Nilsson, M., Lehrach, H., and Brookes, A.J. (2011) Targeted enrichment of genomic DNA regions for next-generation sequencing. *Briefings in Functional Genomics* **10**: 374-386.

Michielse, C.B., Hooykaas, P.J.J., van den Hondel, C., and Ram, A.F.J. (2005) Agrobacterium-mediated transformation as a tool for functional genomics in fungi. *Current Genetics* **48**: 1-17.

Motoyama, T., Imanishi, K., and Yamaguchi, I. (1998) cDNA cloning, expression, and mutagenesis of scytalone dehydratase needed for pathogenicity of the rice blast fungus, Pyricularia oryzae. *Bioscience Biotechnology and Biochemistry* **62**: 564-566.

Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M., and Kang, S. (2001) Agrobacterium-mediated transformation of Fusarium oxysporum: An efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* **91**: 173-180.

Nakayashiki, H., Hanada, S., Quoc, N.B., Kadotani, N., Tosa, Y., and Mayama, S. (2005) RNA silencing as a tool for exploring gene function in ascomycete fungi. *Fungal Genetics and Biology* **42**: 275-283.

O'Meara, T.R., Veri, A.O., Ketela, T., Jiang, B., Roemer, T., and Cowen, L.E. (2015) Global analysis of fungal morphology exposes mechanisms of host cell escape. *Nature Communications* **6**.

Patton, E.E., and Zon, L.I. (2001) The art and design of genetic screens: Zebrafish. *Nature Reviews Genetics* **2**: 956-966.

Penalba, J.V., Smith, L.L., Tonione, M.A., Sass, C., Hykin, S.M., Skipwith, P.L., McGuire, J.A., Bowie, R.C.K., and Moritz, C. (2014) Sequence capture using PCR-generated probes: a cost-effective method of targeted high-throughput sequencing for nonmodel organisms. *Molecular Ecology Resources* **14**: 1000-1010.

Pfannenstiel, B.T., Zhao, X.X., Wortman, J., Wiemann, P., Throckmorton, K., Spraker, J.E., Soukup, A.A., Luo, X.Y., Lindner, D.L., Lim, F.Y., Knox, B.P., Haas, B., Fischer, G.J., Choera, T., Butchko, R.A.E., Bok, J.W., Affeldt, K.J., Keller, N.P., and Palmer, J.M. (2017) Revitalization of a Forward Genetic Screen Identifies Three New Regulators of Fungal Secondary Metabolism in the Genus Aspergillus. *Mbio* **8**.

Reilly, M.C., Kim, J., Lynn, J., Simmons, B.A., Gladden, J.M., Magnuson, J.K., and Baker, S.E. (2018) Forward genetics screen coupled with whole-genome resequencing identifies novel gene targets for improving heterologous enzyme production in Aspergillus niger. *Applied Microbiology and Biotechnology*.

Ridenour, J., Hirsch, R., and Bluhm, B., (2012) Identifying Genes in Fusarium verticillioides Through Forward and Reverse Genetics. In: Plant Fungal Pathogens. Springer, pp. 457-479.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011) Integrative genomics viewer. *Nature Biotechnology* **29**: 24-26.

Sauday, S. (2010) The role of Stl1p in glycerol accumulation in osmotically stressed icewine yeast Saccharomyces cerevisiae K1 V1116.

Schmitt, M.W., Fox, E.J., Prindle, M.J., Reid-Bayliss, K.S., True, L.D., Radich, J.P., and Loeb, L.A. (2015) Sequencing small genomic targets with high efficiency and extreme accuracy. *Nature Methods* **12**: 423-+.

Sharma, S., Ridenour, J.B., Zaccaron, A., and Bluhm, B. (2017) Genome analyses of a novel, nematophagous fungus in the genus Brachyphoris provides clues on its potential use as biocontrol. *Phytopathology* **107**: 10.

Shen, B., Xiao, J., Dai, L., Huang, Y., Mao, Z., Lin, R., Yao, Y., and Xie, B. (2015) Development of a high-efficiency gene knockout system for Pochonia chlamydosporia. *Microbiological Research* **170**: 18-26.

Shim, W.B., and Dunkle, L.D. (2002) Identification of genes expressed during cercosporin biosynthesis in Cercospora zeae-maydis. *Physiological and Molecular Plant Pathology* **61**: 237-248.

Tam, L.W., and Lefebvre, P.A. (1993) Cloning of flagellar genes in Chlamydomonas-reinhardtii by DNA insertional mutagenesis. *Genetics* **135**: 375-384.

Ward, J.M.J., Stromberg, E.L., Nowell, D.C., and Nutter, F.W. (1999) Gray leaf spot - A disease of global importance in maize production. *Plant Disease* **83**: 884-895.

Williams-Carrier, R., Stiffler, N., Belcher, S., Kroeger, T., Stern, D.B., Monde, R.-A., Coalter, R., and Barkan, A. (2010) Use of Illumina sequencing to identify transposon insertions underlying mutant phenotypes in high-copy Mutator lines of maize. *Plant Journal* **63**: 167-177.

Xu, J., Mo, M.H., Zhou, W., Huang, X.W., and Zhang, K.Q. (2005) Transformation and mutagenesis of the nematode-trapping fungus Monacrosporium sphaeroides by restriction enzyme-mediated integration (REMI). *Journal of Microbiology* **43**: 417-423.

Yang, J., Wang, L., Ji, X., Feng, Y., Li, X., Zou, C., Xu, J., Ren, Y., Mi, Q., Wu, J., Liu, S., Liu, Y., Huang, X., Wang, H., Niu, X., Li, J., Liang, L., Luo, Y., Ji, K., Zhou, W., Yu, Z., Li, G., Liu, Y., Li, L., Qiao, M., Feng, L., and Zhang, K.-Q. (2011) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. *Plos Pathogens* **7**.

Yu, J.H., and Keller, N. (2005) Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437-458.

Zaccaron, M., Sharma, S., and Bluhm, B.H. (2018) MoNSTR-seq, a restriction site-associated DNA sequencing technique to characterize Agrobacterium-mediated transfer-DNA insertions in Phomopsis longicolla. *Letters in Applied Microbiology* **66**: 19-24.

Zhang, R., Patena, W., Armbruster, U., Gang, S.S., Blum, S.R., and Jonikas, M.C. (2014) High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. *Plant Cell* **26**: 1398-1409.

Figures and tables legend

Figure 1. Flowchart followed for the target capture sequencing. Special biotinylated probes were designed to span the edge of the mutagenesis cassette. Pooled libraries were hybridized with the biotinylated probes followed by binding with streptavidin bound magnetic beads (1). The streptavidin-biotin conjugate were pulled down with magnetic beads (2). Final PCR was performed to selectively amplify the reads enriched for cassette-genome break junction (3). The libraries were amplified on the Ion Torrent PGM platform (4). Reads were mapped onto the cassette and then to the SCOH1-5 reference genome assembly to identify the site/s of insertion/s of the mutagenesis cassette.

Figure 2. Screening of mutants on 0.2✕strength PDA at constant light at A-seven days after inoculation and B- 10 days after inoculation. 1: WT, 2 and 3: mutants over-producing cercosporin, and 4: mutant not producing cercosporin, 5: mutant with severe reduction in cercosporin and 6: mutant with reduced cercosporin.

Figure 3. One mutant identified in the forward genetic screen- named CzmRI.6.251 produced cercosporin constitutively, even in the dark. Figure shows the mutant producing cercosporin in V8 agar in dark, 6 days after padding (A), while the wild type *Cercospora zeae-maydis* strain (SCOH1-5) does not (B). Sequencing results show that the mutant strain had the cassette inserted in putative Scytalone dehydratase (*SDH1*) ortholog, which is involved in the production of melanin in many fungal species, including *Magnaporthe oryzae*.

Figure 4. Figure showing two different kinds of insertion identified in the genetic screens- asymmetric and symmetric.

Figure 5. A total of 109 insertions across the 81 individuals have been plotted on the *C. zeae-maydis* scaffolds. Insertions are represented by triangles color coded by the screen. Mutants in screen 1,2 were obtained via PEG-mediated transformation and screens 3,4 were generated using *Agrobacterium*-mediated transformation. Scaffolds numbers are shown inside the empty bars. Blue and black highlights on the scaffolds represent genes and repeats, respectively.

Figure 6. Different classes of genes identified in the screen that putatively regulate cercosporin production.

Figure 7. Quantification of cercosporin levels in different RNAi lines. 10^5 spores from actively growing cultures were plated on to 0.2x strength PDA plates and incubated in constant white light for 7 days. Cercosporin was with 5N KOH, and was quantified by measuring the absorbance at 480 nm. The OD values were converted into concentrations using the formula A= εmcl. The molar extinction coefficient for cercosporin is 23,300 m^{-1} cm^{-1}. Means with same letters do not differ significantly at $P \leq 0.05$ according to Tukey's HSD test.

Figure 8. RNAi lines showing different levels of cercosporin production.

Table 1. Table depicting the number of mutants identified from the screens and the number of insertions found in individual mutants.

Table 2. Table depicting the types of insertions identified in different mutants. Asymmetric insertion is one where in only one border of the mutation cassette was identified, while symmetric mutation is the one where in both the borders of the mutation cassette was identified in the mutants.

Table 3. Table depicting the genes that were selected for validation of the screen along with the putative function of the genes, the phenotype of the mutation, and the construct used to transform the RNAi line.

Table 4. List of primers used for generating RNA silencing constructs.

Table 5. List of Mutants identified via probe-capture technique, with their respective phenotype, number/s and site/s of insertion, position- upstream or downstream with distance from the start codon, and the putative gene/s associated or disrupted.

Figures and tables

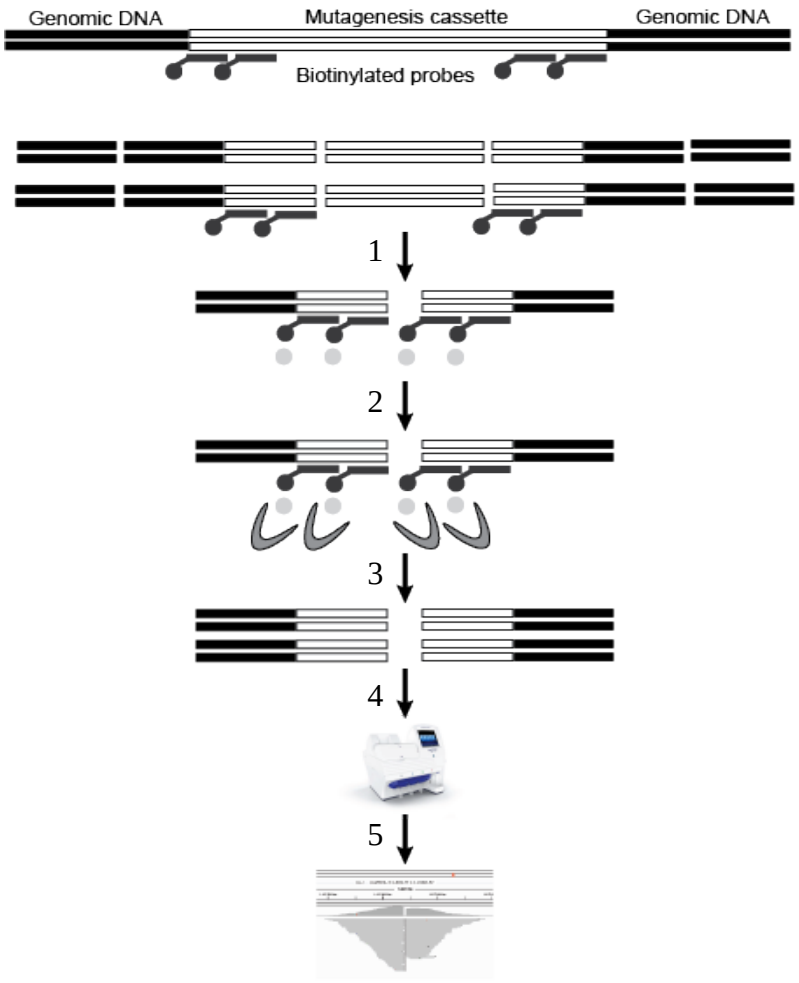


Figure 1

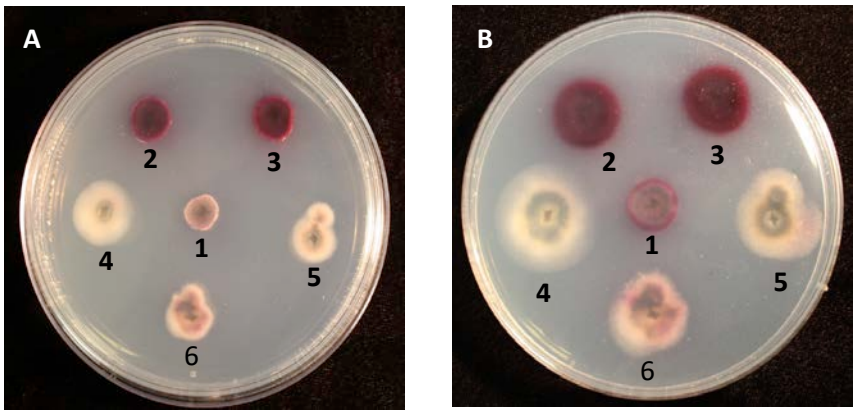


Figure 2

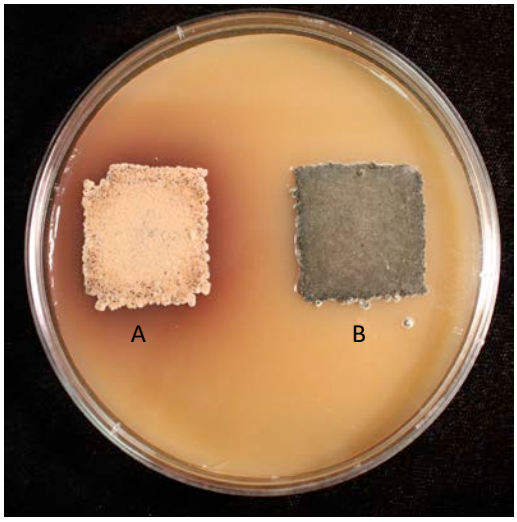


Figure 3

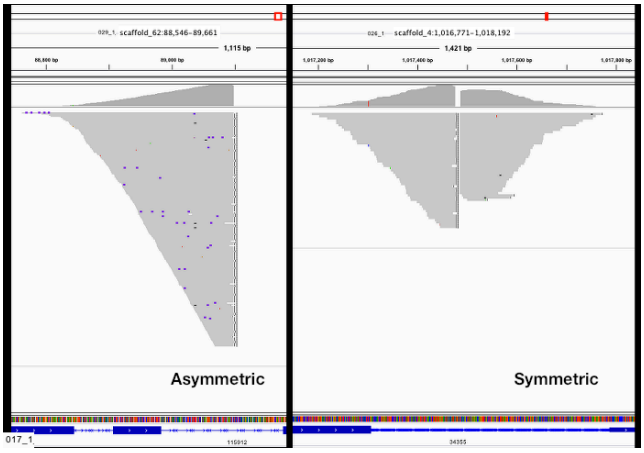


Figure 4

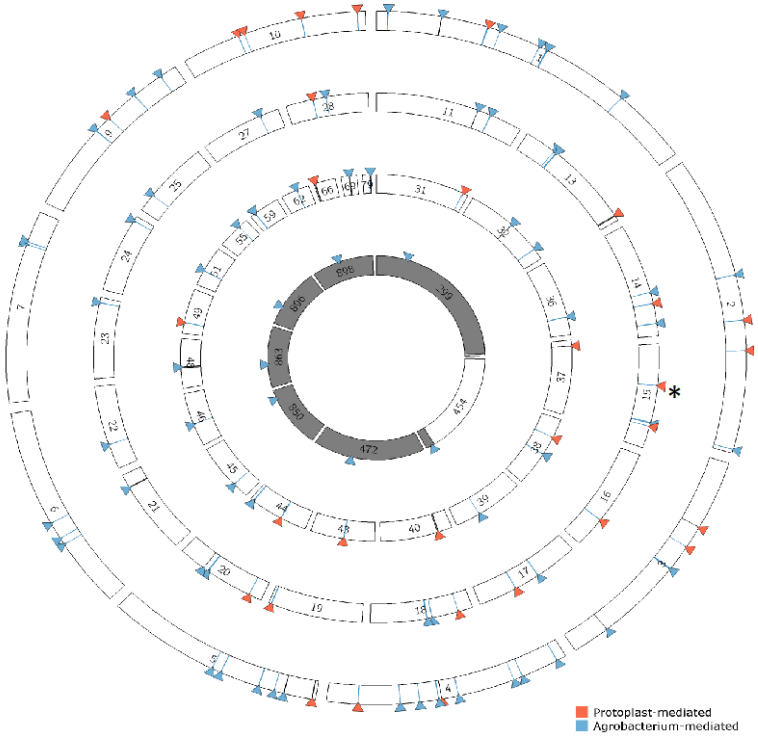


Figure 5

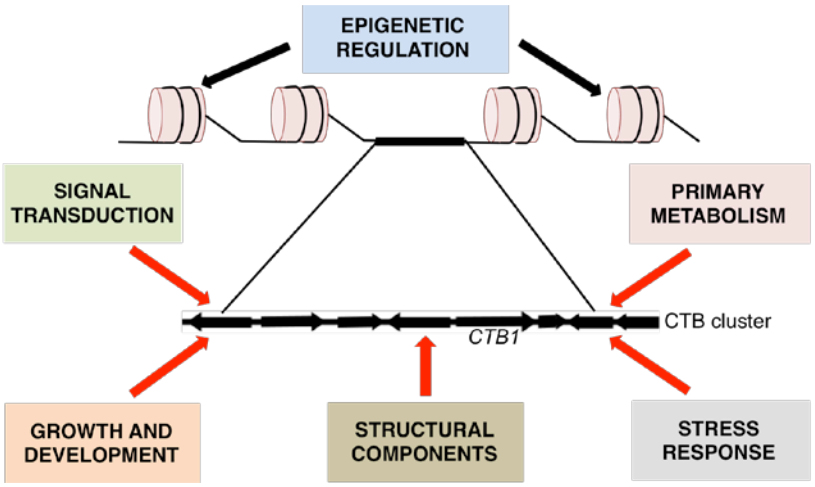


Figure 6

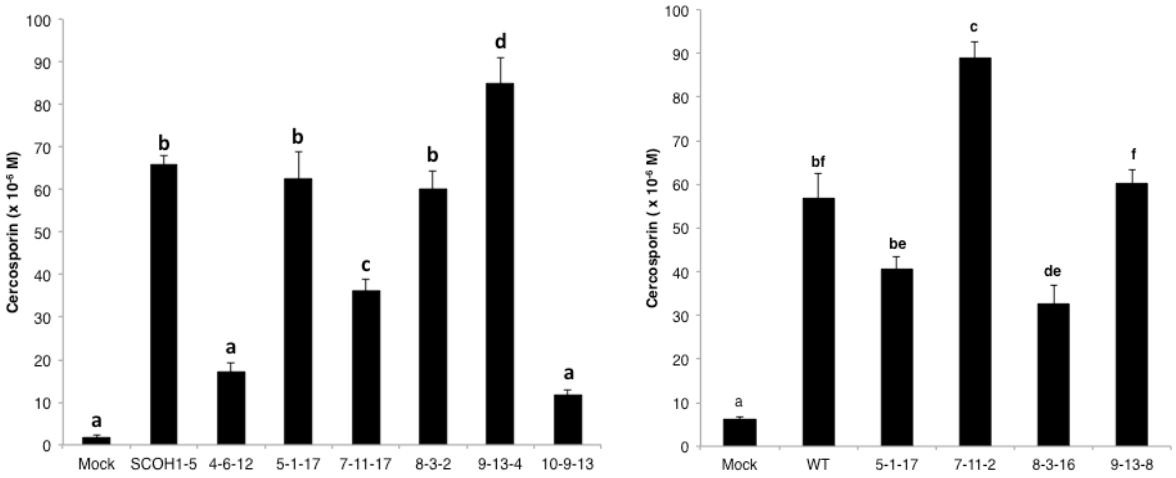


Figure 7

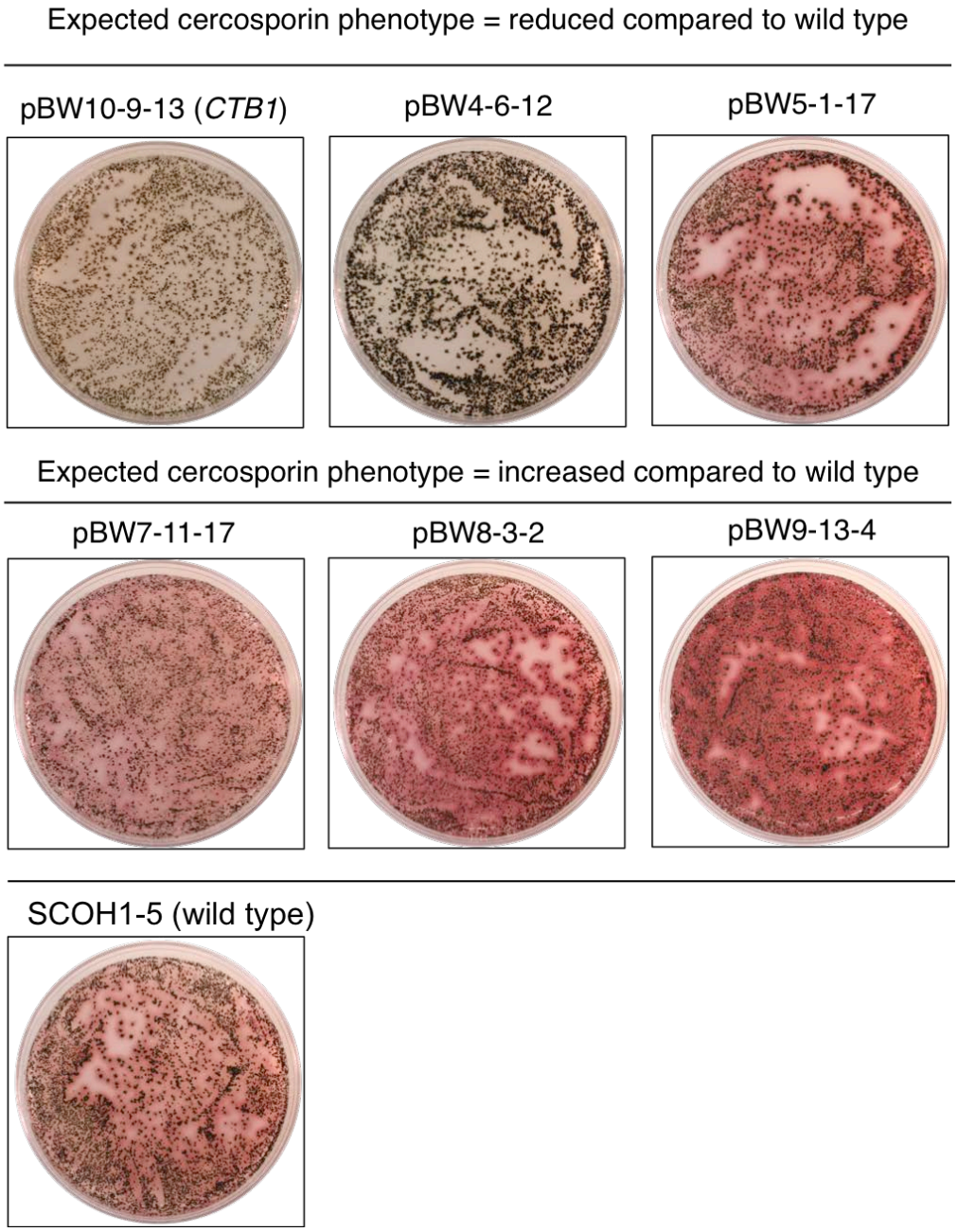

Expected cercosporin phenotype = reduced compared to wild type
pBW10-9-13 (*CTB1*)
pBW4-6-12
pBW5-1-17
Expected cercosporin phenotype = increased compared to wild type
pBW7-11-17
pBW8-3-2
pBW9-13-4
SCOH1-5 (wild type)

Figure 8

Table 1

	Single	Multiple	No Data	Total
Protoplast-mediated	22	4	3	29
Agrobacterium-mediated	27	22	3	52
Total	49	26	6	**81**

Table 2

	Asymmetric	Symmetric	Total
Protoplast-mediated	16	15	31
Agrobacterium-mediated	38	40	78
Total	54	55	**109**

Table 3

Mutant	Cercosporin phenotype	Putative function	Silencing vector
CZMATMT2.277	No production	*CTB1*	pBW10-9-13
CZMRI6.333	No production	Chromatin modification	pBW4-6-12
CzmRI6.197	Reduced production	Conserved hypothetical	pBW5-1-17
CZMRI6.274	Increased production	Aromatic ring hydroxylase	pBW7-11-17
CZMRI6.285	Increased production	Melanin biosynthesis	pBW8-3-2
CZMRI6.251	Increased production	Ergosterol biosynthesis	pBW9-13-4

Table 4

Primer name	Sequence
pSilent F1	TACGAATTCGAGCTCGGTACCCGCTGCAGGAATTCATGCCAGTT
pSilent R1	GGTCGACTCTAGAGGATCCCCGCGTAATACGACTCACTATAGGGC GAATTG
pS Intron F1	GTACAAGCTTGCTGGAGGATACA
pS Intron R1	CCTTAGCATGCGAAGATCTGCC
Czm_60071 SF1	CGATACCGTCGACCTCGAGGTACAGCTCCAGAACTACTACCCGCA
Czm_60071 SR1	TGTATCCTCCAGCAAGCTTGTACACGGTTCAGCTGTCGTTTCT
Czm_60071 ASF1	GGCAGATCTTCGCATGCTAAGGACGGTTCAGCTGTCGTTTCT
Czm_60071 ASR1	ATCCGGGGCCCAGGTACCACAGGAGCTCCAGAACTACTACCCGC A
Czm_11389 SF1	CGATACCGTCGACCTCGAGGTACTAGCACGCATGACCAGCTAC
Czm_11389 SR1	TGTATCCTCCAGCAAGCTTGTACGCTCTCGTCGCAATTCTACC
Czm_11389 ASF1	GGCAGATCTTCGCATGCTAAGGGCTCTCGTCGCAATTCTACC
Czm_11389 ASR1	ATCCGGGGCCCAGGTACCACAGGTAGCACGCATGACCAGCTAC
Czm_116247 SF1	CGATACCGTCGACCTCGAGGTACTCGTCGACAGACAAGGACTG
Czm_116247 SR1	TGTATCCTCCAGCAAGCTTGTACCGACACCGTAACCTGATGTG
Czm_116247 ASF1	GGCAGATCTTCGCATGCTAAGGCGACACCGTAACCTGATGTG
Czm_116247 ASR1	ATCCGGGGCCCAGGTACCACAGGTCGTCGACAGACAAGGACTG
Czm_91826 SF1	CGATACCGTCGACCTCGAGGTACCAGCAAAGACTGGGAACGTC
Czm_91826 SR1	TGTATCCTCCAGCAAGCTTGTACATCGGATGTCAGGGTTCAAG
Czm_91826 ASF1	GGCAGATCTTCGCATGCTAAGGATCGGATGTCAGGGTTCAAG
Czm_91826 ASR1	ATCCGGGGCCCAGGTACCACAGGCAGCAAAGACTGGGAACGTC
Czm_96848 SF1	CGATACCGTCGACCTCGAGGTACGTGCTGTATCGTGGCTGCTA
Czm_96848 SR1	TGTATCCTCCAGCAAGCTTGTACTGGTGGCAAACACCTTGATA

Table 4 (cont.)

Primer name	Sequence
Czm_96848 ASF1	GGCAGATCTTCGCATGCTAAGGTGGTGGCAAACACCTTGATA
Czm_96848 ASR1	ATCCGGGGCCCAGGTACCACAGGGTGCTGTATCGTGGCTGCTA
Czm_42949 SF1	CGATACCGTCGACCTCGAGGTACCCAGTCAACTGTGCGGACTA
Czm_42949 SR1	TGTATCCTCCAGCAAGCTTGTACGCAAGCGACTCGTAATGACA
Czm_42949 ASF1	GGCAGATCTTCGCATGCTAAGGGCAAGCGACTCGTAATGACA
Czm_42949 ASR1	ATCCGGGGCCCAGGTACCACAGGCCAGTCAACTGTGCGGACTA

Table 5

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
Mutants created via PEG mediated transformation of protoplasts							
1	CzmRI.6.3	None	1	Scaffold 10: 1192340-1192783	Up	152	SANT, DNA Binding, WD40 repeat
2	CzmRI.6.16	None	1	Scaffold 31: 491925-492465	Up	819	Conserved hypothetical protein (Glucoamylase precurssor)
3	CzmRI.6.115	None	1	Scaffold 10: 435833-436071	Up	155	Actin depolymerizing factor
4	CzmRI.6.151	None	1	Scaffold 4: 867952-868617	Down	125	WD40 repeat
5	CzmRI.6.190	None	1	Scaffold 43: 172081-172792	In	0	Bromodomain transcription factor
6	CzmRI.6.329	None	1	Scaffold 40: 82824-83113	In	0	Repeat
7	CzmRI.6.333	None	1	Scaffold 16: 597700-598002	In	0	SNF5/SMARCB1/INI1
8	CzmRI.6.117	Reduced	1	Scaffold 5: 22430-23126	In	0	Pleckstrin-like
9	CzmRI.6.178	Reduced	1	Scaffold 44: 148146-148498	In	0	UTP-glucose-1-phosphate uridylyltransferase
10	CzmRI.6.197	Reduced	1	Scaffold 38: 112986-113566	In	0	Conserved hypothetical protein
11	CzmRI.6.274	Increased	1	Scaffold 2: 1183105-1183636	Up	228	Phenol 2-monooxygenase
12	CzmRI.6.285	Increased	1	Scaffold 44: 328208-328787	In	0	Scytalone dehydratase
13	AT773	Reduced	1	Scaffold 14: 659173-659174	Up	286	HSP (DnaJ domain)
14	AT515	Reduced	1	Scaffold_19: 699717-700014	In	0	Vacuolar protein sorting-associated protein 35
15	AT1164	Reduced	1	Scaffold_66:6821-6822	In	0	Repeat
16	AT1371	Reduced	1	Scaffold 3: 650164-651218	Down	176	Short chain dehydrogenase
17	AT1398	Reduced	1	Scaffold 1: 742529-743075	In	0	Histone Binding SET3 domain

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
18	AT1159	Reduced	1	Scaffold 20: 98847-99153	In	0	Acyl-CoA transferase/carnitine dehydratase
19	AT669	Reduced	1	Scaffold 17: 532061- 532432	In	0	Receptor-activated Ca^{2+}-permeable cation channel
20	AT1193	Reduced	1	Scaffold 28: 206763- 207148	Down	784	Only in Capnodiales/Dothideomycetes
21	AT873	Reduced	1	Scaffold 15: 663338-663339	In	0	GA4 desaturase family
22	CzmRI.6.251	Increased	1	Scaffold 10: 822903-832674	Up	277	ERG5 sterol C-22 desaturase
23	CzmRI.6.230	Reduced	2	Scaffold 37: 33593-34375 Scaffold 49: 61969-62603	Down In	308 0	Repeat Nucleotidyltransferase
24	AT1368	Reduced	2	Scaffold_16:666616-666794 Scaffold_40:279220-279339			
25	AT1108	Reduced	2	Scaffold_9:724186-724248 Scaffold_10:394699-394734	Up Down	243 314	No BLAST hit Putative mannosyl transferase
26	AT1289	Reduced	2	Scaffold_40:310097-310276 Scaffold_56:145647-145704			
27	AT1200	Reduced	2	Scaffold_4:1422157-1422158 Scaffold_13:928786-928787	Down Up	559 322	F1F0 ATP synthase subunit g Repeat
28	AT1337	Reduced	2	Scaffold_29:354778-354851 Scaffold_30:308570-308641			
29	CzmRI.6.91	None	3	Scaffold 3: 489920-490201 Scaffold 15: 331676-332251 Scaffold 18: 111283-111568	Up Up Up	94 1215 708	CAS/CSE, C-terminal Cyclic nucleotide binding Protein with VHS domain

Table 5 (cont.)

Mutants created via *Agrobacterium tumefaciens* mediated transformation							
No	**Mutant Id**	**Cercosporin Production**	**Number**	**Site/s of Insertion**	**Position**	**Distance**	**Putative gene/s associated or disrupted**
30	CzmATMT2.175	None	1	Scaffold_299:994-995	In	0	Repeat
31	CzmATMT2.177	None	1	Scaffold_454:3606-3607	In	0	Repeat
32	CzmATMT2.258	None	1	Scaffold_4:317795-317799	Up	174	Alcohol dehydrogenase, NAD(P)-binding
33	CzmATMT2.277	None	1	Scaffold_15:638449-638461	In	0	Putative *CTB1*
34	CzmATMT5.56	None	1	Scaffold_4:757673-757696	Up	427	Transferase
35	CzmATMT5.86	None	1	Scaffold_15:720954-721434			BRCT
36	CzmATMT5.134	None	1	Scaffold_31:200227-200508			Repeat
37	CzmATMT7.75	None	1	Scaffold_472:2399-2400	In	0	Repeat
38	CzmATMT8.39	None	1	Scaffold_3:1427485-1427497	Down	122	Repeat
39	CzmATMT8.40	None	1	Scaffold_5:714453-714454	In	0	Salicylate hydroxylase
40	CzmATMT8.83	None	1	Scaffold_55:138446-138464	Down	638	No BLAST hit
41	CzmATMT4.105	None	1	Scaffold_1:1104823-1104841	Up	243	Major facilitator superfamily (MFS1)
42	CzmATMT4.152	None	1	Scaffold_14:819105-819106	Down	178	No BLAST hit

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
43	CzmATMT4.154	None	1	Scaffold_32:454222-454234	Up	590	Hat2p/WD40 repeat
44	CzmATMT4.155	None	1	Scaffold_32:454222-454234	Up	590	Hat2p/WD40 repeat
45	CzmATMT4.170	None	1	Scaffold_4:379148-379168	Up	365	Protein of unknown function/DUF 250
46	CzmATMT4.199	None	1	Scaffold_20:460450-460465	Down	1128	HAD-superfamily hydrolase, subfamily IIIA; Polynucleotide kinase 3 phosphatase, central region; DNA 3-phosphatase
47	CzmATMT4.207	None	1	Scaffold_4:1146925-1146942	Up	254	Reticulon
48	CzmATMT4.232	None	1	Scaffold_1:1750322-1750323	In	0	No BLAST hit
49	CzmATMT4.238	None	1	Scaffold_1:1750322-1750323	In	0	No BLAST hit
50	CzmATMT5.79	None	1	Scaffold_48:125683-125708	In	0	Repeat
51	CzmATMT5.87	None	1	Scaffold_27:480309-482658	In	0	VeA domain containing protein
52	CzmATMT7.52	None	1	Scaffold_1:77388-77389	Up	991	SANT DNA-binding, Homeodomain-like
53	CzmATMT7.59	None	1	Scaffold_51:90258-90265	Up	287	Glucose-inactivated glycerol proton symporter STL1
54	CzmATMT7.110	None	1	Scaffold_1:432188-432189	In	0	Repeat

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
55	CzmATMT8.105	None	1	Scaffold_6:533342-533343	Up	55	H (+)-transporting V1 sector ATPase subunit C
56	CzmATMT8.135	None	1	Scaffold_5:279672-279687	In	0	No BLAST hit
57	CzmATMT8.143	None	1	Scaffold_5:211305-211306	Up	43	Repeat
58	CzmATMT8.145	None	1	Scaffold_9:955632-955646	Down	386	No BLAST hit
59	CzmATMT5.116	None	3	Scaffold_850:1858-1873 Scaffold_18:313231-313232 Scaffold_896:626-627	In In In	0 0 0	No BLAST hit Repeat Repeat
60	CzmATMT8.25	None	2	Scaffold_69:46684-46685 Scaffold_9:1172977-1172978	In In	0 0	Repeat Repeat
61	CzmATMT8.62	None	2	Scaffold_4:888735-888736 Scaffold_14:566698-566699	In In	0 0	Carboxylestrase Type B No BLAST hit
62	CzmATMT2.229	None	2	Scaffold_7:1143368-1143369 Scaffold_7:1126877-1126878	In Up	0 1265	No BLAST hit FAD dependent pyridine nucleotide-disulfide oxidoreductase
63	CzmATMT2.235	None	2	Scaffold_11:777209-777253 Scaffold_79:43666-43667	In In	0 0	Repeat Repeat
64	CzmATMT4.80	None	2	Scaffold_5:650829-650830 Scaffold_4:65331-65332	In In	0 0	Acetyltransferase 3 Sepiapterin reductase family protein IRC24

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
65	CzmATMT4.180	None	2	Scaffold_2:2095968-2095969 Scaffold_6:654411-654421	Down Down	155 660	No BLAST hit No BLAST hit
66	CzmATMT5.21	None	2	Scaffold_3:833095-839426 Scaffold_17:335797-335798	In In	0 0	Ketoreductase Karyopherin MSN5
67	CzmATMT5.116	None	2	Scaffold_18:313231-313232 Scaffold_18:351367-351368	In In	0 0	Engulfment and cell motility, Armadillo-type Putative DNA/RNA helicase SEN1
68	CzmATMT7.70	None	2	Scaffold_38:225335-225336 Scaffold_46:134094-134095	Down Down	556 192	Pex11p/ Peroxisomal biogenesis factor No BLAST hit
69	CzmATMT8.28	None	2	Scaffold_1:824467-824472 Scaffold_45:93772-93773	In Up	0 468	Peptidylprolyl isomerase CPR3 No BLAST hit
70	CzmATMT8.30	None	2	Scaffold_898:911-917 Scaffold_4:1017476-1017486	In Down	0 170	Repeat No BLAST hit
71	CzmATMT8.82	None	2	Scaffold_11:877320-877321 Scaffold_11:878511-878512	Down Up	235 238	No BLAST hit Serine/threonine-protein kinase GCN2
72	CzmATMT8.123	None	2	Scaffold_6:484723-484724 Scaffold_62:89097-89098	Up Up	333 79	Spermine transporter No BLAST hit
73	CzmATMT2.209	None	2	Scaffold_20:456957-456962 Scaffold_39:266354-266729	Down Down	438 352	EGF-like region, conserved site. Tetratricopeptide repeat 11

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
74	CzmATMT2.240	None	2	Scaffold_59:30472-30473 Scaffold_59:33186-33187	In In	0 0	GroES-like protein NAD(P)-binding Heterokaryon incompatibility
75	CzmATMT5.77	None	2	Scaffold_2:863680-863681 Scaffold_ 32:264267-264268	Up Down	257 14	DENN-domain protein (regulator of RabGTPase) Repeat
76	CzmATMT5.93	None	2	Scaffold_20:501689-501691 Scaffold_21:580005-580006	Down In	701 0	Repeat Repeat
77	CzmATMT2.293	None	3	Scaffold_28:306476-306479 Scaffold_5:384175-384184 Scaffold_1:1156496-1163787	Up Up In	1713 403 0	Regulator of G-protein signaling Ubiquitin-binding SDF ubiquitin ligase complex subunit MET30 MFS general substrate transporter
78	CzmATMT4.235	None	3	Scaffold_13:242737-242746 Scaffold_13:265402-265403 Scaffold_13:267226-267227	Down Down In	673 623 0	No BLAST hit Calmodulin-dependent protein kinase CMK2 Beta-fructofuranosidase SUC2
79	CzmATMT8.71	None	4	Scaffold_ 23:612911-612921 Scaffold_25:146934-146954 Scaffold_44:329942-329943 Scaffold_863:818-819	In Up Up In	0 1647 124 0	Major facilitator superfamily MFS1 No BLAST hit No BLAST hit R=6

Table 5 (cont.)

No	Mutant Id	Cercosporin Production	Number	Site/s of Insertion	Position	Distance	Putative gene/s associated or disrupted
80	CzmATMT7.51	None	4	Scaffold_9:606120-606121 Scaffold_22:203809-203810 Scaffold_24:596941-597337 Scaffold_36:340626-340627	Up Up Up Up	728 217 66 5	Major Facilitator Superfamily Anp1p SANT DNA domain, Homeodomain Serine/threonine-protein kinase GCN2

CONCLUSIONS

Despite the importance of the economic losses caused due to plant parasitic nematodes on crop plants (Elling, 2013), an effective yet environmentally friendly strategy to control their population is lacking. Alternative methods for effective control are in the development phase and but their application in agricultural settings have certain limitations (Banerjee *et al.*, 2017). Harpin proteins are of bacterial origin (Wei *et al.*, 1992), and have been widely used to induce plant defense against different biotic and biotic factors (Dong *et al.*, 2004, Dong *et al.*, 1999, Dong *et al.*, 2005, Reboutier *et al.*, 2007). Although they are effective in inducing resistance against variety of biotrophic and necrotrophic pathogens, their role against plant parasitic nematodes is understudied. Previous field experiments have established that harpins are capable of reducing nematode populations in soil. Additionally, a fungal isolate, designated as ARF18, was identified that was capable of suppressing reniform and cyst nematode population in the soil. Considering the economic importance of plant parasitic nematodes, harpin proteins and ARF18 have the potential to be effective alternate strategies to curb nematode populations in soil. The overall objective of the dissertation was to establish harpin and ARF18 as control mechanisms of plant parasitic nematode. The study aimed to identify the efficacy of harpin proteins in suppressing the population of reniform and cyst nematodes in soil. Additionally, we also provided a taxonomic placement for the nematophagous fungal isolate ARF18, and sequenced and analyzed its genome to identify its potential as a biological control.

The efficacy of harpin against the reniform and cyst nematodes were studied and described in chapter two. Green house experiments showed that harpin protein was successful in effectively reducing reniform population in soybean without change in phenotype of the plants. However, we did not observe any difference in resistance to cyst nematode. The difference in

response could be due to the reason that infection strategies of cyst and reniform nematodes are different. Soybean seeds treated with harpin also show aggregation of the harpin protein on the seed surface. Many elicitors are capable of interacting with seeds (Worrall *et al.*, 2012). The ability of harpin protein to form coating onto the seed surface could explain its ability of induce resistance. However, it is unclear if harpin is absorbed within the seeds or travels from the seed surface towards the roots to induce the response in plants. To dissect signaling pathways in soybean in response to harpin treatment, RNA sequencing on soybean roots from seeds treated with harpin proteins, both with and without reniform infection was performed. Additionally, transgenic soybean plants to silence salicylic acid production were generated. The hypothesis was that harpin treatment would cause transcriptional changes in soybean root resulting in reduction of reniform population in soil, and the changes would be mediated through salicylic acid signaling. Although the expression of several genes was observed from soybean roots, significant differences in expression of genes between treatments were not observed and the results were inconclusive. The low depth of sequencing on the Ion Torrent Personal Genome Machine platform could have resulted in insufficient reads for statistical analyses.

In chapter four, the nematophagous fungal isolate ARF18 was studied for its growth habit in different nutritional conditions, and its pathogenicity on cyst nematodes. Its genome was sequenced and assembled. This is an important step towards providing public resource, and naming the fungus, which is an important component of biological control of plant parasitic nematodes (Kim & Riggs, 1991, Kim & Riggs, 1995, Wang *et al.*, 2004). The ability of the fungus to penetrate the cysts of *Heterodera glycines* through special appresorium-like structures was identified. Although appresoria, have been reported in several species in orbiliomycetes, this is the first report of the structures in this genus. The genome of ARF18 was sequenced and

assembled which identified several nematode parasitism genes. The genus *Brachyphoris* within orbiliomycetes is a poorly studied genus. This study will not only provide resources for the study of this genus but will also facilitate with the intellectual property rights, especially during commercialization of the fungus as a marketable product.

Molecular genetics has made advancement due to availability of several techniques. Additionally availability of next generation sequencing has supplemented the available technologies in gene identification and characterization. However, the application of functional genomics to devise strategies to manage plant parasitic nematodes is limited. Thus to incorporate molecular genetics as a component of nematode management a high throughput method of mutant generation, screening, and identification of mutation underlying the phenotype was developed. A recently developed in-solution target-enrichment to identify large number of mutants simultaneously was utilized. Although target-enrichment methods have gained popularity recently (Carpenter *et al.*, 2013, Ng *et al.*, 2009, Mercer *et al.*, 2014), the method has not been applied in filamentous fungi especially in nematophagous fungi. As a proof of concept the method was applied on the corn pathogen *Cercospora zeae-maydis*. *C. zeae-maydis* as a system was selected for method development due to ease of handling and manipulation, easy to transform using protoplast and *Agrobacterium,* and the phenotype of cercosporin production is easy to evaluate. From the experiments with random insertional mutants in *C. zeae-maydis,* the method was successful in enriching selected region of the genome, was high throughput and enabled pooling of numerous samples simultaneously. The method development on *C. zeae-maydis* identified several genes required for cercosporin production. The method has potential for widespread applications in molecular genetics of filamentous fungi, including nematophagous fungi, especially to dissect pathways related to secondary metabolism,

nematophagy and pathogenesis. The method developed is easily applicable to non-model organisms, even without a completely assembled genome.

References

Banerjee, S., Banerjee, A., Gill, S.S., Gupta, O.P., Dahuja, A., Jain, P.K., and Sirohi, A. (2017) RNA Interference: A Novel Source of Resistance to Combat Plant Parasitic Nematodes. *Frontiers in Plant Science* **8**.

Carpenter, M.L., Buenrostro, J.D., Valdiosera, C., Schroeder, H., Allentoft, M.E., Sikora, M., Rasmussen, M., Gravel, S., Guillen, S., Nekhrizov, G., Leshtakov, K., Dimitrova, D., Theodossiev, N., Pettener, D., Luiselli, D., Sandoval, K., Moreno-Estrada, A., Li, Y.R., Wang, J., Gilbert, M.T.P., Willerslev, E., Greenleaf, W.J., and Bustamante, C.D. (2013) Pulling out the 1%: Whole-Genome Capture for the Targeted Enrichment of Ancient DNA Sequencing Libraries. *American Journal of Human Genetics* **93**: 852-864.

Dong, H.P., Peng, J.L., Bao, Z.L., Meng, X.D., Bonasera, J.M., Chen, G.Y., Beer, S.V., and Dong, H.S. (2004) Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiology* **136**: 3628-3638.

Dong, H.P., Yu, H.Q., Bao, Z.L., Guo, X.J., Peng, J.L., Yao, Z., Chen, G.Y., Qu, S.P., and Dong, H.S. (2005) The ABI2-dependent abscisic acid signalling controls HrpN-induced drought tolerance in Arabidopsis. *Planta* **221**: 313-327.

Dong, H.S., Delaney, T.P., Bauer, D.W., and Beer, S.V. (1999) Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. *Plant Journal* **20**: 207-215.

Elling, A.A. (2013) Major Emerging Problems with Minor Meloidogyne Species. *Phytopathology* **103**: 1092-1102.

Kim, D.G., and Riggs, R.D. (1991) Characteristics and efficacy of a sterile hyphomycete (ARF18), a new biocontrol agent for *Heterodera glycines* and other nematodes. *Journal of Nematology* **23**: 275-282.

Kim, D.G., and Riggs, R.D. (1995) Efficacy of the nematophagous fungus ARF18 in alginate-clay pellet formulations against Heterodera glycines. *Journal of Nematology* **27**: 602-608.

Mercer, T.R., Clark, M.B., Crawford, J., Brunck, M.E., Gerhardt, D.J., Taft, R.J., Nielsen, L.K., Dinger, M.E., and Mattick, J.S. (2014) Targeted sequencing for gene discovery and quantification using RNA CaptureSeq. *Nature Protocols* **9**: 989-1009.

Ng, S.B., Turner, E.H., Robertson, P.D., Flygare, S.D., Bigham, A.W., Lee, C., Shaffer, T., Wong, M., Bhattacharjee, A., Eichler, E.E., Bamshad, M., Nickerson, D.A., and Shendure, J. (2009) Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* **461**: 272-U153.

Reboutier, D., Frankart, C., Briand, J., Biligui, B., Laroche, S., Rona, J.-P., Barny, M.-A., and Bouteau, F. (2007) The HrpN(ea) harpin from Erwinia amylovora triggers differential responses on the nonhost Arabidopsis thaliana cells and on the host apple cells. *Molecular Plant-Microbe Interactions* **20**: 94-100.

Wang, K.N., Riggs, R.D., and Crippen, D. (2004) Suppression of *Rotylenchulus reniformis* on cotton by the nematophagous fungus ARF. *Journal of Nematology* **36**: 186-191.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., and Beer, S.V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**: 85-88.

Worrall, D., Holroyd, G.H., Moore, J.P., Glowacz, M., Croft, P., Taylor, J.E., Paul, N.D., and Roberts, M.R. (2012) Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytologist* **193**: 770-778.