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

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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

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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. zea-maydis*.

The method identified more than 80 genes that altered cercosporin production in *C. zea-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.
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# TABLE OF CONTENTS

CHAPTER I.................................................................................................................................. 1
INTRODUCTION AND REVIEW OF LITERATURE........................................................................ 1
  Project overview.......................................................................................................................... 2
  Nematodes are important agricultural pests.............................................................................. 2
  Current strategies for controlling plant-parasitic nematodes...................................................... 4
  Application of harpin proteins as inducers of plant defense....................................................... 8
  Utilizing nematophagous fungi as biological control agents against plant-parasitic nematodes.................... 9
  Molecular genetics research in nematophagous fungi................................................................. 10
  *Cercospora zeae-maydis* - a model system to develop tools for molecular genetic studies.......... 11
  References.................................................................................................................................. 16
CHAPTER II.................................................................................................................................. 27
TREATMENT OF SOYBEAN AND COTTON SEEDS WITH HARPIN PROTEIN DEMONSTRATES ITS EFFICACY AGAINST RENIFORM NEMATODES........................................ 27
ABSTRACT................................................................................................................................. 28
INTRODUCTION............................................................................................................................ 29
MATERIALS AND METHODS................................................................................................. 32
  Nematodes culture and collection.............................................................................................. 32
  Extraction of nematodes for inoculum preparation..................................................................... 33
  Seed treatments and planting...................................................................................................... 34
  Inoculation of nematodes into the soil......................................................................................... 35
  Analyses of plant phenotype and nematode population............................................................ 36
  Scanning electron microscopy of soybean seeds soaked in harpin........................................... 37
RESULTS......................................................................................................................................... 38
  Effect of harpin protein treatment on plant height.................................................................... 38
  Effect of harpin protein treatment on plant biomass................................................................. 38
  Effect of harpin protein treatment on nematode population...................................................... 38
  Harpin protein forms an aggregate on the surface of soybean seeds........................................ 39
DISCUSSION.................................................................................................................................. 39
REFERENCES............................................................................................................................... 44
FIGURE LEGENDS..................................................................................................................... 51
FIGURES........................................................................................................................................ 53
CHAPTER III.................................................................................................................................. 61
RNA sequencing of Harpin Treated and Control Soybean Roots Identifies Genes Expressed During Reniform infection............................................................................................... 61
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.
LIST OF PUBLICATIONS (FIRST AUTHOR)

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 cells 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. \( Hs1^{pro-1} \), 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). *Hs1pro-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 *Purpureocillium 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


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 greenhouse 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 vermicform 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 vermicform *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 bucket. 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 greenhouse 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 is unknown.

Plants parasitic nematodes are a serious threat to crop yield and productivity. With the loss of some efficacious chemical nematicides, 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 safety, 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


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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≤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 μg/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 2.7

Figure 2.8
Figure 2.9

Figure 2.10
Figure 2.11

Figure 2.12
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 protein Nematode1 had the maximum read with 621480 reads. Additionally, the number of bases that passed the quality threshold (\( \geq Q20 \)) 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. A 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 log₂ 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 log₂ 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.,
The analyses showed that no reads mapped to the putative \textit{PRI} gene. Surprisingly a total of only about 0.005\% of the total transcripts mapped to the locus. Additionally, the locus for \textit{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 \textit{nahG} (salicylic hydroxylase) is not available in public resources. Previous researches have expressed the gene exclusively in soybean roots through hairy root transformation using \textit{Agrobacterium rhizogenes} (Mitchum, 2016, Youssef \textit{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


Figures and tables legends

**Figure 4.1** DNA ladder. 1% agarose gel showing the integrity of RNA from soybean root samples. Lanes 2-4: Control, lanes 5-7: harpin only, lanes 8-10: reniform nematodes only, and lanes 11-13: harpin + nematodes. Lane 1: 1 kb plus.

**Figure 4.2** QC of cDNA libraries from 12 samples Control-A1, B1,C1; Harpin only D1,E1,F1; Reniform only-G1,H1,A2; and Reniform with harpin B2,C2,D2.

**Table 4.1** Quantification of total RNA from samples using spectrophotometer.

**Table 4.2** Quality of the cDNA libraries from 12 samples measured on the Agilent Tapestation D 1000.

**Table 4.3** Table depicting reads and total bases in libraries from different treatments obtained from Ion Torrent PGM sequencing.

**Table 4.4** Table depicting the number of reads mapped onto soybean genome in different treatments.

**Table 4.5** Number of differentially expressed genes compared across different treatments: CH- control and harpin, CN- control and nematode, HHN- harpin and harpin nematode, and NHN- nematode and harpin nematode.

**Table 4.6** Soybean transformation seeds received from Iowa State University.

**Table 4.7** List of the primers used in the study.
Figures and Tables

Figure 4.1

Figure 4.2
### Table 4.1

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### Table 4.2

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<tr>
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<tr>
<td>C3</td>
<td>381</td>
<td>48.6</td>
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Table 4.3

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<td>81,567,830</td>
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<td>63,949,922</td>
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87
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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, Zygomyccota, 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% glutaraldehyde 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 & Nordbring-Hertz, 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 Dactylellina (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


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](image-url)

**Figure 3.1**
Figure 3.2

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 3.3
### Table 3.1

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<td>TN14_07867A</td>
<td><em>Arthrobotrys musiformis</em></td>
<td>EF113088.1</td>
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</table>
Appendix

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

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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

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REFERENCES


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 enzymes (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 pyreleenequinone 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 polyethylene glycol (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 ATKpn11F and ATKpn1R and purified by precipitating with ethanol and dissolved in dH2O. 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 $1 \times 10^6$ 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 μgml⁻¹ carbenicillin and 100 μgml⁻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 μgml⁻¹ carbenicillin and 100 μgml⁻¹ kanamycin for selection, to an OD₆₀₀ of 0.2. After 24 hours, the OD₆₀₀ of the bacterial culture in the IMM was quantified and diluted with IMM to set the final OD₆₀₀ 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 10⁶ 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 cephaxime, 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 CTBl 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 μg 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, 10⁵ 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 \( A = \varepsilon m C l \), where \( \varepsilon m \) 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-maydis* 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 (*SDHI*) 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. zeae-maydis, CRP1, the homolog of WC1, 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


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 $A= \varepsilon \text{mcI}$. The molar extinction coefficient for cercosporin is 23,300 $\text{m}^{-1} \text{cm}^{-1}$. 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 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

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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


