THE ROLE OF *HETERODERA GLYCINES* BIOTIN SYNTHASE IN THE NEMATODE-SOYBEAN INTERACTIONS

BY

KHEE-MAN KWON

THESIS

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Master's Committee:

Associate Professor Kris Lambert, Chair
Assistant Professor Leslie Domier
Professor Dean Riechers

ABSTRACT

Heterodera glycines, the soybean cyst nematode (SCN), is a plant-parasitic nematode capable of manipulating host plant biochemistry and development. Many studies have shown that the nematode has acquired genes from bacteria via horizontal gene transfer events (HGTs) that have the potential to enhance nematode parasitism. A recent allelic imbalance analysis identified two candidate virulence genes, which also appear to have entered the SCN genome through HGTs. One of the candidate genes, *H. glycines* biotin synthase (*HgBioB*), contained sequence polymorphisms between avirulent and virulent inbred SCN strains. To test the function of avirulent and virulent *HgBioB* alleles, a complementation experiment using mutant *Escherichia coli* with these two *HgBioB* alleles was conducted. Here we report that avirulent nematodes produce an active biotin synthase while virulent ones contain an inactive form of the enzyme. Moreover, we conclude from the sequencing analysis of SCN field populations that in nature the *HgBioB* gene contains a diverse mixture consisting of both avirulent and virulent alleles, but the virulent forms are more prevalent. We hypothesize that a lack of *HgBioB* activity within the virulent SCN could allow the nematode to evade a dethiobiotin-related toxin defense mechanism in host plants. Specifically, we showed that all soybean cultivars accumulate detectable levels of α-methyldethiobiotin (α-MDB), a dethiobiotin-related toxin found in bacteria. This is the first report of detecting α-MDB from soybean or any plant. Future work will determine if there is a significant difference in α-MDB levels between resistant and susceptible soybean cultivars.

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DEDICATION

To my loving family: my dad (Dr. Oh-Seung Kwon), my mom (Hye-Soon Chang), and my little brother (Luke Kwon) for their love and support.

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CHAPTER 1

Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops in the world, widely grown for protein meal and vegetable oil (Hartman et al., 2011). The United States is the world's top producer of soybean accounting for 34% of world soybean production. The phytoparasitic soybean cyst nematode (SCN), *Heterodera glycines*, is an obligate plant parasite that poses a serious threat to soybean production worldwide. It is the most damaging pest of soybean in the United States and is estimated to cause more yield losses than any other disease (Koenning and Wrather, 2010; Wrather et al., 2010). Yield losses are attributed to the fact that these nematodes inject a complex mixture of proteins and metabolites into the plant root cells (Niblack et al., 2006) and transform them into a metabolically active feeding structure called a syncytium (Hussey, 1989). The syncytium becomes a nutrient sink where the nematode feeds throughout its life cycle (Jones, 1981; Endo, 1998). This disrupts the plant's vascular system and prevents normal functioning of the root system leading to lower soybean yields.

What makes matters worse is the nematode's ability to persist in soils for 10 or more years even in the absence of a soybean host (Riggs, 2004). The most common and practical control strategy for SCN is to use SCN resistant soybean cultivars and rotate to non-host crop plants (Niblack et al., 2006). Although the management of SCN has been initially successful thanks to the use of resistant cultivars, the repeated use of the same resistance source has selected for virulent nematode populations that can reproduce on resistant cultivars (Mitchum et al., 2007; Mitchum, 2016; Niblack and Riggs, 2004). The increasing prevalence of these virulent SCN populations adapted to this resistance source could mean that the resistance mechanism is

becoming less effective. Understanding how SCN evade or suppress host plant resistance at the molecular level may allow the development of more effective sources of SCN-resistant soybean.

To this end, tremendous effort was put in the past decades to understand the complex interactions between the nematode and its host plant. Because plant-parasitic nematodes use their stylet, a protrusible needle-like structure in their mouthpart, to pierce plant cell walls and inject proteinaceous substances during feeding cell formation, these secreted proteins originating from the esophageal glands, two subventral and one dorsal, are thought to be involved in manipulating host plant biochemistry, development, and defenses (Davis et al., 2000; Hussey, 1989; Lambert et al., 1999; Smant et al., 1998). Initial work focused on the purification of secretory proteins originating from these esophageal glands. The first gene to be characterized encoded β-1,4-endoglucanse or cellulase expressed in the esophageal gland, but such approaches for isolation were slow and cumbersome and did not result in the identification of many proteins (Smant et al., 1998). A major breakthrough occurred when chorismate mutase (CM) from *Meloidogyne javanica* was discovered by incorporating a novel strategy combining the use of microscopic surgical techniques, to excise nematode esophageal gland regions, coupled with polymerase chain reaction that enabled the identification of secretory proteins from nematode esophageal glands (Lambert et al., 1999). Subsequently, other researchers applied similar approaches to isolate genes specifically expressed in the esophageal glands culminating in the identification of many secreted effector proteins. It is now believed that nematode effector mechanisms can range from modulating host plant susceptibility to directly inducing host defense responses (Hewezi and Baum, 2013).

Not all nematode secretory proteins, however, originate from the esophageal glands and some of the genes that encode secretory proteins can act as (a)virulence genes. Plant-parasitic

nematodes that can grow and reproduce on cultivars considered resistant are called virulent, while those that cannot are called avirulent nematodes (Roberts et al., 1995). It is not well understood how virulent nematodes evade or suppress host defense responses; however, some nematode genes have a potential to act as virulence genes that can help the nematode suppress host plant defenses and increase susceptibility on resistant crops. For example, an esophageal gland-secreted protein/enzyme CM is thought to suppress host defense by altering chorismate levels required for conversion to chorismate-derived compounds such as indole-3-acetic acid and salicylic acid (Doyle and Lambert, 2003; Lambert et al., 1999). Some SCN CM genes contain polymorphisms correlating with virulence on resistant soybean cultivars (Bekal et al., 2003; Lambert et al., 2005), also implicating these genes in SCN virulence. Similarly, a cuticle-secreted lipid-binding protein Gp-FAR-1 from *Globodera pallida* may be able to inhibit the host's jasmonic acid signaling pathway (Prior et al., 2001). On the contrary, some nematode genes may act as avirulence genes, which may help the nematode evade host plant defenses when those genes are absent. For instance, the SPRYSEC effector protein Gp-RBP-1 secreted from an esophageal gland in *G. pallida* (Sacco et al., 2009), MAP-1 secreted from amphids in *M. incognita* (Semblat et al., 2001), and a SNARE-like protein (*HgSLP-1*) secreted from an esophageal gland in *H. glycines* (Bekal et al., 2015) appear to act as avirulence genes. Although not a secreted protein, Mj-Cg-1 from *M. javanica* is also thought to function as an avirulence gene (Gleason et al., 2008).

Some of the secreted proteins are closely related to bacterial proteins, suggesting that they have been acquired from bacteria through horizontal gene transfer events (HGTs) (Bekal et al., 2015; Bird et al., 2003; Davis et al., 2000; Lambert et al., 1999; Smant et al., 1998). While these studies focusing on secreted proteins and HGT candidates have been productive for

understanding the molecular basis of nematode-host interactions, this means that non-secreted proteins, enzymes, and metabolites have been overlooked; hence, horizontally transferred genes coding for non-secreted proteins/enzymes with bacterial homology could also play a critical role in SCN biology and (a)virulence.

Studies on non-secreted nematode proteins have been few in number until Craig et al. (2008) showed that a certain group of non-secreted HGT proteins can include enzymes involved in vitamin biosynthesis of the nematode. A bioinformatics screen of the SCN genome revealed a hypothetically horizontally transferred gene pair, *HgSNO* and *HgSNZ*, which code for a functional pyridoxal 5'-phosphate synthase, the enzyme responsible for producing vitamin B_6 (Craig et al., 2008). The discovery of these two genes was of great significance because it was the first example of an entire metabolic pathway horizontally transferred to a nematode. Another screen in SCN discovered seven additional hypothetically horizontally transferred genes involved in vitamin B_1, B_5, and B_7 biosynthesis and their corresponding salvage pathways (Craig et al., 2009). More recently, a whole genome allelic imbalance analysis, conducted for the purpose of identifying single nucleotide polymorphisms (SNPs) associated with SCN genes implicated in virulence, found two candidate virulence genes, *HgSLP-1* and biotin synthase (*HgBioB*), both of which are thought to have been acquired through HGTs (Bekal et al., 2015). *HgBioB* is a non-secreted protein/enzyme in *H. glycines* responsible for catalyzing the conversion of dethiobiotin (desthiobiotin or DTB) to biotin (vitamin B_7). Furthermore, *HgBioB* contained SNPs that generated amino acid polymorphisms between avirulent and virulent inbred SCN strains, suggesting the allelic forms of *HgBioB* may have different activity (Bekal et al., 2015).

We postulated that these sequence polymorphisms could alter *HgBioB* activity and play an important role in SCN virulence. The observation that the nematode does not have the complete biotin biosynthetic pathway, but only the last key enzyme required for biotin synthesis (Craig et al., 2009), suggests SCN is capable of scavenging the precursor DTB from the plant and converting it into biotin to aid its growth. Thus, we hypothesized that virulent nematodes might produce a more active form of *HgBioB* than their avirulent counterparts because it seemed reasonable that a nematode's ability to produce more biotin might counteract a plant resistance mechanism that restricts the nematode from accessing free biotin or its immediate precursor. To support or refute this hypothesis, we tested the function of the two *HgBioB* alleles to ascertain if any functional differences could be detected between the virulent and avirulent forms of the enzyme.

The rest of the thesis is structured as follows: Chapter 2 gives background information on the biology of SCN, as well as the history and function of biotin and biotin synthase; Chapter 3 describes where the materials are obtained from and how the experiments are conducted; Chapter 4 provides the results and interpretations of data; Chapter 5 discusses the findings of this research; and, finally, Chapter 6 concludes the thesis and provides future directions.

CHAPTER 2

Background

Biology of the Soybean Cyst Nematode

SCN is an obligate plant parasite that must feed on a living host plant to complete its life cycle. It is included in the genus *Heterodera*, one of the three genera that are sedentary endoparasites considered the most economically important group of plant-parasitic nematodes (Williamson and Gleason, 2003). The life cycle of SCN **(Figure 2.1)** starts with an egg which serves as the reproductive unit as well as the survival structure (Niblack et al., 2006). Within the egg, the first-stage juvenile develops and molts to a second-stage juvenile (J2). Eggs hatch as J2, which can then find a host plant for infection. To successfully infect a host, the J2 uses a stylet, a protusible needle-like structure, during invasion and feeding to pierce plant cell walls and inject a mixture of proteins and metabolites into the plant root cells (Hussey, 1989; Niblack et al., 2006). This proteinaceous mixture, thought to originate from the esophageal glands, manipulates the host plant biochemistry, so that the nematode can evade or suppress host defenses (Davis et al., 2000; Hussey, 1989; Niblack et al., 2006), and transforms the root cells into highly metabolically active feeding cells called syncytia (Endo, 1998; Jones, 1981). These syncytial cells develop through cell wall breakdown and cellular fusion (Mitchum et al., 2012) which provides a place for the sedentary nematode to feed throughout its life cycle. After successful formation of a feeding site, the J2 molts to the third- and fourth-stage juveniles (J3 and J4) within the host roots before reaching adult stage. It is at J3 when sexual dimorphism occurs to produce males and females. When males become adults, they leave the roots in search for females to mate with. When females become adults, their body becomes a white spheroid or lemon-shaped

structure (Niblack et al., 2006). After sexual reproduction and fertilization, the female can produce up to 600 eggs (Sipes et al., 1992), oftentimes making its body too large to remain attached to the roots. Some of the eggs are released into a gelatinous matrix produced by the female. This gelatinous matrix, primarily composed of carbohydrate, contains antimicrobial compounds (Niblack and Karr, 1994), to protect the eggs from predation and desiccation. Still, most eggs are kept inside the female body; when the female dies, its body becomes a harden cyst to act as a survival structure which will protect hundreds of viable eggs inside. These eggs inside the cyst can remain viable for over ten years (Inagaki and Tsutsumi, 1971) even in the absence of a host plant (Riggs, 2004), making it almost impossible to eradicate this pest.

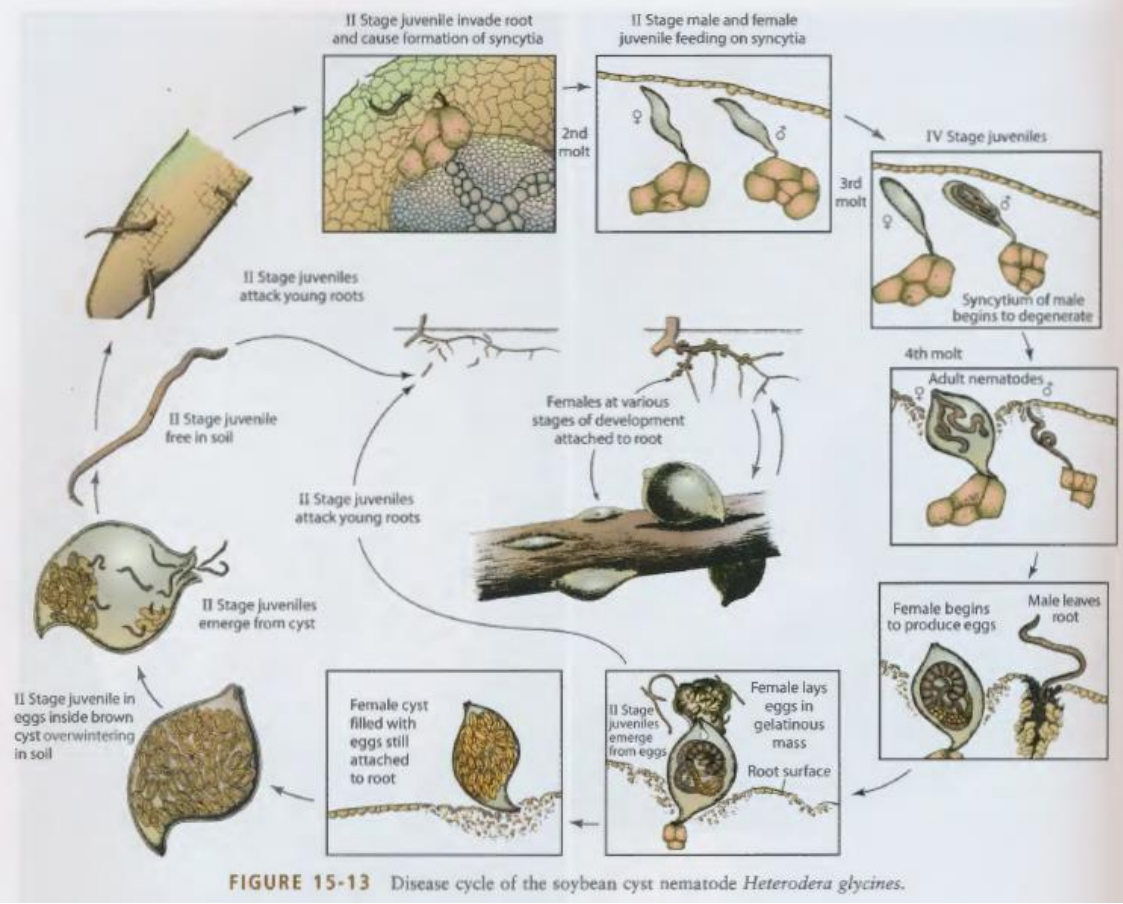


Figure 2.1. SCN life cycle (Source: Agrios, 2005).

Biotin and Biotin Synthase

Biotin was first extracted from yeast as an unknown, but essential growth factor for yeast and named *bios* (Wildiers, 1901). Then biotin was isolated from egg yolk (Kögl and Tönnis, 1936), and from liver and named vitamin H (*haut*: meaning skin in German) (du Vigneaud et al., 1941). Biotin was also found to be an important cofactor for rhizobia, hence the name coenzyme R (Allison et al., 1933). Later, it turned out that all these discoveries were about the same molecule biotin.

As a member of the water-soluble vitamin B family, biotin (or vitamin B_7) is an essential cofactor for biotin-dependent enzymes including certain carboxylases, decarboxylases, and transcarboxylases, required for fatty acid, amino acid, and carbohydrate metabolic processes (Alban et al., 2000; Attwood and Wallace, 2002; Cronan, 2014; Knowles, 1989). During these processes, biotin acts as a carboxyl carrier to transfer a carboxyl group between metabolites (S Paparella et al., 2014). Biotin is also involved in cell signaling, chromatin structure, and epigenetic gene regulation (Zempleni, 2005), and a more recent discovery shows that biotin plays a role in bacterial virulence (Feng et al., 2014).

Although a critical enzyme cofactor necessary for all three domains of life (Depeint et al., 2006a; Waldrop et al., 2012; Zempleni et al., 2008), biotin is only produced in microorganisms and plants (Alban, 2011; Hall and Dietrich, 2007). In most of these organisms, biotin biosynthesis is highly conserved **(Figure 2.2)** starting with pimeloyl-CoA (ACP) in a four-step pathway, consisting of 7-keto-8-aminopelargonic acid (KAPA), 7,8-diaminopelargnoic acid (DAPA), dethiobiotin (DTB), and biotin (Alban, 2011; Lin and Cronan, 2011; Streit and Entcheva, 2003). In *Escherichia coli*, the four key enzymes responsible for biotin biosynthesis

are encoded in a gene cluster containing bioF (KAPA synthase), bioA (DAPA synthase), bioD (DTB synthetase), and bioB (biotin synthase) (Alban, 2011).

Biotin synthase (bioB) is especially important among these four enzymes because it catalyzes the last reaction, conversion of DTB into biotin, which also happens to be the rate-limiting step (Taylor et al., 2008). Biotin synthase inserts a sulfur atom between the two carbon atoms, C6 and C9, on DTB and thereby closes the thiophane ring to generate biotin (Cronan, 2014). The mechanism behind this reaction is not well understood, and the fact that biotin synthase is an S-adenosyl-methionine (Adomet)-dependent radical enzyme makes it an even more complicated enzyme (Berkovitch et al., 2004; Fugate and Jarrett, 2012; Jarrett, 2003). Moreover, biotin synthase is of special interest because animals do not have the ability to synthesize biotin (Hall and Dietrich, 2007), and they depend on dietary intake. Yet, SCN, an animal and a parasite which should have no problem acquiring vitamins from its host (Craig et al., 2008), contains the biotin synthase gene (Craig et al., 2009). SCN does not have the complete biotin biosynthesis pathway, but a partial pathway that encodes biotin synthase (Craig et al., 2009). The presence of this incomplete *de novo* biotin biosynthesis pathway implies that biotin has an important function in SCN and suggests the nematode is scavenging DTB from its host.

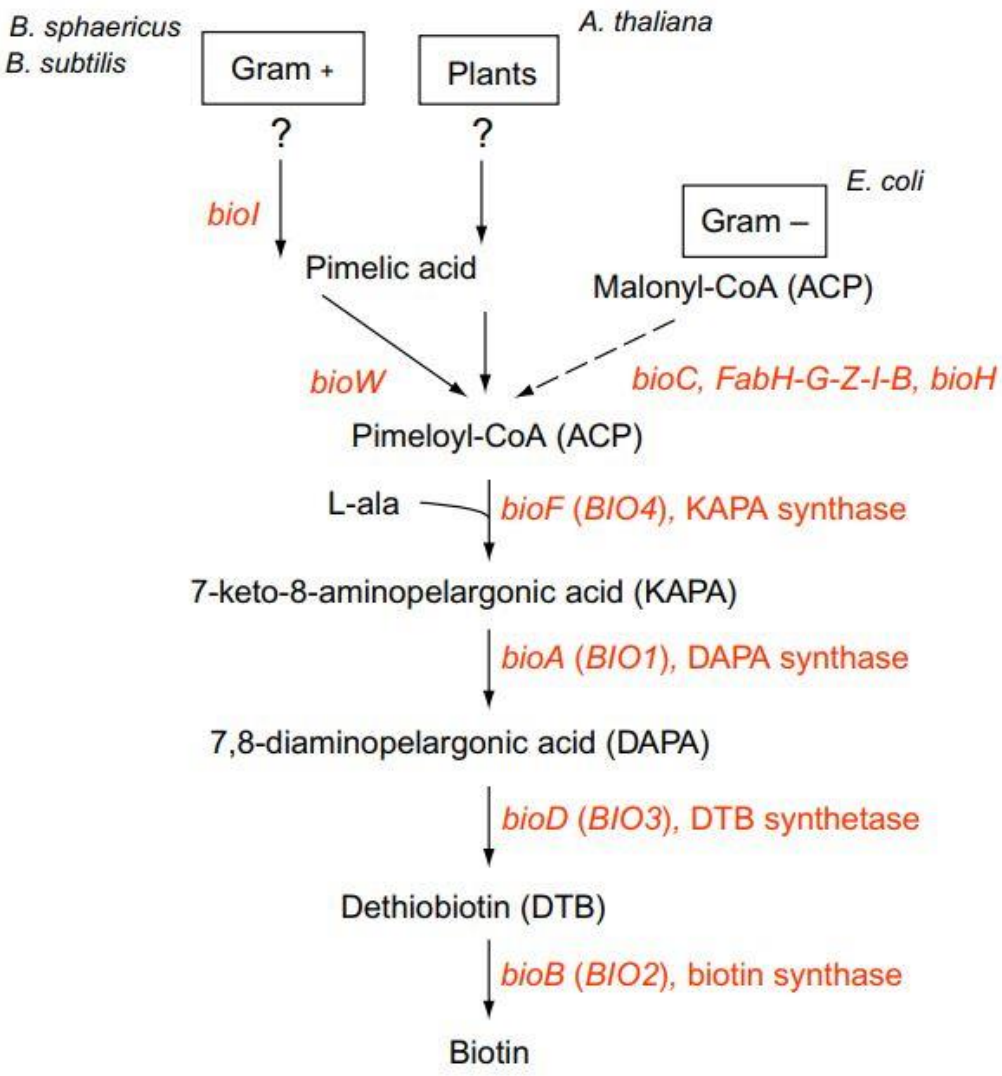


Figure 2.2. Biotin synthesis pathway in bacteria and plants (Source: Alban, 2011).

CHAPTER 3

Materials and Methods

Construction and Cloning of Recombinant Plasmids containing *HgBioB*

Two different synthetic DNA fragments *HgBioB*-avr (avirulent) and *HgBioB*-vir (virulent) that code for *HgBioB* in inbred SCN strains TN10 (avirulent) and TN20 (virulent), respectively, were optimized for prokaryotic expression (Life Technologies, Carlsbad, CA). Also, two different mutagenized forms of *HgBioB*-vir, *HgBioB*-vir-M1 (virulent, mutagenized allele 1) and *HgBioB*-vir-M2 (virulent, mutagenized allele 2), were prepared by generating (1) an alanine to proline mutation (M1) at amino acid position 24 (A24P); and (2) a glutamine to arginine mutation (M2) at amino acid position 44 (Q44R), respectively. M1 was produced via site-directed mutagenesis with a pair of inverse polymerase chain reaction (PCR) primers **(Table 3.1)**, BioB-mut1-F and BioB-mut1-R, generated following the manufacturer's instructions for In-Fusion cloning (Clontech, Mountain View, CA), using *HgBioB*-vir as the template. M2 was prepared by custom-ordering the synthetic DNA fragment (Life Technologies).

To perform In-Fusion cloning (Clontech), two pairs of inverse PCR primers **(Table 3.1)** were designed for the insert and the vector so that they would share a 15-bp homology with each other. All four DNA fragments were amplified using the inverse PCR primers BioB-F and BioB-R; and pBAD.LIC.8A cloning vector (Addgene plasmid #37501) was amplified with inverse PCR primers BioB-Vec-F and BioB-Vec-R. All PCRs were done using an iProof HF Mastermix (Bio-Rad, Hercules, CA) following the manufacturer's recommended PCR conditions. The resulting PCR products were gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), joined together using an In-Fusion HD Cloning Kit (Clontech), and transformed

into Stellar Competent Cells (Clontech) following the manufacturer's instructions. Cells containing successful transformants were screened on Luria-Bertani (LB) agar plates with ampicillin (100 mg/ml), and a single colony was picked to inoculate 5 ml of LB broth with ampicillin (100 mg/ml) which was incubated overnight at 37C. Recombinant plasmid DNA was extracted from the overnight culture using a QIAprep Spin Miniprep Kit (Qiagen) and the resulting plasmids were sequenced for verification at the University of Illinois Roy J. Carver Biotechnology Center.

Table 3.1. Oligonucleotides for Plasmid Construction and Sequencing Analysis.

Primer Name	Sequence
BioB-F	5'-GAA GGA GAT ATA GAT ATG CCT CCG CCT ATT GGT AGC-3'
BioB-R	5'-TTA TGG AGT TGG GAT TTA CAG ATT CAG GGT CAC TTT TTC ATC GT-3'
BioB-mut1-F	5'-CCA TTT CCG GAA CTG ATT TTT CGT GCA CAG AAT GTT C-3'
BioB-mut1-R	5'-CAG TTC CGG AAA TGG CAG GCT AAA AAC GCT CAG TGC-3'
BioB-Vec-F	5'-ATC CCA ACT CCA TAA GGA TCC CTT G-3'
BioB-Vec-R	5'-ATC TAT ATC TCC TTC TTA AAG TTA AAC AAA ATT ATT TCT AGA TGT AGA TGT TAG CC-3'
BioB-flank-F1a	5'-GGA GAG GAA TGA TAT GAT GAA-3'
BioB-flank-R1a	5'-CAT CTT CTG CTT CTG TTC TG-3'

Complementation of ΔbioB *Escherichia coli* with Avirulent and Virulent Alleles of *HgBioB*

A kanamycin-resistant *E. coli* vitamin B_7 auxotroph (K-12 Keio Collection: JW0785-1) was obtained from the Coli Genetic Stock Center at Yale University and used to prepare electrocompetent cells as described by Dower et al., (1988) and Hanahan, (1983). Four different recombinant plasmids—pBAD.LIC.8A:: *HgBioB*-avr, pBAD.LIC.8A:: *HgBioB*-vir, pBAD.LIC.8A:: *HgBioB*-vir-M1, and pBAD.LIC.8A:: *HgBioB*-vir-M2—were generated and

independently transformed into the kanamycin-resistant ΔbioB mutant electrocompetent cells. For control purposes, an empty vector (pBAD.LIC.8A) lacking any inserts was also transformed into the electrocompetent cells. The resulting transformants were screened on LB agar plates with ampicillin (100 mg/ml) and kanamycin (50 mg/ml), and a single colony was picked to inoculate 10 ml of LB broth with ampicillin (100 mg/ml) and kanamycin (50 mg/ml) for overnight incubation at 37C. The overnight cultures of these three strains were thoroughly washed in liquid M9 minimal media to remove trace amounts of biotin that may be present, and then streaked onto M9 minimal media plates containing ampicillin (100 mg/ml), kanamycin (50 mg/ml), and either avidin or biotin. Plates containing avidin (1 mg/ml) were prepared to sequester trace amounts of biotin in the M9 media. Positive control plates were M9 media supplemented with biotin (1 M), and a negative control plate consisted of ΔbioB *E. coli* transformed with an empty pBAD.LIC.8A vector plated on M9 media lacking biotin. Some plates were also treated with 2% arabinose to induce *HgBioB* expression and monitor its potential effect on *E. coli* growth. All plates were initially incubated at 28C for 48 hrs, and then allowed to grow at room temperature for 10 days. *E. coli* growth was monitored daily.

Nematode Culture and DNA Extraction of SCN Field Populations

A collection of soil samples containing diverse SCN field populations from Iowa was a kind gift from Dr. Gregory Tylka at Iowa State University (Ames, IA). Cysts from each field population were purified as described by Niblack et al., (1993) and crushed over a 0.25-mm (60 mesh) sieve to release eggs. Eggs were suspended in 2% carboxymethyl cellulose solution for an easy delivery of an approximately equal number of eggs during inoculation, and used to inoculate soybean cv. Essex plants, previously pre-germinated and planted into 50-ml Falcon

tubes with the absorbent wick described in Bekal et al., (2015). Inoculated plants were grown for eight weeks to allow eggs to hatch and infect the plants. The resulting cysts, approximately 2-200 cysts from each plant, were harvested in 2-ml cryotube vials, and stored at -80C until use. The cyst-containing vials were placed in a pre-cooled rack in liquid nitrogen and two stainless steel beads (3.2 mm diameter) were added into each vial. These frozen cysts were then freeze-fractured with a bead beater homogenizer and total nucleic acid was extracted using an RNeasy Mini Kit (Qiagen).

Sequencing and Statistical Analysis of Different SCN Populations

From each extracted 50-µl DNA sample, 2.5 µl was used as a template for amplification using a GenomiPhi Kit (GE Healthcare, Piscataway, NJ) following the manufacturer's protocols. The amplified DNA was treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) for the degradation of primers and nucleotides that may interfere with downstream applications. A set of PCR primers **(Table 3.1)**, BioB-flank-F1a and BioB-flank-R1a, flanking the polymorphic region of the *HgBioB* gene was designed for PCR amplification and subsequent sequencing analysis. All PCRs were done using a CloneAmp Hi-Fi PCR Premix (Clontech) following the manufacturer's recommended conditions. The resulting PCR product was gel-purified using a QIAquick Gel Extraction Kit (Qiagen) and then sent for sequencing at the University of Illinois Roy J. Carver Biotechnology Center. The sequences were aligned to the original *HgBioB* sequence using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). The relative chromatogram peak heights corresponding to amino acid positions 24 and 44 were measured to estimate the percentage of each polymorphic nucleotide(s).

Peak heights were statistically analyzed using both descriptive and inferential methods. First, a box plot was generated to compare the median values and understand the distributional characteristics of these alleles at both polymorphic regions, and then a Wilcoxon signed rank test was performed using the SAS Proc Univariate procedure (SAS Institute, Cary, NC) to test if the differences between avirulent and virulent alleles were statistically significant. For the signed rank test, a new variable diff was set as the difference between the virulent and avirulent alleles (diff = vir – avr) at each amino acid for each SCN sample prior to running the Proc Univariate procedure on that variable. Finally, a Spearman rank-order correlation was conducted using the SAS Proc Corr procedure (SAS Institute) to calculate the coefficients for the relationship between the two variables, amino acid positions 24 and 44, for each set of virulent and avirulent pairs.

Metabolite Extraction from Resistant and Susceptible Soybean Roots

Seeds from four soybean cultivars—SCN-resistant cv. Peking (PI 548402) and PI 88788; and SCN-susceptible cv. Essex and Lee 74—were surface-sterilized in 10% bleach solution for 15 min and thoroughly washed three times in sterile distilled water, and germinated under sterile conditions in autoclaved Magenta culture boxes which contained glass beads (3 mm diameter), distilled water, and a filter paper mounted inside.

The sterile seeds were gently placed on top of the filter paper to keep them moist for imbibition and germination. After 5 days, the soybean roots were dissected, collected in a 50-ml tube, and stored at -80C. The frozen roots were chopped into smaller pieces and transferred into a mortar and pestle pre-chilled in liquid nitrogen. Liquid nitrogen was added to the root debris and ground with a pestle into a fine powder. A pre-chilled methanol solution containing a final

solution of 1 µg/µl 6-(2,5-dioxo-pyrrolidin-1-YI)-hexanoic acid (Sigma-Aldrich, St. Louis, MO), used as an internal standard for LC-MS/MS, was added to the sample and gently mixed. The samples were placed on a shaker for 30 min at room temperature and centrifuged at 3,000 × g for 10 min to pellet the organic materials. Finally, the resulting supernatant was lyophilized overnight using a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA).

Synthesis of α-Methyldethiobiotin

An α-MDB analytical standard for LC-MS/MS was chemically synthesized and provided by Dr. Hua Li at Korea Institute of Science and Technology (KIST) (Seoul, South Korea). Alpha-MDB was prepared from D-desthiobiotin (Sigma-Aldrich) in three steps using a modification of the preparation of α-methylbiotin (α-MB) described in Dixon et al., (2002). Briefly, D-esterification of D-desthiobiotin was carried out and, subsequently, α-methylation with KHMDS as a base was performed in the presence of TMEDA and HMPA to give α-MDB methyl ester in 39% yield with a 3.7:1 mixture with the starting ester. This α-MDB methyl ester was a 1:1 mixture of two diastereomers meaning that α-methylation was not stereoselective. Hydrolysis of the esters using trifluoroacetic acid gave α-MDB as a 1:1 diastereomeric mixture at α–position containing 21% of unmethylated D-desthiobiotin, or α-MDB with a purity of 79%.

LC-MS/MS Analysis of Soybean Root Metabolites

Prior to the LC-MS/MS analysis, the freeze-dried extracts were reconstituted in 0.6 ml of 50% methanol, sonicated for 10 min, and centrifuged at 16,000 × g for 10 min. The samples were then diluted 1/20 with 0.1% formic acid in water (to a final volume of 12 ml) and injected by an autosampler. The LC-MS/MS analysis of α-MDB and DTB in the root extracts was

conducted by the Doping Control Center at KIST with a nano UHPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) coupled to a mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Scientific). A pre-column (Acclaim PepMap 100, nanoviper fitting, C18; 75 μm Diameter × 200 mm Length, 3 μm particle size, 100 Å; Thermo Scientific) and an analytical column (EASY-Spray Column, PepMap, nanoviper fitting, C18; 75 μm × 150 mm, 3 μm, 100 Å; Thermo Scientific) were used for trap and analytical column, respectively. The HPLC mobile phases consisted of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The column oven was set at 40C, and the HPLC flow rate was set at 350 nl/min, with the following gradient: 4% B for 3 min; 4-60% B for 17 min; 60-95% B for 2 min; 95% B for 3 min; 95- 4% B for 0.1 min; and 4% B for 9.9 min. The MS was performed using full scans (m/z 300-1800) at a resolution of 70,000 (at m/z 200) and parallel reaction monitoring MS/MS scans at a resolution of 17,500 (at m/z 200). HCD collision energy was 28%. Data were processed using the Xcalibur software (Thermo Scientific) with a mass tolerance window of ±5 ppm.

CHAPTER 4

Results

Complementation of ΔbioB *E. coli* with Avirulent and Virulent Alleles of *HgBioB*

We had observed differences in the predicted amino acid sequences between avirulent and virulent *HgBioB* alleles, and thus hypothesized that such differences could lead to an altered enzyme function. To test for *HgBioB* function, we opted to use genetic complementation of a BioB *E. coli* mutant. Moreover, we wanted to learn if amino acid(s) changes, P24A or R44Q, which differ between the *HgBioB* alleles, would alter BioB enzyme function.

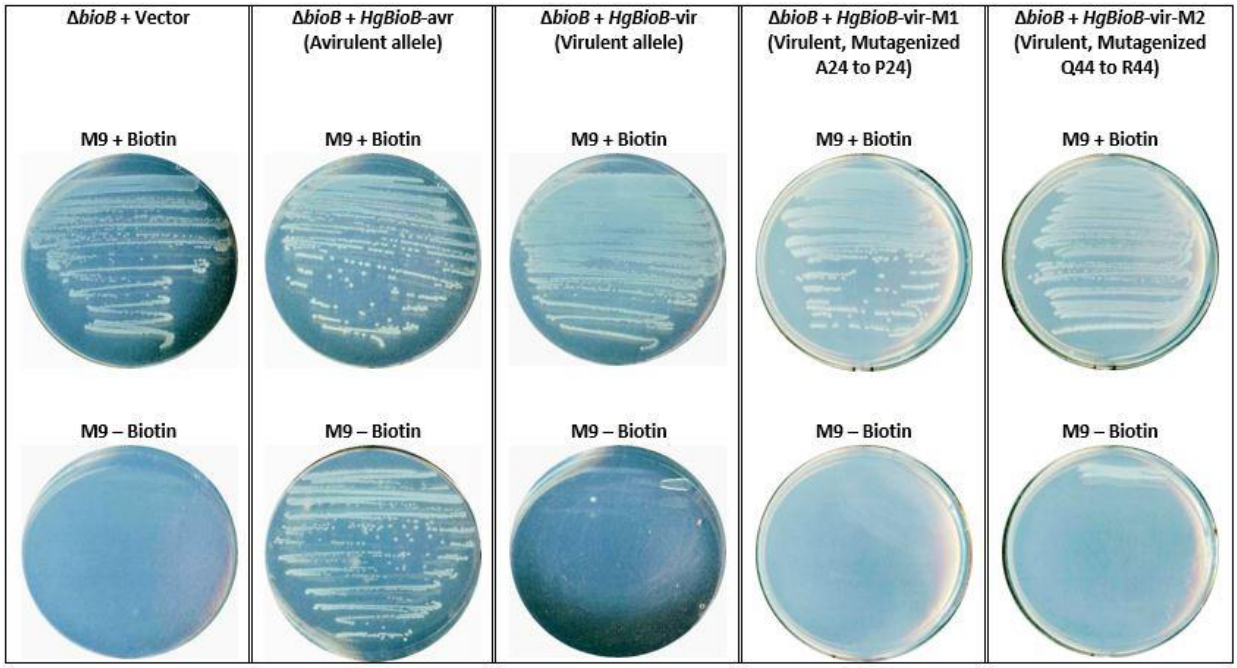
To conduct this experiment, four plasmid constructs were generated to express the different forms of *HgBioB*; the virulent allele (*HgBioB*-vir), the avirulent allele (*HgBioB*-avr), and the two single mutant alleles (*HgBioB*-vir-M1 and *HgBioB*-vir-M2). These plasmids, along with an empty vector control, were transformed into mutant *E. coli* lacking endogenous BioB function and were then allowed to grow on minimal agar with and without supplemented biotin.

Within 48 hours after streaking the *E. coli* containing the BioB constructs onto minimal media plates, all five biotin-supplemented plates (minimal media +biotin; positive controls) showed vigorous growth of *E. coli* as expected. Among the five plates lacking biotin (minimal media alone), however, growth only occurred for the bacteria containing the avirulent allele (*HgBioB*-avr). The other four constructs, *E. coli* carrying the empty vector (negative control), the virulent allele (*HgBioB*-vir), and the two mutagenized forms of the virulent allele (*HgBioB*-vir-M1 and *HgBioB*-vir-M2) failed to complement the growth of the mutant *E. coli* **(Figure 4.1A)**.

This complementation test indicates that avirulent SCN produces an active biotin synthase while virulent SCN contains an inactive form of the enzyme. Additionally, site-directed

mutagenesis experiments confirmed that the presence of both amino acids, a proline at position 24 (P24) and an arginine at position 44 (R44), are required for a functional BioB activity **(Figure 4.1A, Figure 4.1B)**. If either of these two amino acids was changed to a different one, the enzyme lost its ability to complement the mutant, meaning that the *HgBioB* protein has become inactive. This experiment was repeated and produced the same results.

(A)



(B)

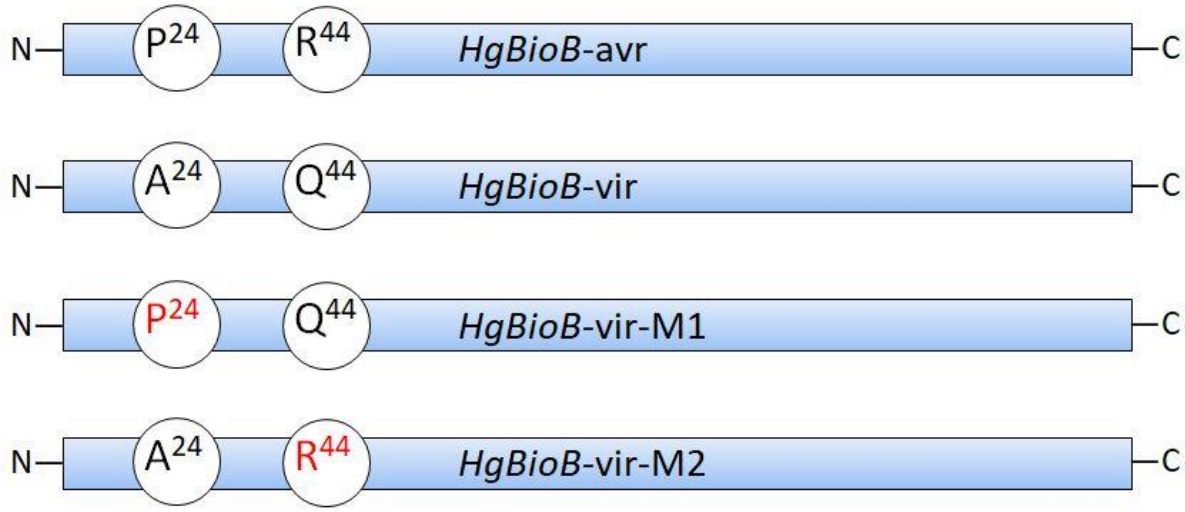


Figure 4.1. Complementation by expression of *HgBioB* from low copy number plasmids in a Δ*bioB E. coli* strain. (A) The expression of the *HgBioB*-avr allowed to restore growth on biotin-free M9 minimal medium, whereas the expression of the *HgBioB*-vir failed to grow. The two mutagenized forms of the virulent allele *HgBioB*-vir-M1 and *HgBioB*-vir-M2 also failed to complement the growth of the mutant *E. coli* showing that both a proline at position 24 and an arginine at position 44 are required for a functional BioB activity. (B) Diagram showing amino acids, from N-terminus to C-terminus, at positions 24 and 44. *HgBioB*-vir was mutagenized to produce *HgBioB*-vir-M1, by generating an alanine to proline mutation at position 24 (A24P); and *HgBioB*-vir-M2, by generating a glutamine to arginine mutation at position 44 (Q44R).

Sequencing, Statistical Analysis, and Confirmation of SNPs from SCN Field Populations

Since our preliminary studies were based on just two inbred SCN strains, we were interested to learn if sequence variation in the *HgBioB* gene was common in different field populations of SCN. Four inbred SCN strains were extreme and carried single homozygous forms **(Table 4.1)**. In contrast, sequencing results from 30 SCN field populations showed that in most SCN populations the *HgBioB* gene was not present in a single homozygous form, but rather in a diverse mixture consisting of both SNPs resulting in both active and inactive forms of *HgBioB* **(Table 4.1)**.

Statistical analysis of the field sequencing data was conducted by generating a box plot and comparing the median values. This analysis showed that the *HgBioB*-vir alleles were present in a higher percentage than the *HgBioB*-avr alleles at both polymorphic regions (i.e., more A24 than P24; more Q44 than R44) **(Figure 4.2A, Figure 4.2B, Table 4.2)**. This trend was also supported by conducting a Wilcoxon signed rank test, the nonparametric alternative to the paired *t*-test, which was chosen because the polymorphic nucleotide percentage data for avirulent and virulent alleles were not normally distributed. The Wilcoxon signed rank test results showed that *HgBioB*-vir allele ranks were statistically significantly higher than *HgBioB*-avr allele ranks for both amino acid positions 24 ($Z = 105$, $p < 0.05$) and 44 ($Z = 121.5$, $p < 0.01$). Therefore, from both descriptive and inferential statistical results, we concluded that the virulent alleles are more prevalent than the avirulent alleles in the field sites tested.

In addition, it is interesting to note that these SNP changes occur simultaneously in pairs, at about the same frequency, between amino acid positions 24 and 44. For example, if a SNP in *HgBioB* results in a P24 then it would be paired with an R44; if it were an A24 then it would be paired with a Q44. This linkage of SNP frequency was supported by testing the correlations

between the virulent allele pair (i.e., A24 and Q44) as well as the avirulent allele pair (i.e., P24 and R44) using a Spearman rank-order correlation, the nonparametric alternative to the Pearson product-moment correlation, chosen because the assumptions of normality could not be met. The Spearman correlation results showed that there was a positive correlation for both virulent ($r = 0.8683$, $n = 30$, $p < 0.001$) and avirulent ($r = 0.9184$, $n = 30$, $p < 0.001$) pairs. Scatterplots for both avirulent and virulent pairs also summarize that there is a positive linear relationship between amino acid positions 24 and 44 **(Figure 4.3A, Figure 4.3B)**. The strong, positive correlation between these positions suggests that these amino acid changes must both occur in the same protein to exert their desired, but unknown function. Under rare circumstances, there are also alternative SNPs that result in a threonine at position 24 (T24) and a proline at position 44 (P44), again suggesting an important but unknown function of these two amino acids in the *HgBioB* enzyme.

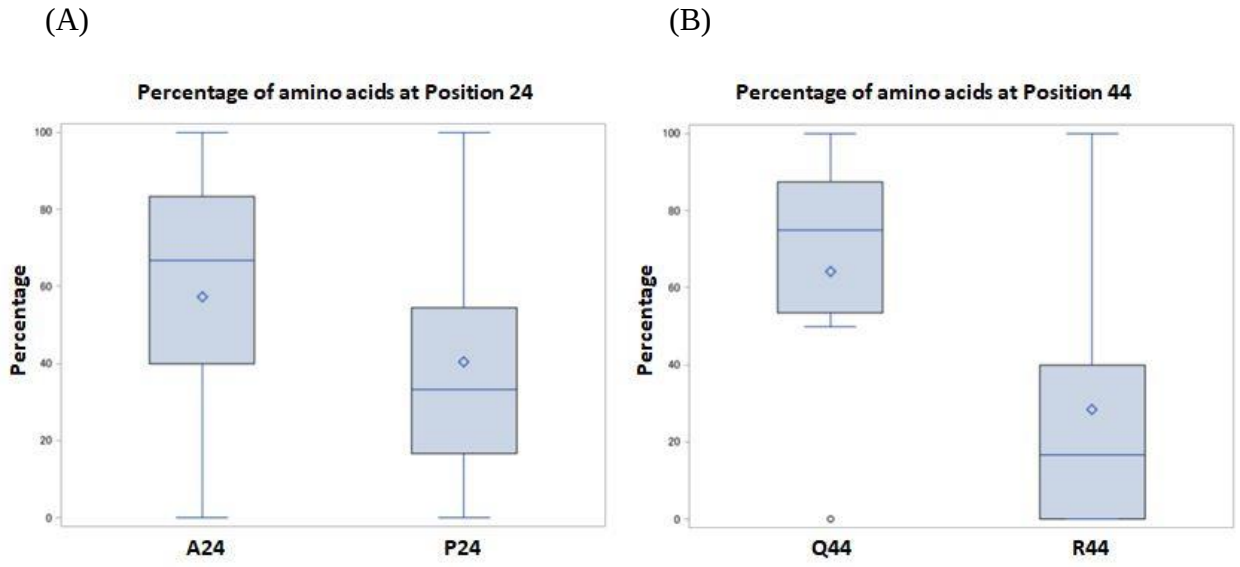


Figure 4.2. Box plots showing the percentage of amino acids at positions 24 and 44.
(A) There are more virulent alleles at amino acid position 24. (B) Likewise, there are more virulent alleles at amino acid position 44.

Table 4.1. Sequencing Analysis of Nematode Field Populations.

SCN Populations	24th Amino Acid			44th Amino Acid		
	Threonine (**A**CT) %A	Alanine (**G**CT) %G	Proline (**C**CT) %C	Glutamine (C**A**A) %A	Arginine (C**G**A) %G	Proline (C**C**A) %C
257		**100.00**		**100.00**		
Hg0 A		60.00	40.00	53.33	40.00	
Hg0 B	33.33	66.67		**100.00**		
OP 25 g **			**100.00**		**100.00**	
OP 20 g **			**100.00**			**100.00**
OP 50 g **	33.33		66.67			**100.00**
TN 20 g **		**100.00**		64.29	21.42	
Carbondale 6		75.00	25.00	75.00	25.00	
Carbondale 30			**100.00**		**100.00**	
C 104 *	10.00	40.00	50.00	87.50	12.50	
C 432 *		83.33	16.67	**100.00**		
EC 138 *		66.67	33.33	83.33	16.67	
EC 146 *		66.67	33.33	75.00	25.00	
EC 406 *		60.00	40.00	75.00	25.00	
NC 228 *		75.00	25.00	87.50		12.50
NC 238 *		66.67	33.33	60.00	20.00	20.00
NC 305 *		**100.00**		**100.00**		
NC 408 *		50.00	50.00	60.00	40.00	
NE 336 *		83.33	16.67	87.50	12.50	
NE 433 *		**100.00**		**100.00**		
NW 138 *		75.00	25.00	85.71	14.28	
NW 209 *		57.14	42.85	66.67	33.33	
NW 308 *		75.00	25.00	83.33	16.67	
NW 421 *		66.67	33.33	83.33	16.67	
SC 349 *		75.00	25.00	83.33	16.67	
SE 132 *		60.00	40.00	66.67	33.33	
SE 148 *			**100.00**		**100.00**	
SE 217 *			**100.00**		**100.00**	
SW 207 *		40.00	60.00	50.00	50.00	
SW 242 *		45.45	54.54	60.00	40.00	
SW 251 *		85.71	14.28	**100.00**		
WC 216 *			**100.00**		**100.00**	
WC 229 *		83.33	16.67	90.91	9.09	
WC 350 *		90.90	9.09	**100.00**		

* denotes SCN field populations from Iowa.

** denotes inbred SCN strains.

Table 4.2. Statistical Analysis of Nematode Field Populations.

	amino acid position 24		amino acid position 44	
	Alanine (A24)	Proline (P24)	Glutamine (Q44)	Arginine (R44)
Mean	57.28	40.46	64.07	28.47
Std Dev	33.51	32.88	36.02	33.21
Median	66.67	33.33	75.00	16.67
Min	40.00	9.09	50.00	9.09
Max	100.00	100.00	100.00	100.00

(A) (B)

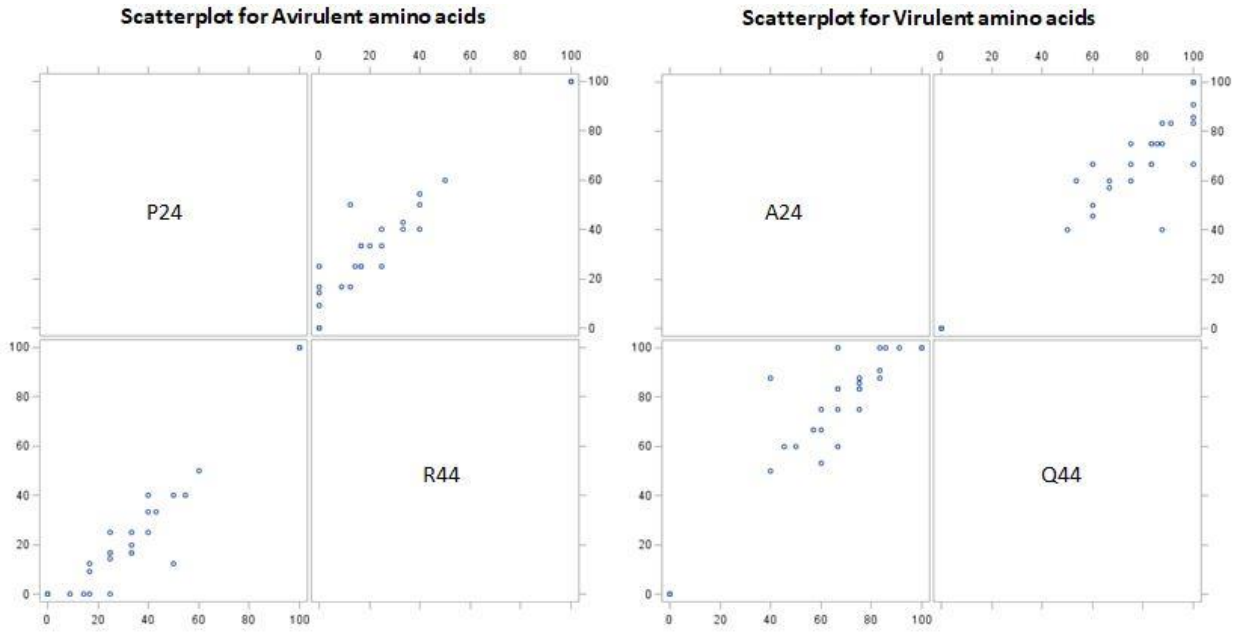


Figure 4.3. Scatterplots showing the avirulent and virulent amino acid pairs.
(A) The avirulent pair shows a positive linear relationship between amino acid positions 24 and 44. (B) Similarly, the virulent pair also shows a positive linear relationship between amino acid positions 24 and 44.

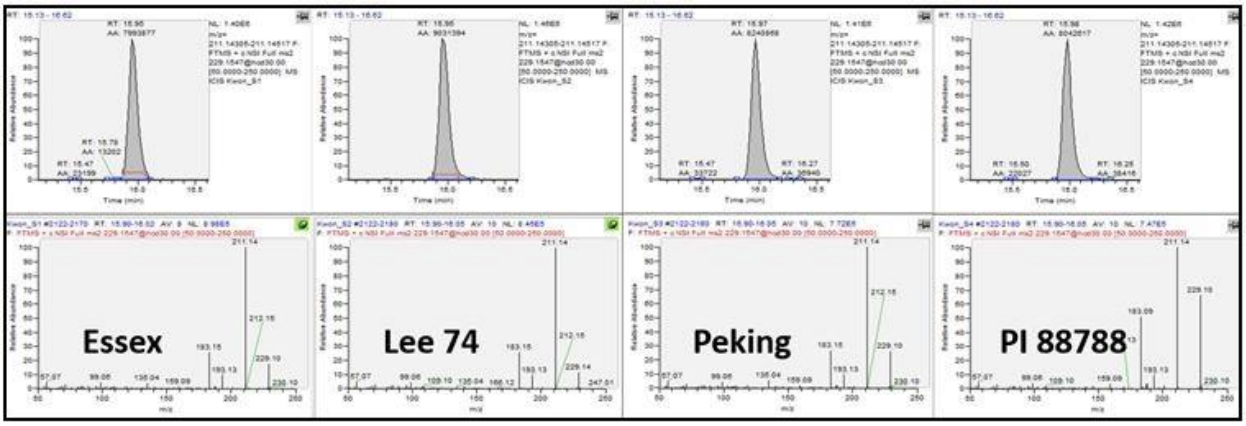
LC-MS/MS Analysis of α-Methyldethiobiotin (α-MDB) from Soybean Roots

We wanted to understand how a lack of *HgBioB* activity within the virulent SCN might aid the nematode. Our hypothesis was that having an inactive form of biotin synthase would be beneficial to a virulent SCN if part of the soybean defense mechanism is to deploy a dethiobiotin

(DTB)-related toxin, where virulent nematodes expressing a nonfunctional *HgBioB* would be protected from a soybean-produced antimetabolite. This class of antimetabolite would only become toxic if converted by an active biotin synthase. Hence, we speculated that this potential antimetabolite might be alpha-methyldethiobiotin (α-MDB) because it was the only DTB-related toxin reported in the literature (Hanka et al., 1972; Yahagi et al., 1974).

While α-MDB has not been detected in plants before, it seemed like a logical antinematode defense compound; thus, we attempted to detect this toxin, also called an antimetabolite, in soybean. To accomplish this, we opted to use nano-scale liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) and detected α-MDB as well as DTB in four soybean cultivars tested **(Figure 4.4A, Figure 4.4B)**. The ions at m/z 211.14 (α-MDB) and m/z 197.13 (DTB) were the most intense, indicating that both metabolites are naturally produced in both resistant and susceptible soybean cultivars. However, we could neither quantify nor compare the metabolite concentrations among the four soybean cultivars because of the small volumes of reagents used in the analysis.

(A)



(B)

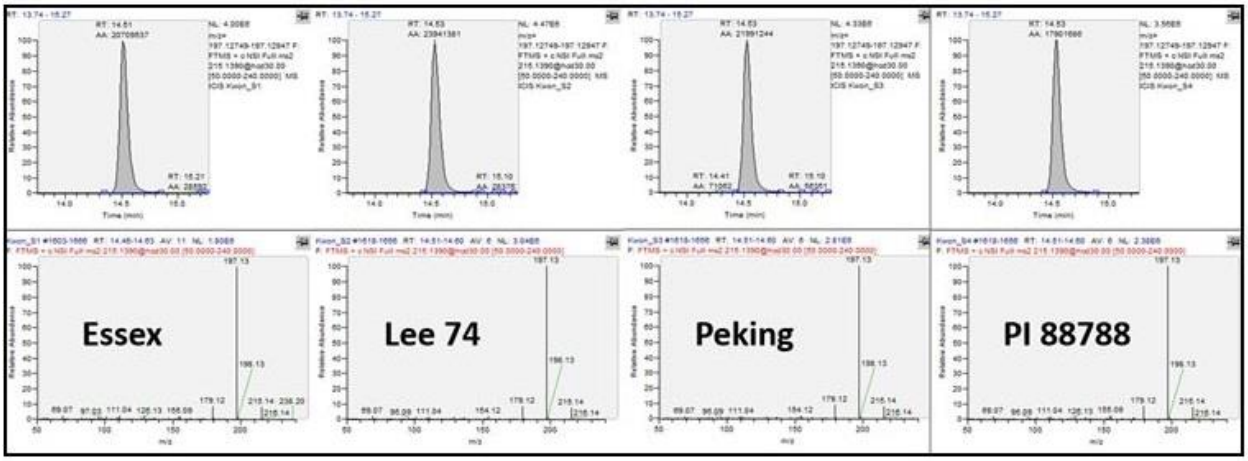


Figure 4.4. LC-MS/MS Analysis of α-Methyldethiobiotin (α-MDB) from Soybean Roots.
(A) The ions at m/z 211.14 correspond to α-MDB. (B) The ions at m/z 197.13 correspond to DTB. This indicates that both metabolites are naturally produced in both susceptible (cv. Essex and Lee 74) and resistant (cv. Peking and PI 88788) soybeans.

CHAPTER 5

Discussion

In this study, we compared the enzymatic activities of biotin synthase genes from virulent and avirulent inbred SCN strains and showed that the *HgBioB*-vir from virulent nematodes lacked activity while the *HgBioB*-avr from avirulent nematodes was fully active, suggesting *HgBioB* is important in SCN virulence.

The presence of this incomplete *de novo* biotin biosynthesis pathway implies that biotin has an important function in SCN and suggests the nematode is scavenging biotin from its host (Craig et al., 2009). Although free biotin can be present at high levels in the plant cells, unlike animal cells which have more protein-bound biotin (Alban, 2011), it remains unknown if sufficient free biotin is available to SCN as they feed from syncytial cells of high metabolic activity. It is possible that SCN, a filter feeder that does not consume plants via chewing, cannot obtain enough biotin if it is bound to proteins. That is, their prevalence for withdrawing nutrients via feeding tubes, acting as a molecular sieve (Böckenhoff and Grundler, 1994), may limit their vitamin ingestion, and having the biotin synthase gene, or other vitamin B biosynthetic genes, could be a selective advantage.

We previously predicted that virulent nematodes might gain a competitive advantage by producing a more enzymatically active biotin synthase (Bekal et al., 2015) and utilizing the precursors to expand its salvage pathways (Craig et al., 2008; Craig et al., 2009). In our complementation study, however, only the avirulent allele of *HgBioB* (*HgBioB*-avr) successfully complemented the growth of an *E. coli* strain containing a deletion of bioB (ΔbioB). This

showed that avirulent inbred SCN produces an active biotin synthase while virulent inbred nematodes contain an inactive form of the enzyme, thereby refuting our hypothesis.

However, it is possible that the inbred nature of the nematodes selected for a rare *HgBioB* allele that was inactive. To assess how common *HgBioB*-vir alleles are in natural populations of SCN, the *HgBioB* gene was PCR-amplified and sequenced from a series of field SCN populations. The sequencing data showed that both SNPs were present in the field SCN populations, resulting in a mixture of both active and inactive forms of *HgBioB.* In most SCN populations the virulent forms were prevalent, mirroring the increased prevalence of virulent SCN described by many nematologists (Mitchum et al., 2007; Niblack et al., 2008; Niblack and Riggs, 2004; Tylka and Mullaney, 2015). This implies that an opposing selection for *HgBioB* activity is occurring in the wild. There also seems to be an important, but unknown reason why the SNP changes occur concurrently in pairs with similar frequencies between amino acid positions 24 and 44. We initially speculated that these amino acid sequence polymorphisms might affect the overall biotin synthase activity if these SNPs were located close to the conserved regions responsible for substrate-binding or catalytic activity of the enzyme. However, these SNPs were not in the conserved $C^{53}xxxC^{57}xxC^{60}$ motif (C, cysteine; x, any amino acid), a key characteristic of biotin synthase and other enzymes in the radical S-adenosyl-methionine superfamily (Berkovitch et al., 2004; Frey and Booker, 2001; Jarrett, 2003). Both the presence of sequence polymorphisms at amino acid positions 24 and 44, and the evidence from the site-directed mutagenesis indicating the requirement for both proline and arginine at those specific locations (i.e., P24 and R44) further stress the importance of these SNPs in spite of their unknown functional role in the *HgBioB* enzyme. Since our sequencing data showed that these SNPs did not occur randomly but changed in a coordinated fashion, these SNPs might lead to a

complete change in protein function other than biotin synthase. It is even possible that these SNPs might convert the *HgBioB* enzyme into a toxin-binding protein that may protect the virulent nematode from host defense mechanisms.

The most puzzling aspect of this study is that virulent nematodes still express the *HgBioB* protein (Craig et al., 2009), even if it is an inactive form of the enzyme. For a virulent SCN, expressing a nonfunctional biotin synthase would only make sense if, for some reason, it were disadvantageous to produce an active *HgBioB.* A possible explanation for losing this enzyme activity might be that the nematode is avoiding a plant deployed DTB-related toxin, which only works when the parasite has an active biotin synthase. In this scenario, a potential DTB-related antimetabolite produced inside the roots gets consumed by the nematode, acts as a substrate for biotin synthase and, if converted, becomes toxic to the nematode. This would be harmful to avirulent nematodes which produce an active enzyme, though it would not affect virulent nematodes thanks to their inability to process this antimetabolite. Virulent SCN might evade this DTB-related toxicity and have a survival advantage; however, it may become unhealthy, or even grow and reproduce slower, due to the absence of biotin scavenging ability of an active *HgBioB.*

Because α-MDB was the only reported DTB-related antimetabolite (Hanka et al., 1972; Yahagi et al., 1974) that would become toxic if converted to α-methylbiotin (α-MB) by an active biotin synthase, we speculated that α-MDB could be this potential antimetabolite produced by soybean. Certain bacterial species, including some of *Streptomyces* spp., have been found to produce biotin antimetabolites, such as α-dehydrobiotin, α-MDB, and α-MB, which have antibacterial properties (Hanka et al., 1966; Hanka et al., 1969; Hanka et al., 1972). Among these biotin analogs, α-MDB, also known as libramycin A, is a fat-soluble and weakly acidic substance reported to have an antimicrobial effect against some bacteria and fungi (Hanka et al., 1972;

Yahagi et al., 1974). Structurally speaking, α-MDB is a DTB molecule with a methyl group attached to the alpha carbon (C_α), the first carbon next to the carbonyl carbon. Due to its structural similarity to DTB, biotin synthase may also catalyze α-MDB and convert it into α-MB **(Figure 5.1)**; only in this instance, α-MB becomes the active form of the toxin, unlike the normal biotin molecule. These two biotin analogs, α-MDB and α-MB, are very similar to the regular biotin in that they all have the carboxyl group necessary for binding to biotin-dependent carboxylases (S Paparella et al., 2014). However, once incorporated into a biotin-dependent enzyme, the enzyme's carboxylase activity is inhibited due to the structural modification of biotin (Piffeteau et al., 1980). Inactivation of essential carboxylase activities will not only hinder vital metabolic processes, but also fail to maintain mitochondrial function leading to mitochondrial toxicity (Depeint et al., 2006b; Rodríguez-Meléndez et al., 2001). If some of the bacteria are able to produce these biotin antimetabolites, it is also possible that some plants might do a similar thing to deter microbial/plant-parasitic nematode infections.

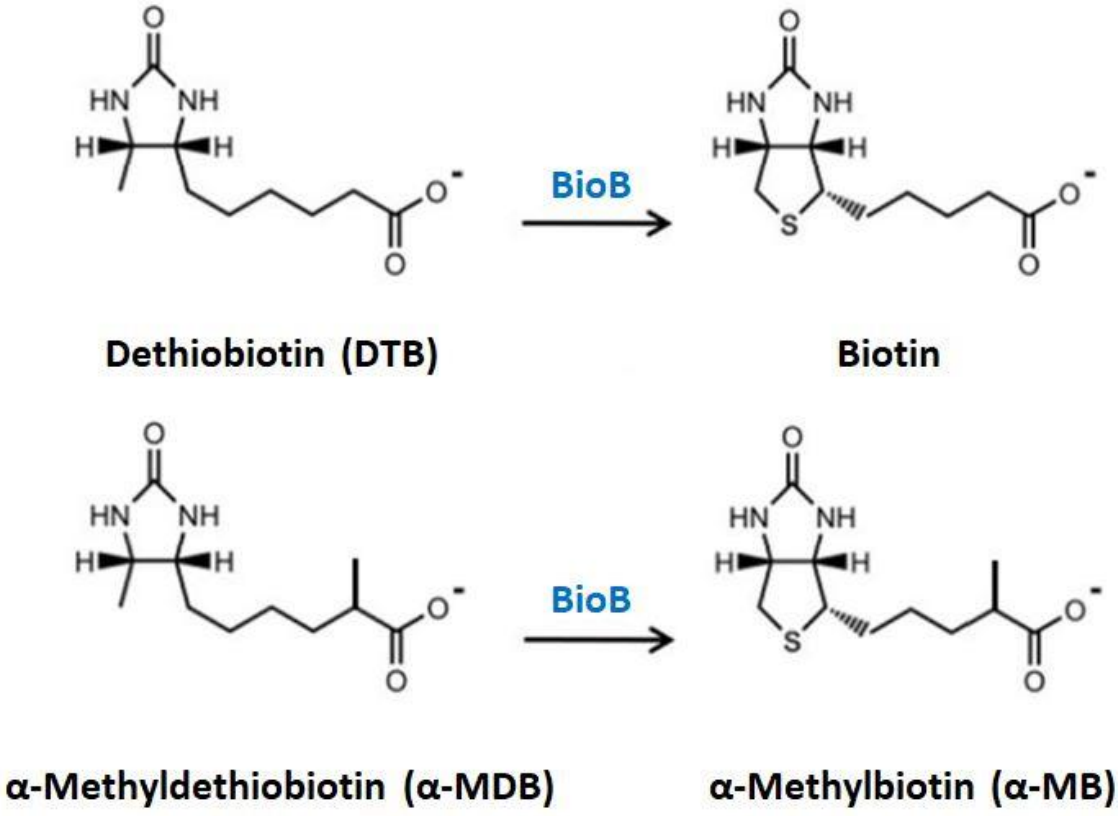


Figure 5.1. Reaction schemes for biotin synthesis and α-Methylbiotin (α-MB) synthesis.
Similar to the conversion of DTB to biotin, biotin synthase may also catalyze the conversion of α-MDB to α-MB. This is due to the structural similarity between α-MDB and DTB; α-MDB is a DTB molecule with a methyl group attached to the alpha carbon. Upon conversion, α-MB becomes the active form of the toxin, unlike the normal biotin molecule.

Our LC-MS/MS analysis confirmed the presence of α-MDB in the roots from both resistant and susceptible soybean cultivars. This is the first report of soybean or any plant producing α-MDB, which could be another possible plant chemical defense mechanism. The chance of these metabolites originating from bacterial contamination can be ruled out because the seedlings were surface-sterilized and aseptically germinated. The fact that both α-MDB and DTB were detected in soybean, and the observation that the nematode only has the last key enzyme in the biotin biosynthetic pathway, further support the argument that SCN is scavenging the precursor DTB to convert it into biotin to improve its nutritional status. However, we now

hypothesize that there is a tradeoff—either produce an active biotin synthase for a higher biotin availability but with a higher chance of α-MDB poisoning in the host plant, or have an inactive form of the enzyme for a higher chance of survival within the plant but with a lower vitamin nutritional status. In other words, the avirulent nematode may have more access to biotin, but it would harm itself if the host has a high level of α-MDB, which would be converted to α-MB and eventually "cripple" its biotin-dependent carboxylases. On the contrary, the virulent nematode might have biotin deficiency due to its nonfunctional enzyme; however, this could be a great survival strategy if α-MDB cannot be converted to α-MB, or if it can be sequestered by the inactive enzyme functioning as an α-MDB-binding protein.

Because we opted for a highly sensitive detection method that is not very quantitative due to the small volumes of reagents used in the analysis, we could not make quantitative conclusions from this data regarding the level of α-MDB or DTB content in certain soybean cultivar(s). Yet, these analytes were detectable and the presence of these biotin analogs in soybean is of potentially great significance for understanding how nematodes might evade host defense mechanisms. Moreover, the production of α-MDB inside soybean might be another possible plant chemical defense mechanism to deter SCN infection. By developing and deploying soybean varieties with a high level of α-MDB, it might be possible to select for the virulent SCN populations in agricultural fields that carry the virulent/inactive alleles of *HgBioB*. These virulent nematodes would survive and reproduce on resistant cultivars, though they are likely to suffer from a lower nutritional status due to biotin deficiency, which could lead to future generations of virulent SCN populations having lower female indices, possibly resulting in the production of fewer viable eggs, and ultimately minimizing soybean yield losses affected by these devastating pathogens.

CHAPTER 6

Conclusions and Future Directions

In this study, a complementation experiment using mutant *E. coli* with avirulent and virulent *HgBioB* alleles was conducted to test the function of these two *HgBioB* alleles. We showed that avirulent nematodes produce an active biotin synthase while virulent ones contain an inactive form of the enzyme. We also conclude that in SCN field populations the *HgBioB* gene contains a diverse mixture containing both avirulent and virulent alleles, with the virulent alleles being more prevalent in nature. Furthermore, we showed that all soybean cultivars accumulate detectable levels of α-MDB, a dethiobiotin-related toxin found in bacteria, perhaps explaining why the virulent SCN would have the inactive form of *HgBioB*. We hypothesize that the virulent SCN lacking *HgBioB* activity could evade a dethiobiotin-related toxin defense mechanism in host plants.

In the future, it would be interesting to see if different soybean varieties produce differing levels of α-MDB. It would be necessary to determine if there is a significant difference in α-MDB levels between resistant and susceptible cultivars because resistant cultivars might have a higher amount of α-MDB, possibly contributing to their resistance traits. It would also be interesting to compare α-MDB levels before and after SCN infection to see if soybean produces more α-MDB after infection. Moreover, an Hg-Type test should be conducted along with the sequencing analysis for the SCN field populations to see if there is a positive correlation between the female index and the percentage of virulent alleles present within each field population. Furthermore, RNA interference (RNAi) knockdown experiments, although extremely challenging for certain organisms, have been shown to successfully work on some plant-parasitic

nematodes. If RNAi would successfully knockdown the active form of the *HgBioB* allele on the avirulent SCN, we expect that this avirulent nematode would behave more like a virulent SCN.

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