PEST MANAGEMENT FOR SCN BIOASSAYS AND CREATION OF NEW RNAI CONSTRUCTS FOR NEMATODE SUPPRESSION

by

CHAD R. BRADY

B.S, Kansas State University, 2010

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Approved by:

Major Professor
Dr. Harold Trick

Copyright

Abstract

The object of this study was to find a target sequence for the known *Heterodera glycines* Y25 sequence that contained no homology to any known *Glycine max* genes so homologous endogenous soybean gene expression will not be effected. In addition, in attempt to improve the accuracy of SCN bioassays performed in greenhouse settings, applications of a variety of insecticides with differing modes of action were applied to screen for any detectable effects on the SCN populations. The full-length sequence of the Y25 gene was blasted against the *G. max* genome using the National Center for Biotechnology Information blast database and a portion of the gene was found to contained no homology to the *G. max* genome. A rapid hairy root assay was used to screen for resistance to *H. glycines.* The sequence was transformed into *Agrobacterium rhizogenes* using a modified heat shock method. The transformed *A. rhizogenes* were used to inoculate soybean seedlings. The inoculated seedlings developed hairy roots expressing the target sequence. Upon finishing the hairy root assay it was discovered that there were no detectable differences across any of the treatments or the controls. It was neither proved nor disproved that the new target sequence containing no homology to the *G. max* genome was as effective as the original target. Further investigation will need to be conducted to show the level of control for the new target sequence.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	
AS	Acetosyringone
dsRNA	Double stranded RNA
GOI	Gene of Interest
LB	Lysogeny broth
MS	Murashige and Skoog Salts
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNAi	RNA interference
SCN	Soybean Cyst Nematode

Acknowledgements

I would like to thank my advisor Dr. Harold N. Trick for his guidance and financial support during my studies in addition to providing me the opportunity to study at Kansas State University. I would also like to thank Mr. Timothy C. Todd for allowing me to use his laboratory and his valuable expertise in statistics to help with experimental design and data analysis. In addition I would like to thank Mr. Thomas Oakley for sharing his lab space and teaching me valuable techniques that I needed to work with soybean cyst nematode. I am grateful to all the members of the laboratory for their direct and indirect assistance with my studies.

I am extremely grateful to Dr. Jiarui Li for all of the help and guidance that he gave me. This work would not have been possible without Dr Li. I am thankful that he was always willing to answer my endless questions and his ability to explain concepts, techniques, and theories in great detail.

I would also like to thank Jessica Rupp who gave me endless encouragement to complete my writing. She also gave me many tips and suggestions for effective writing and time management. Thank you for being my voice of reason.

Dedication

I would like to dedicate this work to my wonderful wife Amanda, for her kindness and devotion and for her endless support through it all; her love will always be dear to me.

Chapter 1 - Literature Review

Importance of Soybean

Soybean production is an integral part of North American agriculture; in 2011 the US soybean production was valued at almost $35.8 billion (USA, 2012). Since 1980 the World Agricultural Outlook Board has published a monthly World Agricultural Supply and Demand Estimates (WASDE) report and these reports provide forecasts of the supply and demand covering major global crops. The July 11, 2012 WASDE report estimated that the global soybean production to be 235.88 million metric tons (MMT) or approximately 8.67 trillion bushels during the 2011/2012 growing season. During this same growing period 177.37 MMT of soybean meal was produced worldwide, that is the equivalent to 8.23 billion bushels. Additionally, 41.23 MMT of soybean oil was produced during the 2011/2012 season. During this same growing season approximately 102 million hectares were planted to soybean worldwide (United States, 2012). The three largest producers of soybeans during that period were the United States, Brazil, and Argentina producing 83.2, 65.5 and 41 million metric tons, respectively.

Soybeans are a highly nutritious food consisting of 446 Cal for every one hundred grams of mature raw soybean seeds (United States, 2011). The 235.88 MMT of soybean harvested during the 11/12 growing season can be converted to a nutritional value of 1,052 trillion Cal. Soybeans are the only crop plant that is able produce protein that is equal in quality to animal protein sources (Hartman *et al.*, 2011). Soybean serves as a valuable human food source, including soy nuts, soy milk, and tofu (Singh, 2010). Tofu is an excellent alternative to milk, and cheese products providing a rich source of protein, vitamins and minerals for those that are lactose intolerant. Soybean is also used in instant

foods and bakery products. Oils extracted from soybeans have a variety of uses. These include edible uses such as mayonnaise, dressing, salad oil, cooking oil, and shortening. In addition to edible products, soybean oil can also be used in a variety of industrial products such as lubricants, biodiesel, candles, adhesives, auto polish, and a variety of cleaning agents. Bandyopadhyay-Ghosh and colleges have been able to convert soy oil to soy foam that is comparable to other foam products produced from petrochemicals (Bandyopadhyay-Ghosh *et al.*, 2010). This provides the industry with renewable and biodegradable alternative petrochemical based foams.

Soy products are also a very effective feed source. High fiber diets are required in ruminants and the hulls of soybean make an excellent source of this required fiber. Soybean is additionally used in a variety of other animal feeds including fish and pet foods.

Oil can be extracted for soybeans when processed correctly. These oils have a variety of uses including edible applications. Some of the edible uses include mayonnaise, salad oil, dressing, cooking oil, and shorting. In addition to edible products soybean oil can also be used in a variety of industrial products such as lubricants, biodiesel, candles, adhesives, auto polish, and a variety of cleaning agents.

Impact of Pathogens on Soybean Production

Soybean is victim to many different pathogens that cause economic and yield losses. In 2010 Koenning and Wrather expressed the importance of concentrating limited resources on researching management of the most important pathogens that cause the most extensive damage. They compiled estimates of soybean yield potential losses caused by diseases over a four-year period. During this four-year period they estimated

that pathogens caused a loss of over 1.6 billion bushels (Koenning and Wrather, 2010). For example, seedling diseases as a whole has the second greatest economic impact on soybean yields with an average yield loss of a little over 46 million bushels during 2006 to 2009. *Phytophthora sojae* (Phytophthora root and stem rot), the third most damaging soybean pathogen, caused yield losses in most states with an average yield loss of over 43 million bushels (Koenning and Wrather 2010). *Fusarium virguliforme*, the causative agent of sudden death syndrome, was the fourth most damaging biological pathogen of soybean resulting of an annual yield loss of over 26 million bushels (Koenning and Wrather, 2010). Of the 23 different diseases included in the data set, soybean cyst nematode (SCN) consistently causes more damage than any other disease. In fact, SCN causes more damage than the second, third, and fourth most damaging pathogens combined. These facts demonstrate the importance of controlling pathogens in general and, more importantly, SCN to minimize future yield losses.

Soybean cyst nematode, *Heterodera glycines* originated in China and was first discovered in North Carolina in 1954 (Winstead *et al.*, 1955). Since then SCN has spread to every soybean producing state in the United States (Schmitt *et al.*, 2004) and has been been spread globally in all soybean producing areas have efficiently (Schmitt *et al.*, 2004). This makes soybean cyst nematode an important yield limiting factor not just for US producers but across the entire globe. Losses due to the soybean cyst nematode accounted for over $120 million in the US alone (Koenning and Wrather, 2010).

Current control strategies

There are three main practices producers have used for nematode control with limited success and each has their own challenges. First, rotation to non-host crops can be used to decrease initial populations. In the absence of the host the number of eggs and juveniles decrease relatively quickly (Schmitt *et al.*, 2004). However, in the first year of a host deployment SCN populations have the potential to rapidly increase. Rotations to non-host crops can lower the SCN population enough to increase yields. In a study conducted by Koenning it was found that a rotation to a non-host for one or two years increased soybean yields by 10% to 40% when compared to yields from similar infested fields of monoculture soybean. (Koenning *et al.*, 1993)

A second cultural practice that has been implemented is the use of nematicides. However, nematicides are very expensive for producers to use (Carter, 2001), making the cost prohibitive for lower value crops. Nematicides have also been found to contaminate ground water. In Hawaii 1,2-Dibromo-3-chloropropane (DBCP), a nematicide previously used in pineapple production, was detected in several wells on Oahu and Maui (Oki and Giambelluca, 1987). Many of the nematicides, such as DBCP, that were used in the past have been banned from use because of health concerns. (EPA, 2007)

Third, Plant resistance has also used to minimize losses caused by soybean cyst nematode. The major concern with genetic resistance is that many of available resistant varieties have a limited genetic background. Almost 96% of the current resistant cultivars come from the same source of resistance, PI 88788 (Faghihi and Ferris, 2012). The majority of North American breeders are using the major resistance gene *rhg* 1 (Concibido *et al.*, 2004). It is risky having a major gene so widely used. If a population of SCN overcomes a resistant variety containing *rhg* 1, all other varieties that rely on *rhg*

1 will be ineffective. This does not leave producers with many other options for plant resistance.

Transgenic Approaches to Disease Resistance

The idea of pathogen-derived resistance (PDR) was published by Sanford and Johnston in 1985 (Sanford and Johnston, 1985). They believed that the genetic material of a pathogen could be used against itself. They believed that unique and essential molecules produced by a pathogen could be used against it. They proposed that if a host cell genome expressed a dysfunctional pathogen-derived gene product it could potentially inhibit the pathogen (Sanford & Johnston, 1985).

In 1986 Abel published work on transgenic tobacco plants over-expressing the viral coat protein of *Tobacco mosaic virus* (TMV). These plants were found to be resistant or have delayed symptoms when challenged with TMV (Abel *et al.*, 1986). The Dougherty lab working on pathogen derived resistance to *Tobacco etch virus* when they discovered that the mere presence of non-translated RNA cause resistance. This disproved their initial hypothesis that the *Tobacco etch virus* coat protein molecule caused the silencing event that led resistance of *Tobacco etch virus* (Lindbo and Dougherty, 2005).

The mechanism that causes RNAi to cause silencing events is that double stranded RNA must enters into a cell where it interacts with dicer (Bernstein *et al.*, 2001). The double-stranded RNA is cleaved by dicer forming small interfering RNA strands (siRNA). The sizes of these siRNAs are usually 19-22 base pair sequences (Hamilton *et*

al., 2002). dsRNA from an exogenous sources often triggers the cleavage and degradation of sequence specific mRNA (Cullen, 2004).

Application of this technology can be found in the market today in the form of transgenic papaya transformed to contain the coat protein gene of *Papaya ringspot virus* (Fitch *et al.*, 1992). This resistance was originally believed to be triggered by the expression of the viral coat proteins. This was later discovered that the resistance was actually due because of RNAi (Gonsalvase, 1998). In addition papaya virus resistant transgenic summer squash is also used. "The commercial release of squash cultivars derived from lines ZW-20 and CZW-3 has demonstrated the stability and durability of the engineered resistance over more than a decade." (Fuchs and Gonsalves, 2007). Additional RNAi derived resistance was reported in 2005 when Nicola-Negri *et al.* reported the application of a RNAi silencing event in *Nicotiana benthamiana* that conferred resistance to *Plum pox virus* (Nicola-Negri *et al.*, 2005)

Fire was able to show that by expressing RNA segments in the reverse (antisense) orientation to genes in *C. elegans* they could effectively silence that target gene. They also found that a fraction were able to transmit the defective trait to subsequent generations (Fire *et al.*, 1991). Fire *et al*. discovered that silencing a specific gene was possible through the injection of double stranded RNA into the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998). Maeda found that instead of injecting dsRNA into *C. elegans* soaking the nematodes in a solution containing dsRNA, it was possible to elicit a specific phenotype. This demonstrated that a suitable delivery method could be found in the oral uptake of dsRNA harnessing RNAi (Maeda *et al.*, 2001). This has coerced many researchers to pursue the use RNAi as a possible control for plant parasitic nematodes.

Some researchers are targeting parasitism genes to try and prevent yield losses. The theory behind targeting parasitism genes is that if the RNAi is able to shut down required parasitism genes, the soybean cyst nematode will not be able to form or maintain a feeding site. Huang and colleagues (2006) were able to engineer *Arabidopsis* to be resistant to a variety of root-knot nematode species by targeting a specific root-knot nematode parasitism gene through the expression of dsRNA to silence the (Huang *et al.*, 2006). However, the specificity of this target prevented it from being able to control *Heterodera schachtii*, a more distant species of nematode.

Some researchers have chosen parasitism genes essential for parasitism to be established and maintained for targeting. Four different parasitism genes were targeted by Sindhu *et al.* using a plant-expressed RNAi strategy in *Arabidopsis thaliana.* Decreases in *H. schachtii* reproduction ranging from 23% to 64% were achieved for these four targets (Sindhu *et al.,* 2008). Other researchers have been targeting genes necessary for reproduction, embryo development and overall fitness. Steeves *et al.* was able to suppress the rate of reproduction of SCN on soybean. This was done by targeting a gene that coded for a major sperm protein, a crucial gene for the nematode to complete its life cycle, though the use of plant expressed RNAi strategy preceding the work of Sindhu *et al.* (Steeves *et al.* 2006). Steeves was able to generate soybean plants that expressed double stranded RNA targeting a major sperm protein in SCN. A reduction of 49% in numbers of cysts per gram root was observed by Steeves *et al.* Additionally, reductions in numbers of eggs per gram root of 65% were observed (Steeves *et al.* 2006). Steeves *et al.* also found that the nematode offspring that came from nematodes feed on the MSP transgenic plants produced 75% fewer eggs per gram root when compared to the control

(Steeves *et al.* 2006). Other essential gene targets have been shown to be effective at controlling SCN (Li *et al.,*2010). Targeting the Y25 gene in SCN has previously been shown to decrease cysts per gram root by 62% in addition to a reduction of eggs per gram root of 81% and a suppression of eggs cyst per gram root 48% in hairy root assays (Li *et al.*, 2010).

Difficulties of greenhouse SCN bioassays

To assess any potential nematode resistance gene, it is critical to test its effect on nematodes in bioassays. For SCN, bioassays are most commonly performed in greenhouse settings. Greenhouse space is more readily available than growth camber space allowing for a larger number of total plants that can be tested in a given SCN bioassays. Greenhouse bioassays have more variables than growth chamber bioassays. Both greenhouse temperature and humidity are largely affected by outside greenhouse temperatures. Greenhouses using evaporative cooling systems are only capable of cooling the greenhouse by about 10° C. As the greenhouse is cooled by evaporative action humidity is additionally increased. Greenhouse humidity is also largely affected by seasonal conditions. Winter conditions are drier and cooler. The cooler temperatures do not call for evaporative action which prevents the greenhouses from increasing humidity above ambient levels. Growth chambers can better control multiple growing conditions including temperature, humidity, and amount of light. Both greenhouse and growth chambers can suffer from common greenhouse pests such as thrips, mites, aphids, and white flies. This is a variable that has previously been unaddressed.

The goal of this study was twofold. The first was to discover common chemical control practices that controlled common greenhouse pests without effecting nematode

populations of SCN bioassays. The second goal in this study was to target essential genes in the nematode; the theory is that targeting essential genes will be durable and consistent. Targeting more conserved essential genes may also give a broader spectrum of resistance to a large variety of nematodes. The targets selected are conserved between *C. elegans* and *H. glycines.* It is believed that a gene that is conserved between two different species will be conserved across different SCN races/HG types and potentially a broader range of nematode. This is important for the durability and application of the transgene.

Chapter 2 - Compatibility of foliar insecticides and *H. Glycines* bioassays

Introduction

Standardized methods for assessing soybean cyst nematode *(Heterodera glycines*) resistance in soybeans (*Glycine max*) have been developed, with the assignment of resistance or susceptibility based on relative numbers of females/cysts that develop on the roots (Niblack, 2009). However, severe infestations of greenhouse pests such as thrips, aphids, mites, and white flies can compromise bioassay results due to the impact of these aboveground pests on soybean plant health. It has been found that the presence of above ground insect herbivory actually facilitates root feeding nematode populations (De Deyn *et al*., 2007). It has also been found that chewing insect herbivores cause positive plant-mediated interactions for root feeding nematodes while sap-feeding insects cause negative interactions (Kaplan *et al.*, 2009). Above ground herbivory has the potential to both aid and inhibit nematode populations thus aiding an additional variable in standard greenhouse bioassays.

Chemical control of insect pests in the greenhouse raises additional concerns about the direct and indirect effects that even foliar-applied insecticides, through runoff, may have on *H. glycines* development or reproduction. While the non-target effects of direct exposure to insecticides commonly used to control crop pests have been extensively studied for entomopathogenic nematodes (Head, 2001), information on the compatibility of conventional insecticides and greenhouse bioassays involving root-parasitic nematodes is lacking. Nematode species can differ in their susceptibility and directional response to chemical pesticides. Studies have shown, for instance, that some

pesticides can actually increase the activity of infective juveniles of entomopathogenic nematodes (Gaugler, 1991).

Materials and Methods

The objective of the present study was to investigate the effect of foliar-applied insecticides on *H. glycines* reproduction in greenhouse bioassays. In two independent trials, a total of eight different insecticides including abamectin (Avid), bifenazate (Floramite), spiromesifen (Judo/Forbid), spinosad (Conserve), imidacloprid (Marathon II), chlorfenapyr (Pylon), hexythiazox (Hexagon), dinotefuran (Safari), and an abamectin+hexythiazox mixture were used to treat 'Lee' soybean plants grown in D40 Deepots (Stuewe and Sons, Inc., Corvallis, OR) containing soil infested with *H. glycines* HG type 7. Initial nematode population densities averaged 25,000 and 15,480 eggs/100 cm^3 soil for trials 1 and 2, respectively. All insecticides were applied at label recommended concentrations with a hand sprayer at a medium mist for maximum coverage. There was no attempt to shield the soil from overspray or insecticide run-off from the sprayed foliage.

In addition to the insecticide compounds used, each trial consisted of three application timing treatments: group 1 consisted of plants that were treated with insecticides 10 days after planting to day 35; group 2 consisted of plants that were treated with insecticides from 35 days after planting to day 70; and group 3 consisted of plants that were treated with insecticides from 10 days after planting to day 70. After the first initial spray application, all treated soybean plants were sprayed with insecticides every 10 days. Non-sprayed 'Lee' plants served as controls for each group. The experimental

design was a split-plot with insecticide treatments as whole plots and application treatments as subplots. Whole plots were arranged in a randomized complete block with three replications. Plants were destructively harvested to obtain cyst and egg counts after 35 days for group 1 and 70 days for groups 2 and 3. Females and cysts were dislodged from roots with water spray and collected on a 250-µm-pore sieve. Cysts were mechanically ruptured to release eggs and second-stage juveniles. Cyst and egg counts were $\log_{10}$-transformed to minimize heterogeneity of variances prior to statistical analysis using SAS PROC MIXED.

Total cyst and egg counts on a per pot basis averaged 109 and 15,117, respectively, across insecticide treatments and trials. No effects ($P \leq 0.05$) on nematode reproduction were observed for any of the insecticide treatments, regardless of application regime. Mean numbers of *H. glycines* cysts and eggs on a per g dry root weight basis are reported for insecticide treatments in both trials in Figure 1.

There were three different groups of plants tested. Group 1 consisted of plants that were treated from day 10 to day 35. Group 2 consisted of plants that were treated from day 35 to day 70. Group 3 consisted of plants that were treated form day 10 to day 70. This experiment consisted of racks of 20 containers which had 3 seeds in each container. The containers were arranged in 4 rows of 5, this setup had 6 containers that were surrounded on all sides by other containers. These 6 containers that were surrounded were used for cyst and egg counts. They were used to prevent skewed data collection from the borders. Each rack was sprayed 10 days after planting, then again every 10 days. Group 1 was broke down for cyst and egg counts after 35 days. The plants that were removed were replaced with plants from group 2 which had been grown

under the same conditions but were not sprayed. Group 3 remained in the racks for all of the sprays. After 75 days both groups 2 and 3 were broken down for cyst and egg counts.

Results and Discussion

It was discovered that for all insecticides there were no detectable difference for nematode reproduction rates between the treated and non-treated samples (Table2-3). However, there were detectible statistical differences between the three timing groups. This was expected due to varying lengths of time allowing for varying nematode to reproduce. Neither above soil visible differences nor any statistical differences of the nematode cyst and egg counts were observed. Of the varying active ingredients some have been found to be effective nematicides such as avermectins (Putter et al, 1981). Avid (abamectin) has been granted a 24(c) Special Local Need label to control sting and ring nematodes for North Carolina. It was important to use a variety of pesticides because the pests that cause problems can change from screening to screening. It was also important to use pesticides with differing modes of action because the different modes of action may have varying effects on nematodes.

The current data points to no differences between the reproductive rates of nematode populations that feed on untreated soybeans and those that feed on soybeans treated with foliar applied insecticides/miticides. This is relevant since it would allow researchers to use these compounds at any point during the trial with no concern of adding an additional variable that would affect SCN count data concerning resistance. This also may open opportunities for a broader range of pesticide applications. Avid, which is known to have nematicidal properties, did not show any effect on the nematode

population However, more data needs to be collected to give a definitive answer as to whether or not the reproductive rates would be affected by higher application rates, different modes of application, or a soil applied pesticides. Because there was no significant insecticide runoff this experiment only tested indirect interactions between the insecticides and the nematode populations. It is feasible that the insecticides could have caused physiological changes in the plants that influenced nematode reproduction or egg hatching. However, since no differences in nematode populations were detected, such speculations are moot. This experiment had no attempt to shield spray runoff, but the amount of insecticide that entered the soil is most likely negligible. A future experiment could be performed to check for direct interactions by soaking the nematode eggs in the diluted insecticide before inoculating the soil with stated eggs.

In conclusion, our work demonstrated compatibility between eight different commonly used insecticides and *H. glycines* greenhouse bioassays. This result alleviates concerns of non-target effects for a range of contemporary insecticides available for control of greenhouse pests during *H. glycines* bioassays and screening trials. Better control of greenhouse insect pests may, in turn, lead to more uniform results in nematode trials.

Table 2-1 Foliar-applied insecticides/miticides used in *Heterodera glycines* greenhouse trials.

Common Name	Trade Name	Primary Target Site of Action
Abamectin	Avid	Gama amino butyric acid
Bifenazate	Floramite	Compounds of unknown mode of action
Spiromesifen	Judo/Forbid	Inhibitors of acetyl CoA carboxylase
Spinosad	Conserve	Nicotinic acetylcholine receptor allosteric activators
Imidacloprid	Marathon II	Nicotinic acetylcholine receptor agonists
Chlorfenapyr	Pylon	Uncouplers of oxidative phosphorylation
Hexythiazox	Hexagon	Mite growth inhibitors
Dinotefuran	Safari	Nicotinic acetylcholine receptor agonists

Table 2-2 Fixed-effects analysis of variance for log_{10}-transformed numbers of *Heterodera glycines* cysts and eggs recovered from insecticide-treated and non-treated Lee soybean.

		F Value			
	DF	Cysts/pot	Cysts/g root	Eggs/pot	Eggs/g root
Application	2, 10	1.20	7.15*	8.09**	18.00**
Insecticide	9, 24	0.89	0.63	0.67	0.54
Application × Insecticide	18, 282	1.02	1.61	0.88	1.63

* $P \leq 0.05$, ** $P \leq 0.01$.

Table 2-3 Log_{10}-transformed numbers of *Heterodera glycines* cysts and eggs recovered from insecticide-treated and non-treated Lee soybean.

	Means ± standard error			
	Cysts/pot	Cysts/g root	Eggs/pot	Eggs/g root
Insecticide				
Abamectin	1.97 ±0.09	2.16 ±0.10	4.10 ±0.13	4.30 ±0.14
Abamectin + Hexythiazox	2.09 ±0.13	2.27 ±0.13	4.21 ±0.18	4.39 ±0.18
Spinosad	1.81 ±0.13	2.01 ±0.13	3.88 ±0.18	4.08 ±0.18
Bifenazate	2.01 ±0.13	2.18 ±0.13	4.13 ±0.18	4.30 ±0.18
Hexythiazox	2.05 ±0.13	2.19 ±0.13	4.22 ±0.18	4.36 ±0.18
Spiromesifen	2.09 ±0.13	2.26 ±0.13	4.08 ±0.18	4.25 ±0.18
Imidacloprid	2.09 ±0.09	2.23 ±0.10	4.25 ±0.13	4.40 ±0.14
Chlorfenapyr	2.14 ±0.13	2.27 ±0.13	4.17 ±0.18	4.31 ±0.18
Dinotefuran	1.91 ±0.13	2.09 ±0.13	3.90 ±0.18	4.08 ±0.18
Control	1.92 ±0.09	2.11 ±0.10	4.07 ±0.13	4.26 ±0.14
Application				
Days 10-35	2.10 ±0.09	2.39 ±0.09	4.28 ±0.10	4.58 ±0.12
Days 35-70	1.95 ±0.09	2.15 ±0.09	4.06 ±0.10	4.24 ±0.12
Days 10-70	1.97 ±0.09	2.00 ±0.09	3.95 ±0.10	4.00 ±0.12

Figure 2-1 Soybeans in SCN bioassay 13 days after planting in random block design.

Figure 2-2 Soybeans in SCN bioassay 20 days after planting in random block design.

Some of the content of this chapter appeared in Brady, C. R., Li, J., Todd, T. C., Oakley, T. R., and Trick, H. N. 2012. Compatibility of foliar insecticides and soybean cyst nematode bioassays. Plant Health Progress doi:10.1094/PHP-2012-0409-01-BR. http://www.plantmanagementnetwork.org/sub/php/brief/2012/bioassay/bioassay.pdf

Chapter 3 - Comparison of Nematode Responses of Different RNAi Target Sequences within the Y25 Gene Using Hairy Root Bioassays of Soybean

Introduction

Soybean cyst nematode (SCN) is the number one yield limiting pathogen in the United States decreasing yields in 2010 by 118,129,000 bushels (Wrather and Koenning, 2012). It is important that producers have more options to control the pathogen. Rotations incorporating non-host crops and resistant varieties currently represent the primary strategy for managing nematode populations in infested fields. The practice of applying nematicides to control the SCN populations historically has been very costly and introduces toxic compounds into the environment. Plant resistance is a reliable control practice that, along with being cost effective, is also environmentally friendly. The issue with plant resistance is that it relies on a narrow host genetic background to control diverse pathogen populations. New sources of resistance need to be discovered and deployed to replace current resistant sources that are breaking down.

Novel approaches to pest management may provide the necessary options to complement traditional strategies. One promising approach is RNA interference (RNAi). The principle behind RNAi is that double stranded RNA enters into a cell where it encounters dicer (Bernstein *et al.*, 2001). Dicer cleaves the double-stranded RNA creating small interfering RNA molecules (siRNA) that are usually 19-22 base pair sequences (Hamilton *et al.*, 2002). The cleavage and degradation of sequence specific mRNA is often triggered by dsRNA from an exogenous source (Cullen, 2004). It was discovered that double stranded RNA injected into the nematode *Caenorhabditis elegans*

had the ability to silence a specific gene (Fire *et al.*, 1998). Maeda found that when *C. elegans* was merely soaked in a solution containing dsRNA, rather than injection, it was possible to elicit a specific phenotype, demonstrating that oral uptake was a suitable delivery method for harnessing RNAi (Maeda *et al.*, 2001). This led to the attempt to use RNAi as a potential control of plant parasitic nematodes. Some researchers chose to target genes that were essential for parasitism to be established and maintained. (Sindhu *et al.*, 2008) targeted four different parasitism genes with a plant-expressed RNAi strategy in *Arabidopsis thaliana* and were able to demonstrate decreases in *Heterodera schachtii* reproduction ranging from 23% to 64%. Other researchers chose to target essential genes that were required for the nematode to be able to complete the lifecycle. Prior to Sindhu's work, Steeves *et al.* were using a similar plant-expressed RNAi strategy to suppress the rate of reproduction of SCN on soybean through the targeting of the essential gene that coded for a major sperm protein (Steeves *et al.* 2006). Steeves *et al.* observed a reduction of 49% in numbers of cysts per gram root. They also observed a reduction in numbers of eggs per gram root of 65% (Steeves *et el*., 2006). Additionally, Steeves et al. discovered that offspring of nematodes that feed on the MSP transgenic plants produced 75% fewer eggs per gram root compared to the control (Steeves *et al.* 2006). These experiments above show promise for potential long-term control of plant parasitic nematodes.

Y25, another promising candidate for RNAi targeting encodes a beta subunit of the coatomer one (COPI) complex, which is important for cellular movement of vesicles. The COPI complex is responsible for moving proteins from the endoplasmic reticulum to the Golgi complex over long distances (Stephens *et al.*, 2000). RNAi assays using *C.*

elegans showed that Y25C1A.5 was required for fertility, adult viability, osmoregulation, and general health (Kamath *et al.*, 2003).

Targeting the Y25 gene in SCN has previously been shown to decrease cysts per gram root by 62% in addition to a reduction of eggs per gram root of 81% and a suppression of eggs cyst per gram root 48% in hairy root assays (Li *et al.*, 2010). Once the target dsRNA sequence was transformed into stable lines, however, it was noticed that those lines exhibited negative phenotypes. Many plants had a stunted growth pattern with low yields. Most of the plants either did not produce seed or produced very low numbers of seeds (Li *et al.*, unpublished). These phenotypes could be caused by either a problem in during the tissue culture process such as somal-clonal variation or could be caused by "off target effects" caused by homology between the target sequence and the soybean plant genes.

The goal of this study was to determine whether the stunted phenotype was caused by off-target effects, whereby the original target sequence had homology to soybean genes. An additional goal was to find a new target sequence in the SCN Y25 gene that was at least as effective as the original Y25 target but contained no known homology to the soybean genome. It is critical that the new construct can still provide good levels of resistance to SCN or it will be of less value for future control application. Four treatments were used in this experiment: four constructs and one empty vector. The first construct used was the original Y25 construct that was previously proven to provide good control of SCN in previous experimentation (Li *et al.*, 2010) but had a negative phenotype in stable lines (Li, *et al.* unpublished). The second construct used (Y25-1) was a new construct that had zero homology to soybean. The third construct used (Y25-4)

was a new construct that had had more homology to the soybean genome than the original construct. This third construct would theoretically cause similar negative phenotypes in stables if the phenotype was caused by an off target silencing event. Two controls were selected for this study, the first being plants that contained positively transformed hairy roots containing an empty vector. This control was selected as a negative control that was used as a susceptible check. The second control was plants that had positively transformed roots and were expressing dsRNA that targeted a different organism. This control was selected to make sure that the mere presence of dsRNA was not contributing to resistance but that the dsRNA had to have a specific target in the nematode.

Materials and Methods

In Silica Analysis

The full length DNA sequence for the SCN Y25 (Figure 3-1) gene was recovered by sequencing SCN DNA using Y25 primers. This sequence was then blasted using a BLASTN algorithm against the entire completed soybean genome to find areas of homology. A target was then selected using sequences from the 5' portion of the SCN Y25 gene which had little homology with the soybean homolog. Primers for sequence amplification were designed using Primer 3 program (http://frodo.wi.mit.edu/). The sequence of "CACC" was added to the forward primer for directional cloning using pENTR directional cloning TOPO Cloning kit (Invitrogen, Carlsbad, Ca).

SCN DNA isolation

DNA was isolated using a protocol modified from the original protocol from the Fire lab for isolating genomic DNA from *C. elegans*. To 500 µl aliquot of nematodes in

a 15 ml tube 4.5 ml of worm lysis buffer was added. To the 15 ml tube 200 µl of 20 mg/ml Protease K was added and vortexed. One hundred µl of RNaseA was added to the tube. The tube was incubated for 60 minutes at 65° C. During this incubation time the tube was vortexed 5 times. As the worms disintegrated the solution became clear. After the incubation period 5 ml of TE saturated phenol:chloroform was added and vortexed aggressively for one minute. Tubes were centrifuged for 10 minutes at 10,000 rpm. The aqueous layer was extracted and transferred to a new 15 ml tube. To this tube 500 µl of 3M NaOAc was added. Aliquots were then made by pipetting 300 µl volumes in 1.5 ml microfuge tubes. To each tube 750 µl of ice cold 95% EtOH was added and mixed by inversion. Tubes were then centrifuged at 4 ° C for 5 minutes at 10,000 RPM. The pellet was then washed with 70% EtOH and allowed to dry. The pellet was then resuspended in 50 µl of TE and stored at -80° C.

Construct Preparation

Target Y25-1 was amplified through PCR using Y25-1 forward and reverse from Race 3/HG type 7 SCN isolated DNA. Samples were run on Labnet Multigene thermocycler (MidSci, St. Louis, Mo) using the forward and reverse primers (Table 3-3) with the first cycle at 94 °C for 5 minutes followed by second cycle of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 45 seconds repeated 32 times. The last extension cycle was at 72 °C for 10 minutes. PCR products quantified on the Nanodrop model N-1000 (Nanodrop, Wilmington, DE) and were also run on 0.8% agarose gel to confirm the presence of the desired product. The PCR product was mixed with the pENTR™ Directional TOPO® vector using kit (Invitrogen, Grand Island, NY). The TOPO® Cloning Reaction was performed by mixing 2 µl of fresh PCR product, 1 µl of

salt solution, 1 µl TOPO® vector and 1 µl of sterile water so that the final volume was 5 µl. The reaction was gently mixed and incubated for five minutes at room temperature. Vials containing One Shot® (Invitrogen, Grand Island, NY) chemically competent *E. coli* cells had 2 µl of the TOPO® cloning added and were mixed gently. Tubes were incubated on ice for 30 minutes. Tubes were transferred to 42 °C water bath for 30 seconds. Tubes were immediately transferred to ice and had 250 µl of LB media (Table 3-2) and were allowed to incubate at 37 °C for one hour without shaking. Contents of tubes were evenly spread over LB plates containing 50 mg/l Kanamycin and allowed to incubate overnight in 37 °C.

Recovered colonies were streaked onto fresh LB plates containing 50 mg a.i./l kanamycin sulfate with autoclaved toothpicks and allowed to incubate overnight at 37 °C. Colonies were transferred to 5 ml culture tubes containing 2 ml of liquid LB media containing 50 mg a.i./l kanamycin sulfate. Tubes were placed on a shaker incubator running at 200 rpm for 16 hours at 37 °C. Plasmids were isolated using OMEGA E.N.Z.A. Plasmid Miniprep kit with provided protocol (Omega Bio-Tek, Inc., Norcross, GA). One ml of the contents of the tube were transferred to 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for one minute at room temperature. Tubes were decanted. The remainder of the culture tubes contents were added to the microcentrifuge tubes and again pelleted at 13,000 rpm for one minute at room temperature. The pellets were completely resuspended with a pipette using 250 µl of Solution I/RNase A solution. To each tube 250 µl of solution II was added and gently mixed by inverting sever times. Tubes were allowed incubate at room temperature for two minutes. To each tube, 350 µl of Solution III was added and immediately inverted several times until a flocculent white

precipitate formed. Tubes were centrifuged at 13,000 rpm for 10 minutes at room temperature. While tubes were being centrifuged the HiBind® DNA Miniprep Columns were prepared. This step was done by adding 100 µl of Equilibration Buffer to each column before centrifuging the tubes at 13,000 rpm for 1 minute at room temperature. From the microcentrifuge tubes, 800 µl of clear supernatant was removed and added to the columns without disturbing the pellets. Columns were centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through liquid was discarded and 500 µl of Buffer HB was added to each column. Columns were centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through liquid was discarded and 700 µl of DNA Wash Buffer that had been diluted with absolute ethanol was added to each column. Columns were centrifuged at 13,000 rpm for 1 minute at room temperature. Flow-through liquid was removed and the columns were centrifuged at 13,000 rpm for 2 minutes at room temperature to dry the column matrix. Columns were placed into new 1.5 mil microcentrifuge tubes and had 40 µl of Elution Buffer added to each column. Columns were centrifuged at 13,000 rpm for 1 minute at room temperature. To try and capture any remaining DNA each column had an additional 40 µl of Elution Buffer added. Columns were centrifuged at 13,000 rpm for 1 minute at room temperature. Yield and quality of nucleic acids was determined using Nanodrop model N-1000 (Nanodrop, Wilmington, DE).

LR cloning reactions were performed to clone the target gene fragments into destination vector pANDA35HK behind the 35S promoter. Desired plasmids were produced by using the Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Grand

Island, NY). A mixture of 300 ng of the entry clone containing the GOI, 300 ng of the destination vector, 2 µl of 5x LR clonase™ reaction buffer, and TE buffer was added to bring volume to 8 µl. Next 2 µl of LR clonase™ enzyme stored in the -80 ° C freezer was added. The mixture was then vortexted and allowed to incubate at 25° C for 60 min. Next 2 µl of proteinase K solution was added to terminate reaction. The mixture was then briefly vortex and allowed to incubate for 10 min at 37° C.

Escherichia coli Transformation

The LR cloning product was used to transform competent Top 10 *E. coli* containing using a heat shock protocol. Competent *E. coli* cells were removed from -80° C freezer and allowed to thaw on ice. Two µl of PCR product was added to the 1.5 µl tube containing the competent *E. coli* cells. Tubes were gently mixed by tapping with finger and placed back on the ice for 30 minutes. Next the reaction was placed in a 42° C water bath for 90 sec then placed back on ice for an additional 2 minutes. One ml of antibiotic free liquid LB media is placed in the tube. Tubes are placed into shaker running at 200 rpm housed in 37° C incubator for one hour. Tubes were spun down for 1 minute at 10,000 rpm. One hundred µl of liquid was removed and retained in pipette. The remaining liquid was poured off and the pellet was re-suspended with retained liquid. The remainder of the solution was evenly applied to solid LB plates containing 50 mg a.i./l kanamycin sulfate. Plates were incubated overnight at 37° C. The colonies recovered from the transformation were streaked onto fresh LB plates containing 50 mg a.i./l kanamycin sulfate and 50 mg a.i./l hygromycin B for increased growth. PCR was performed to amplify both the sense and antisense portion of the gene fragment. Samples were run on Labnet Multigene thermocycler (MidSci, St. Louis, Mo) using Y25-1 F with

M13 R and Y25-R with M13 F (Table 3-3) The first cycle was run at 94.0 °C for 5 minutes followed by second cycle of 94.0 °C for 30 seconds, 56.0 °C for 30 seconds, 72.0 °C for 45 seconds repeated 32 times. The last extension cycle was at 72.0 °C for 10 minutes. PCR products were run on .8% agarose gel to confirm the presence of the desired arm orientation of the Y25-1 PCR products were also submitted to the KSU sequencing lab for confirmation of arm orientation and sequence similarity.

Agrobacterium rhizogenes Transformation

The Constructed vector was transferred into competent *Agrobacterium rhizogenes* containing K599 through heat shock transformations as described below. Competent *A. rhizogenes* cells were removed from the -80 °C freezer and allowed to thaw at room temperature (27° C). 1 µg of plasmid DNA was added to the 1.5 µl tube containing the competent *A. rhizogenes* cells. Tubes were gently mixed by tapping with finger and replaced back on the ice for 30 minutes. Tubes were dropped into liquid nitrogen for 5 minutes, and then placed on dry heating block set at 37° C for 25 minutes. One ml of antibiotic free liquid LB was placed into the tubes. Tubes were placed on a shaker for 3 hours at 150 rpm at 27°C. Cells were spun down for 3 minutes at 13,000 rpm. Liquid was poured off and the pellet was re-suspended with 200 µl of LB media. One hundred µl was evenly applied using a flamed glass wand to LB plates containing 50 mg a.i./l hygromycin B and 50 mg a.i./l kanamycin sulfate. Plates were allowed to incubate for 3 days in the dark at 27° C. Single colonies were picked with autoclaved toothpicks and streaked onto fresh LB plates containing 50 mg a.i./l hygromycin B and 50 mg a.i./l kanamycin sulfate. Plates were allowed to incubate for 24 hours in the dark at 27° C.

PCR was run on the colonies to check for GOI using perspective primers (Table 3-3). Two positive colonies were picked using a sterile pipette and each placed into a separate 5 ml culture containing 1 ml of LB liquid media with 50 mg a.i./l hygromycin B and 50 mg a.i./l kanamycin sulfate. Tubes were placed on a shaker for 3 hours at 150 rpm at 27°C. Tubes were removed and 850 µl of the contents were placed into tubes containing 150 µl glycerol stock and dropped into liquid nitrogen for 5 minutes. Tubes were removed from the liquid nitrogen and immediately placed into the -80 °C freezer stock.

Hairy Root Transformation

Seedling Preparation

Soybean seeds from variety KS4607 were surface sterilized with chlorine gas as described below. KS4607 was chosen for three reasons. It is a line that has desirable agronomic traits. KS4607 was also select because of its prior relative ease of stable transformation (unpublished). It is also susceptible to SCN which would aid in the search of new resistance. Seeds were placed in petri plate inside of a desiccator under a fume hood. Petri plate lids were removed and placed to the side in the desiccator. Next 3.3 ml of HCL was abruptly added to a 250 ml beaker inside the desiccator containing 100 ml of 5.25% by weight commercial bleach. The lid to the desiccator was immediately replaced and left for 16 hours. Plates containing seeds were removed from desiccator and placed under laminar flow hood with the petri plate lids removed for 30 min. This allowed any lingering chlorine gas to escape from the plates. Seeds were transferred to new plates containing Fisher brand P8 filter paper dampened with 0.5 OMS pH 5.7 (Table 3-2) and

allowed to germinate for 4 days under the hood with continuous light at 27°C. Root tips were removed using a flame sterilized scalpel and forceps.

Agrobacterium rhizogenes Preparation

A. rhizogenes was removed from the -80° C stock and streaked onto solid LB plates containing 50 mg a.i./ l of both and hygromycin B and kanamycin sulfate. Plates were placed in the dark for 24 hours at 27° C. Single colonies were picked using autoclaved toothpicks and streaked onto new solid LB plates containing 50 mg a.i./ l hygromycin B and 50 mg a.i./l kanamycin sulfate. Plates were placed into the dark again for 24 hours at 27° C. PCR was performed on each of the colonies to confirm the presence of the GOI. Colonies were transferred from the plate using a pipette tip and placed into 5 ml culture tubes containing 1 ml of liquid LB 50 mg a.i./l hygromycin B and 50 mg a.i./l kanamycin sulfate. Tubes were placed on a shaker at 150 rpm for 16 hours at 27°C. Contents of the culture tube were transferred to a 50 ml tube containing 5 ml of liquid LB with 50 mg a.i./l hygromycin B and 50 mg a.i./l kanamycin sulfate and placed on a shaker for 6 hours. Shaker ran at 200 rpm in constant light at 27°C. One ml of the contents was placed into 1.5 ml tube and centrifuged at 13,000 rpm for 1 min. The supernatant was removed and an additional 1 ml of contents was added. This was repeated until all of the contents from the 50 ml tube had been centrifuged into the 1.5 ml tube. The pellet in the 1.5 ml tube was re-suspended using 1 ml 0.5X PB buffer. The optical density of the bacterial suspension was adjusted to be between 0.5 and 0.8 O.D. at 600nm using Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Rochester, New York).

Inoculation

Hypocotyls were injected with 6 µl of a suspension of *A. rhizogenes* containing the pANDA35HK vector using a glass chromatography syringe, in addition to the injection a droplet of the suspension was placed on the cut site. Inoculated seedlings were placed into petri plates containing filter paper dampened with 0.5 liquid OMS pH 7.0 and 100 mg/L acetosyringone. Plates were placed into the dark at 27° C for 3 days to allow *A. rhizogenes* bacterial transformation to take place. Seedlings were transplanted into hairy root selection media. Hairy root media containing 500 mg/l Timentin® 200 mg a.i/l kanamycin sulfate was placed into GA-7 containers (Magenta corp., Chicago, IL.) Each container had 4 seedlings, one placed in each corner. Roots were allowed to proliferate for 3 weeks to select for only transformed roots.

SCN Bioassay

Positive transformed seedlings were transplanted to D40 Deepots (Stuewe and Sons, Tangent, OR) containing a 50/50 sand soil mixture infested with approximately 10,000 eggs from SCN race 3/HG type 7 Each pot contained only 1 transplanted seedling. There were 4 plants per treatment per rack with a total of 6 racks used. The entire experiment consisted of 5 treatments containing 24 plants in each treatment for a total of 120 plants tested. At the time of transplanting multiple root tip samples were taken from each transplant. The samples were collected and placed into 1.5 ml microcentrifuge tubes and immediately dropped into liquid nitrogen. These samples were used to isolate RNA for RT-PCR amplification perspective primers (Table 3-3). The plants were placed in large clear bins and covered with clear lids. Bins were grown in a

growth chamber with 16 hour photoperiod at 25° C. To harden off the plants the lids remained on the bins for the first 24 hours before the lids were slid open approximately 1 inch. After an additional 24 hours the lids were placed on top of the bins cover three fourths of the bin opening. Lids were moved to only cover half the bin opening after another 24 hours. Lids were completely removed from the bins 96 hours after transplantation. The plants were grown in the infested soil for 5 weeks with a very careful watering regime. They were watered every 24 hrs with a small amount of water. It was important to balance the needs of the sensitive plants with the needs of the nematodes. Too much water would have been detrimental to the nematodes but not enough would kill the plants so great care was taken in the watering process. Plants were broken down to extract cysts. This was done by briefly soaking roots in cool water (Figure 3). Roots were sprayed with cool water over an 840-µm-pore sieve over a 250-µm-pore sieve to collect cysts (Figure 4). The first sieve caught larger debris and the second sieve caught cysts but allowed water and smaller debris pass through. Cysts were gently washed from 250-µm-pore sieve into 50 ml beaker (Figure 5 and 6). Beakers were decanted over a a 250-µm-pore sieve to remove some excess debris. Cysts were gently washed into 50 ml beaker. Cysts were counted using a custom made counting dish before being mechanically ruptured to release eggs and second stage juvenile. Eggs and juveniles were diluted and counted using a nematode counting slide. Washed roots (Figure 7) were placed in paper bags and dried for at least 7 days at 48 °C before being weighed.

Statistics

Cyst and egg counts were normalized by taking root weights into account then $\log_{10}$ transformed to minimize heterogeneity of variances prior to statistical analysis using PROC MIXED (SAS Institute Inc., Cary, NC).

TRIzol RNA Isolation

RNA was isolated from root tip samples mentioned above. Each sample was ground in a 1.5 ml microcentrifuge tube. Immediately after tissue was ground 1 ml of TRIzol (company Name, city, State) was added to the tube. Samples were incubated for 5 minutes at room temperature. Next, each tube had 0.2 ml of chloroform: IAA 24:1 added and were shaken by hand vigorously. Samples were incubated for 3 minutes at room temperature and then centrifuged at 12,000 x g for 15 minutes at 4° C. The upper aqueous layer was removed and placed into a fresh 1.5 microcentrifuge tube. RNA was precipitated from this layer by mixing with 0.5 ml of isopropyl alcohol. Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4° C. Supernatant was removed and discarded. RNA pellets were washed with 1 ml of 75 % ethanol briefly vortexed and centrifuged at 7,500 x g for 5 minutes at 4° C. Ethanol was then removed, the pellets were allowed to air dry briefly for 10 minutes, re-suspended in 100 µL of nuclease-free water and allowed to incubate at 60° C for 10 minutes.

Concentration of total RNA was checked using Nanodrop model N-1000 (Nanodrop, Wilmington, DE). RT-PCR was performed using the Promega Reverse Transcription System (Promega, Madison, WI). RNA samples were diluted to 0.1 µg/µl and placed into a 0.5 microcentrifuge tube. Samples were incubated at 70° C for 10

minutes and then immediately placed on ice for 5 minutes to break up the secondary structures of the RNA. To each RNA sample, 10.5 µl of a reverse transcription mixture was added containing 4 µl $MgCl_2$, 2 µl Reverse Transcription 10x buffer, 2 µl of 10M dNTP mixture, 0.5 µl Recombinant RNasin Ribonuclease inhibitor, 1 µl AMV reverse transcriptase, and 1 µl random primers. The samples were heated to 42° C for 60 minutes followed by 95°C for 5 minutes to inactivate AMV.

The product cDNA was screened for both housekeeping gene Ribosomal S21 and the GOI. PCR was performed using Rib F and Rib R primers to check for the housekeeping gene. Primers Y25 F and Y25 R were used to check for the presence of the original Y25 target. Primers Y25-1 F and Y25-1 R were used to check for the presence of the new Y25-1 target. Primers WSMV F and WSMV r were used to check for the presence of the control WSMV target. All samples were run on Labnet Multigene thermocycler (MidSci, St. Louis, Mo) with the first cycle ran at 94 °C for 5 minutes followed by second cycle of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 45 seconds repeated 32 times. The last extension cycle was at 72 °C for 10 minutes. PCR product was run on 0.8% agarose gel to check for presence of both the housekeeping gene and the GOI. Each sample was checked for the presence of the housekeeping gene to make sure the quality of the RNA isolation was acceptable and the presence of the desired GOI.

Results and Discussion

The original line of stable Y25-expressing plants produced from a single transformation event by Dr. Li was characterized by their stunted growth patterns. Most plants either produced abnormal flowers with decreased fertility (unpublished). The

plants that had viable flowers produced pseudopods or had very low seed production with low germination rates. The original target had very little homology with the soybean genome, but it did contain a continuous sequence of 19 base pairs (TGCAAAATTTGTCATTGGA) that were 100% homologous to a sequence in the soybean genome with no known function (Figure 3-1 and Table 3-4). This is of great concern because Qui *et al.* (Qui *et al.,* 2005) found that siRNA sequences ranging in length from 17 to 29 base pairs could cause silencing events. Of the targets ranging in size from 17 to 29 base pairs the targets with sequences shorter than 21 nucleotides had an increased chance of causing off-target effects. To determine if this sequence homology was the cause of the negative phenotype an additional target sequence of the Y25 gene was engineered with no known homology to the soybean genome. The full-length sequence of Y25 was blasted using National Center for Biotechnology Information. The nucleotide blast program of the basic local alignment search tool (BLAST) was used, along with the blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to find similarities as small as 7 base pairs. Upon blasting the full-length sequence of the SCN Y25 gene against the entire know soybean genome, it was discovered that the 5' end of the SCN Y25 gene contained less homology with the soybean genome than the 3' end (Figure 1). A new target was then selected that contained no homology with the known soybean genome (Figure 1). In addition, a new target was selected at the 3' end of the gene containing more homology than the original target sequence. The hypothesis to be tested was that the target with more homology would be more likely to cause an off-target silencing event that would show a negative

phenotype based on the assumption that homology to the soybean genome was the cause of the negative phenotypes in the stable Y25 lines.

All five constructs were inoculated every day for seven days. Approximately 31 seedlings were inoculated per construct per day for a total of about 220 inoculated seedlings each day. This number varied slightly based on germination rate of the soybean seeds. A total of about 1100 seedlings were inoculated during the seven-day period. Of the 1100 inoculations at least 25 positively transformed root systems for each construct were recovered for use in bioassays. Any remaining positive root systems were autoclaved and discarded.

There were no distinguishable phenotypic differences of the shoots or leaves while plants grew in culture. Transformed seedlings were easily distinguished from non-transformed seedlings based on strong root growth of transformed seedlings in the selection media (Figure 3-2). Non-transformed roots had great difficulty growing in the selection media since they did not have resistance to kanamycin sulfate that the transformed roots had. It did come as a surprise after RNA was isolated from positive root systems and the GOI was not found to be expressing in all the plants at the time of transplant. To rule out the possibility of PCR error the ribosomal S21 gene was used to make sure the RNA that was isolated was of good quality. The ribosomal S21 gene is always expressed in living cells so it serves as a quality check to insure that the RNA isolated is of acceptable quantity and quality. It was found that all of the samples had bands for the ribosomal S21 gene showing that the process of RNA isolation was without error. However, not all samples contained bands for all for the GOIs from the samples found to be expressing the ribosomal S21 gene.

Transplanting plants to infested soil was performed over a six day period allowing for one block of 20 plants per day. A total of 120 plants containing transformed roots were transplanted during this six day period. There were no distinguishable phenotypic differences noted during bioassays in SCN infested soil. However, it was noticed that the plants appeared to have a slight chlorosis appearance in later stages of the bioassay. This chlorosis may have been caused by lack of nitrogen since the plants were not fertilized in addition to the fact that the soil had been steamed before SCN inoculation, soil was not re-inoculated with rhizobium after steaming. If steamed soil is used for future bioassays it will be important that the soil be inoculated with rhizobium to ensure that nitrogen deficiency is not a problem. Application of nitrogen fertilizers has been shown to interfere with the SCN hatch, penetration and cyst development (Baker *et al.*, 1971). The fact that no distinguishable phenotypic differences were detectable presents a concern that the hairy root system may not be appropriate to screen for negative phenotypic traits.

Bioassays were completed in growth chambers and not greenhouse because of problems with previous attempts to perform bioassays in greenhouses. The environmental controls of the greenhouses in the summer are not sufficient to cool them where often the greenhouse could exceed 40° C even though the temperature controls were set to 25° C. The winter does not yield good conditions either; the greenhouses have humidity levels that are much too low for successful hairy root assays during the winter months. New transplants are very sensitive to both temperature and humidity in addition they require a hardening period to acclimate the plants to the new growing conditions. However, it was discovered after the completion of the bioassays that the temperature did not follow the programmed settings correctly. Temperatures varied

greatly and did not have the normal pattern of 25° C during light periods and 23° C during dark periods. These varied temperatures may have effected GOI target expression. In addition the varying temperature may have played a role in the above noted chlorosis.

Bioassays were broken down over a 6-day period allowing for one block of 20 plants per day. There were no statistical differences in any of the treatments for either cyst or egg counts (Table 3-5). The average cyst count was 125 and the average egg count was 12,246. The average number of cysts per gram of root was 273 and the average number of eggs per gram of root was 29,554. The average root weight for all root samples from bioassay was 0.63 grams.

RNA was isolated from almost all of the transplanted root tip collections. RNA was not isolated from empty vector samples. A total of two samples were lost during centrifugation due to tube failures. A total of 94 RNA isolations were completed. Of the 94 RNA isolations 68 samples had acceptable RNA. The quality of the RNA was deemed to be acceptable based on the detection of the house keeping gene ribosomal S21 of the correct size which is 168 base pairs in length. The correct size product for the housekeeping gene was slightly smaller than the positive control. The positive control was DNA isolated form soybean, it contained both introns and exons for the housekeeping gene. The RNA samples do not have introns therefore, bands that were the same size as the housekeeping gene had DNA contamination. Out of the 68 samples that had acceptable RNA only 56 were found to be expressing GOI target RNA. The proportion of 82% for the number of acceptable RNA isolations to the number of expressing GOI samples were similar to previous experimentation performed by Dr. Li

Removing egg and cyst count data from non-expressing plants did not yield any statistical differences (Table 3-5).

The original purpose of this entire project was to find a new target sequence that had no homology to the known soybean genome and compare that target to the original Y25 target. A new target was produced that had no homology with soybean genome. However, this experiment was unable to definitively declare the new Y25-1 target as effective or ineffective. Nor was it able to confirm that the Y25-4 target containing more homology with the soybean genome caused any detectable phenotypic effects. Further experimentation will need to be completed to confirm or disprove the effectiveness of the new Y25-1 target as a viable control. Currently, Y25-1 is being transformed into stable soybean lines to further investigate its effectiveness as a SCN control.

The new stable plants will need to be screened to make sure they are not only positive for the GOI but are also expressing the dsRNA in the roots. The plants should have their roots checked throughout the plants' lifecycle to confirm that the line is expressing stable dsRNA throughout. Expressing Y25-1 lines also must be screened for the negative phenotype seen in the original Y25 stable lines.

The original purpose of the rapid hairy root assay was to quickly sort through the numerous potential targets of interest to see if the targets showed any potential as a control for SCN. It was important to attempt to sort out the most promising targets because the process to obtain stable lines is a long and labor intensive process. This project has shown that the hairy root system has great variation and is not suitable for comparing relative effectiveness of target sequences. Li observed that the multiple independent integrations had the potential to give rise to variable expression levels of

transgene. He concluded that this would increase the variability in hairy root bioassays (Li *et al*, 2010). There are many assumptions made that may not be true in the hairy root assays. It is assumed that each hairy root is derived from a single incorporation event. It is much more probable that there are multiple incorporation events taking place. This may cause the same root system to have varying levels of expression depending on which event the root was produced from. It is also assumed that each positive transformed root system is expressing the target sequence at approximately the same level. Depending on where in the genome the sequence was incorporated, some areas will have higher rates of expression than areas that incorporated in scaffolding regions. It is assumed that expression of the target sequence is stable and expressed during the entirety of the bioassay. The promoters may be affected by a variety of conditions that would turn the expression on and off such as environmental changes, plant life stage, and genomic location of the incorporated DNA. All these assumption lower the ability to detect subtle differences in effectiveness of useful targets. This system contains much variability, but is well suited as a preliminary test for potential targets. It lacks the statistical power to be used to compare the effectiveness between potential targets because of the high variability. There were no observable differences among treatments that show whether or not roots were transformed. In future experiments it would be of great value to add a reporter gene to the RNAi vector to provide a visual queue as to whether or not the root has been transformed and is expressing. This could be used at both the time of transplantation as well as root breakdown.

A more consistent approach should be taken in the future to compare target GOI effectiveness. After using the hairy root assay for rapid evaluation of new targets the

effective targets should be transformed into stable lines. Once stable lines are recovered each event should be backcrossed to the same agronomic line. Bregitzer found that in barley a single backcross of a transgenic plant with somaclonal variation effectively restored performance to levels similar to the original parent (Bregitzer *et al.*, 2008). After one backcross somaclonal variation should be removed so negative phenotypes would most likely be credited to off target effects. Two backcrosses with non-transformed parent will minimize undesirable effects (Visarada *et al.*, 2009). Backcrosses continued to BC4 would remove the majority of variation. BC4 lines would be almost 97% identical. During each backcross it would be imperative that RNA be extracted from the roots multiple times to check for expression of the target sequence. Selection will have to be based solely on expression because screening for SCN resistances requires sacrificing the plant. After BC4 generations are produced screenings for effectiveness can take place. This would be very useful for detecting smaller differences in effectiveness of targets since all lines would have an almost 97% identical genetic background. This would also allow multiple samples to be taken from the seedling stage through maturity to make sure expression is constant across time. Real-time PCR could be performed to obtain quantifiable levels of expression. Backcrossing transgenic lines while selecting based on expression and desirable phenotypes would yield quality lines containing the desired expressing targets that are ready to be crossed by breeders to current varieties. This would all take approximately 4 years, but at the end of 4 years there would be multiple near isogonics lines that could be thoroughly tested for expression levels and effectiveness. Analysis could be made comparing not only

effectiveness of different target sequences but also comparison of target GOI expression levels to control effectiveness.

Table 3-1: Full length sequence of Y25 .

SCN Y25 full length sequence
CTCCGCATTGGCCAACACTTTGGCCAAATTGGTGTTGCGCTACGCTGAGCTCAA
CAAGGGTGTCCCCTCAACTGTTAATAAATTGGCGAGTGGTGCGCTGCTGCTCAT
CGCTTCAATCATTCATCTTGGCAAGTCGGGCTTGTGCAAACAGCCGATCACTGA
GGACGACTTGGACCGTTTGTCGACCACTGTTCGACTGATTGTTGACCAATGGCC
GAAAGCGGTGGATGTGTTTTTGAGAGAGTGCCGTGCTTCGTTGGAAAGCATGC
TCAAGGCCAAGGGGGACGTGGACCGGCACGAACGCGACACAAAAGCGCCGA
AGAAGAAAATTGTGCAGCCCGACAAGACAATTATGTTCACGCAGCTGTCCAC
ACGCGTGTCAGAAAACGTGACGGACACAAATTTGTTTGATCTTTCGCTTTCCCA
AGCGCTTGGTACTGCACCCAAAACGACCAAATACACCTTTGCCAGCTCCAAAC
TGGGAAAAGTGATTCAGTTAGCCGGCTTTTCGGATCCCGTCTATGCCGAGGCG
TACGTCAACGTCAACCAATATGACATTGTATTGGACGTACTCGTGGTCAACCA
GACTAGCGACACCTTGCAAAATTTGTCATTGGAACTCTCGACCGTTGGCGACCT
TAAATTGGTGGACAAACCCTCGCCGATTACTCTTGCACCTAACGACTTCACTAA
CATCAAAGCCACCGTAAAAGTGTCATCCACCGAAAATGGAGTCATTTTCTCCA
CCATTGCTTATGATGTGCGAGGGTCGACCTCGGATCGAAATTGTGTGTACCTTG
AGGACATTCACATTGACATAATGGATTACATTGTGCCGGGCACTTGCACAGAT
ACAGAATTTCGGAAAATGTGGGCCGAATTTGAATGGGAGAACAAGGTCGGAG
TTGTGACCCCAATTACGGACCTTCGACAATACTTGGACCATTTGTCCGCTCAAA
CAAATATGAAACTGCTGACAACAGACGCGGCATTGGAAGGCGATTGCGGTTTT
CTGGCGGCCAACTTCTGTGCCCATTCCATTTTTGGCGAGGATGCGTTGGCCAAT
GTTTCCGTTGAAAAGGCGGACCCACTGGATCCAATGAGTGCCATTATTGGCCA
TATTCGGATCAGGGCCAAGTCTCAGGGGATGGCACTTTCGCTGGGGGACAAG
ATAAACCACGCGCAGAAGGAACGTAAGCCGGTGGAGAGGGGCGGCGGGGCG
AGGGCAGTGGCGAACGCTGCCGCGAAATGAATGAAGACGAGCAAAACGTTT
AGCAATTTATTGCAAATAATATTTTAATCGCATCATTCACTGTTTTCCATGTTTTT
CTTATTGTTTTTCTCGTCATTGTTAAATTTCTTTGTATTTTGTGATGTGATTTAATA
TATTTCTTAATTTTGGGATTTGTTTACCTAAATTTCAAGCCGATCATTCATTATA
AAAAATGTACTGAAAAAAAAAAAAAAAAAAAAAA

Sequence amplified from DNA isolated from SCN HG Type 7

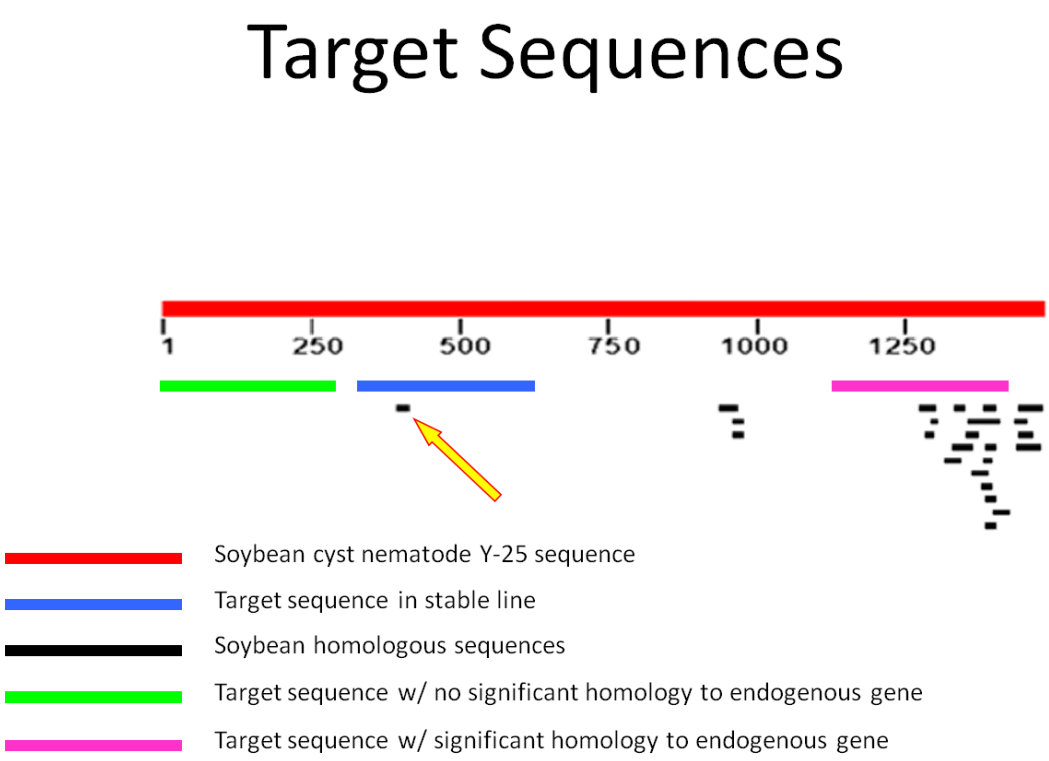

Figure 3-1: Sequence Homology

Sequence analysis comparing the known soybean genome (black bars) with the sequence of soybean cyst nematode Y25 gene (red bars). The blue bar represents the original Y25 sequence. The yellow arrow shows that the original target contained some homology with the known soybean genome. The pink bar represents the new target sequence that contains increased amounts of homology with the soybean genome. The green bar represents the new target sequence that has no known homology to the soybean genome.

Table 3-2 Media Compositions and references

Medium	Composition [1]	Reference
1/2 MS [2]	MS salts, B5 vitamins, MES, 3% sucrose, 0.2 % Gelrite™, pH 5.7	Modified from Murashige and Skoog (1962)
LB [3]	Bacto-tryptone, Bactone-yeast extract, NaCl, pH 7.0	Bertani (1951)
HR [4]	MS Salts, B5 vitamins, MES, NH_4NO_3, KNO_3, Sucrose, Gelrite™	Li et al. (2010)
1/2 PBS	Na_2HPO_4, NaCl	Li et al. (2010)

[1] All media were autoclaved for 20 minutes at 180°

[2] pH 5.7 for seed germination and pH7.0 + AS100 for hairy root formation

[3] 15 g/l of agar for LB plates

[4] Timentin 200mg/l and Kanamycin 500mg/l used for selection

Table 3-3 List of primers used for PCR amplification of genes used in study

Primer	Sequence (5' → 3')	Tm	Size[1] (bp)
Y25 F	GCAGCCCGACAAGACAAT3	55.8	291
Y25 R	TGAGAGTTCCAATGACAAAT	49.2	
Y25-1 F	CACCCTCCGCATTGGCCAACACTTT	64.1	292
Y25-1 R	GTCCACGTCCCCCTTGGCCTTGAG	65.7	
Y25-4 F	CACCCTTGGACCATTTGTCCGCTCAA	63.1	292
Y25-4 R	TGCCCTCGCCCCGCCGCCCCTCTC	75.5	
WSMV F	ACCTATCATACCAAGGGGCTT	61.1	243
WSMV R	TGCGTGTTCTCCCTCACATCATCT	64.1	
RIB F	CTAAGATGCAGAACGAGGAAGG	55.1	168
RIB R	GAGAGCAAAAGTGGAGAAATGG	54.0	

[1] Expected size of PCR amplified products

Table 3-4 Target sequences

Target	Sequence	Size (bp)
Y25 original	GCAGCCCGACAAGACAATTATGTTCACGCAGCTGTCCACACGCGTGTCAGAAAACGT GACGGACACAAATTTGTTTGATCTTTCGCTTTCCCAAGCGCTTGGTACTGCACCCAAAA CGACCAAATACACCTTTGCCAGCTCCAAACTGGGAAAAGTGATTCAGTTAGCCGGCTT TTCGGATCCCGTCTATGCCGAGGCGTACGTCAACGTCAACCAATATGACATTGTATTG GACGTACTCGTGGTCAACCAGACTAGCGACACCTTGCAAAATTTGTCATTGGAACTCT CG	292
Y25-1	CTCCGCATTGGCCAACACTTTGGCCAAATTGGTGTTGCGCTACGCTGAGCTCAACAAG GGTGTCCCCTCAACTGTTAATAAATTGGCGAGTGGTGCGCTGCTGCTCATCGCTTCAA TCATTCATCTTGGCAAGTCGGGCTTGTGCAAACAGCCGATCACTGAGGACGACTTGGA CCGTTTGTCGACCACTGTTCGACTGATTGTTGACCAATGGCCGAAAGCGGTGGATGTG TTTTTGAGAGAGTGCCGTGCTTCGTTGGAAAGCATGCTCAAGGCCAAGGGGGACGTG GAC	292
Y25-4	CTTGGACCATTTGTCCGCTCAAACAAATATGAAACTGCTGACAACAGACGCGGCATTG GAAGGCGATTGCGGTTTTCTGGCGGCCAACTTCTGTGCCCATTCCATTTTTGGCGAGG ATGCGTTGGCCAATGTTTCCGTTGAAAAGGCGGACCCACTGGATCCAATGAGTGCCAT TATTGGCCATATTCGGATCAGGGCCAAGTCTCAGGGGATGGCACTTTCGCTGGGGGA CAAGATAAACCACGCGCAGAAGGAACGTAAGCCGGTGGAGAGGGGCGGCGGGGCG AGGGCA	292
WSMV	ACCTATCATACCAAGGGGCTTCGACAAGGCCGGTGTGCTAAGCATCAACAATATTGTG GCAGCGTGTGATTTCATTATGCGCGGTGCAGATGACACACCAAATTTTGTGCAAGTGC AGAACAGCGTTGCAGTGAACAGGCTACGCGGAATACAGAACAAGCTGTTTGCACAGG CACGACTGAGTGCGGGTACTAATGAGGACAACTCACGTCATGATGCAGATGATGTGA GGGAGAACACGCA	243

List of entire target sequences used for RNAi targeting of genes used in study Y25 original, Y25-1 and Y25-4 all are from *H. glycines* WSMV is from *Wheat streak mosaic virus*.

Figure 3-2: Comparison of transformed roots and non-transformed roots.

A) Plant with a non-transformed root system that did not grow well in selection media
B) Plant with a positive transformed root system that grew successfully in the selection media.

Figure 3-3: Roots soaking in cool tap water

Roots were soaked in water to remove soil from roots.

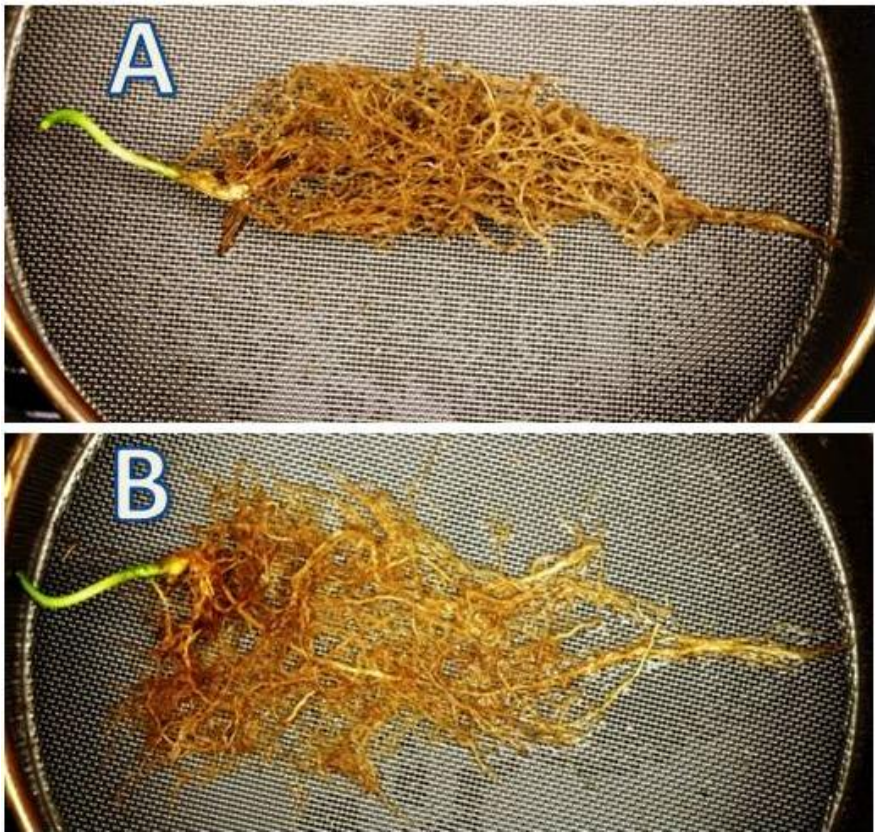

Figure 3-4: Cleaned Roots

A) Roots on sieve before being sprayed with cool tap water
B) Roots after being sprayed with cool tap water

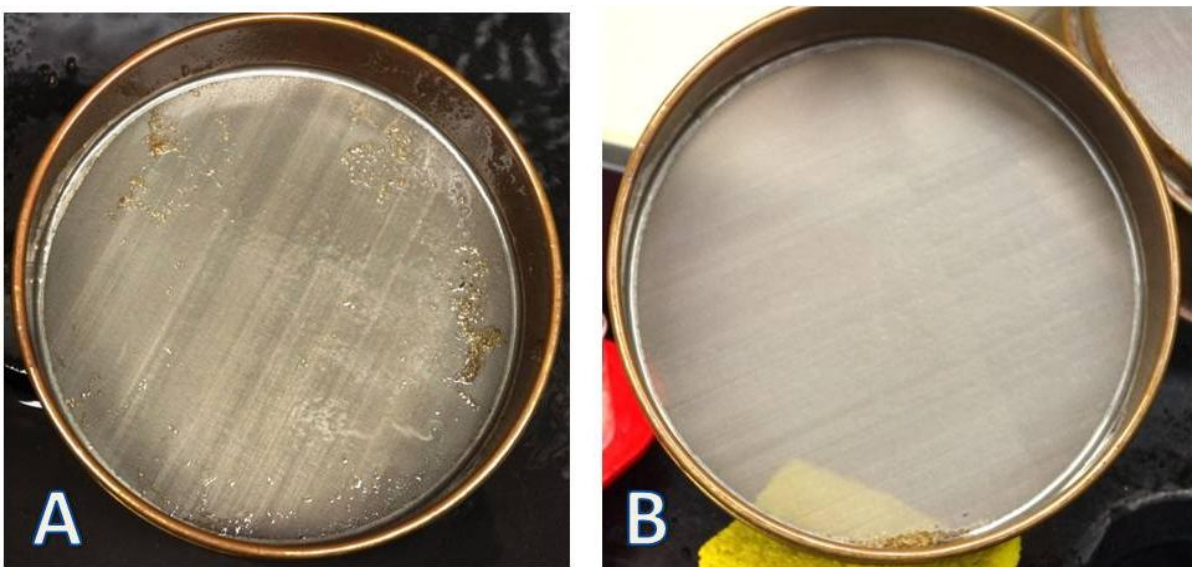

Figure 3-5: Cysts and debris on sieve

A) Cysts and debris were collected on sieve
B) Cysts rinsed to one side before being washed into a 50 ml beaker.

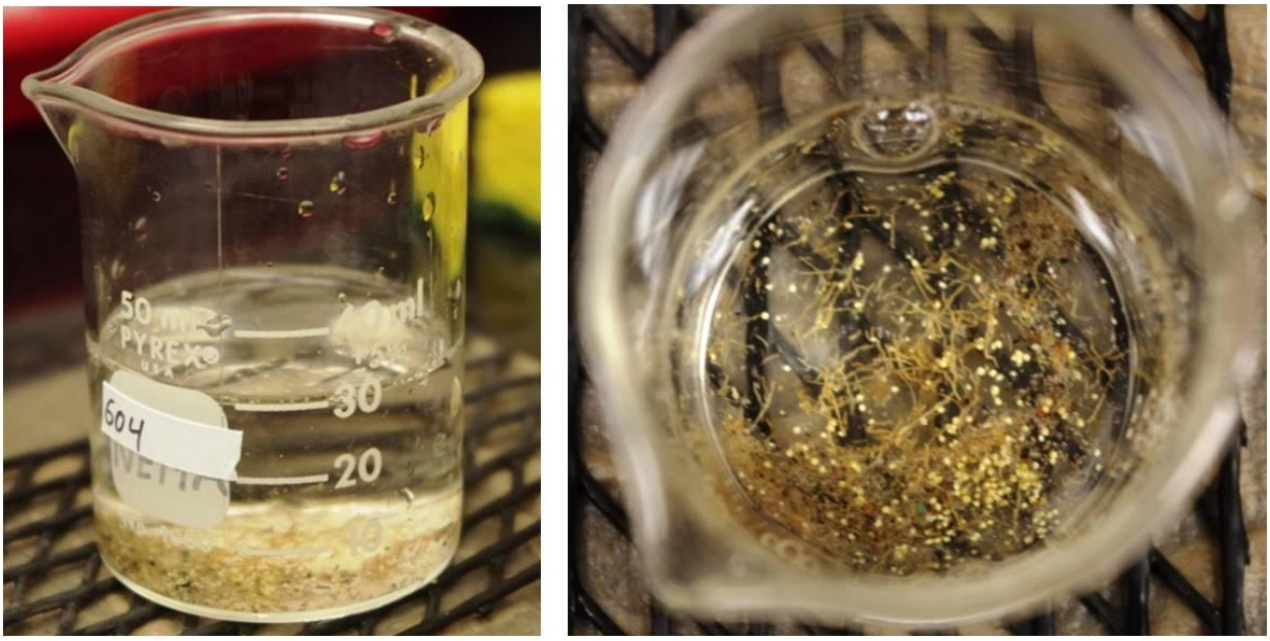

Figure 3-6: Cysts in 50 ml beaker

Cysts and debris caught on sieve and poured into 50 ml beakers. Samples contained much debris and required an additional clean up step.

Figure 3-7: Roots placed on paper towel to begin drying process

Roots were placed into paper bags after drying on a paper towel for a few minutes. Bags were placed into drying room to finish drying down.

Table 3-5 Log transformed comparison of SCN reproduction and expression

Construct (homology)	Expression	Eggs	Cysts	Eggs/g root	Cysts/g root
Y25 (original construct)	Expressing	3.9061	1.7910	4.2226	2.0993
	Non-expressing	4.0451	1.9350	4.2745	2.1705
Y25-1 (No homology)	Expressing	4.0439	1.9700	4.2779	2.2110
	Non-expressing	4.0440	2.0670	4.3265	2.3388
Y25-4 (Increased homology)	Expressing	3.8974	1.8894	4.1798	2.1737
	Non-expressing	4.0557	2.0355	4.3690	2.3449
WSMV (No homology)	Expressing	3.9348	2.1248	4.1834	2.1598
	Non-expressing	4.1117	2.1248	4.4417	2.4612

No statistical differences were found between treatments.

Chapter 4 - Conclusion

The two main objectives of this project were to create new target sequences with no homology to any know soybean genes as well as a construct with more homology than the original target and to find a variety insecticides that were compatible with SCN bioassays. One of the compounding problems of performing greenhouse bioassays of any type is pest control and the buildup of said pests. Previously bioassays performed in greenhouse fell prey to the typical greenhouse pests such as spider mites, thrips, and white flies. This added another layer of variability to the bioassay analysis. In the past bioassays would run for 6 weeks with no pest control. If greenhouse pest were accidentally introduced they had ample time and source material to reproduce to large populations. To remove this variability a study was conducted using common pesticides used in greenhouse applications to screen for any insecticide affects on the soybean cyst nematode population. It was found that routine applications of all the insecticides used had no effect on the nematode populations. This result provides a source of control for the greenhouse pests that can affect the consistency of greenhouse bioassays and removes one more variable in the greenhouse studies.

A target sequence was discovered that contained no significant homology. However, it still remains to be proven whether the new target is any different in effectiveness in the control of SCN. RNAi has been shown to reduce reproduction in a variety of nematodes including plant pathogenic species. There is still potential for the use of RNAi as a control strategy for SCN resistance. It is important to provide producers with new tools to control SCN. It is essential to have more host plant

resistance options in order to protect the existing sources while expanding the possibilities of control.

The hairy root system has its problems and limitations, but they still serve as a key part in the exploration of new potential target sequences in the control of SCN. Future work in comparative analysis of different targets should be done by other means such as on T1 backcrosses. Rapid hairy root assays are just not the right tool for the job of detailed comparisons. Ultimately stable transgenic lines are needed to confirm that the Y25 original sequence may or may not have off target effects that are detrimental to the metabolism of the soybean plant. Stable lines will also be able to prove whether or not other sequences may be used as effective controls without negative phenotypes. In the future co-expression of GFP would give the researcher a visual queue as to the success of expression of the GOI. It was found in the study that expression varied from plant to plant and having a reporter gene in addition to the selectable marker would be of great value in the future. This would also help to reduce the number of escapes.

RNAseq should be used in the future to check for the presence of siRNAs in roots. RNAseq could be used to check for the level of expression at various stages of the hairy root assays to look at the possibility of varying expression levels across time. RNAseq would be an improvement upon the RT PCR that was previously performed. RT PCR is only able to show the presence or absence of the expression of the GOI. It would be interesting to compare the levels of SCN to the amount of siRNAs found in plants. There may be a correlation between the amounts of siRNA found in the plant or there may be a threshold limit that must be reached before SCN control attained. This would allow researchers to know if they should focus their efforts on achieving higher

expression levels of the target siRNAs or if they need to only be concerned that expression is at a certain level for control. RNAseq could help to remove much of the variability of the hairy root system and improve its usefulness.

References

"1,2-Dibromo-3-Chloropropane (DBCP)." *EPA*. Environmental Protection Agency, 06 Nov. 2007. Web. 07 June 2012. <http://www.epa.gov/ttn/atw/hlthef/dibromo-.html>.

Abel, P., R. Nelson, B. De, N. Hoffmann, S. Rogers, R. Fraley, and R. Beachy. "Delay of Disease Development in Transgenic Plants That Express the Tobacco Mosaic Virus Coat Protein Gene." *Science* 232.4751 (1986): 738-43. Web. 12 June 2012.

Alumai A, Grewal PS (2004) Tank-mix compatibility of entomopathogenic matodes Heterorhabditis bacteriophora and Steinernema carpocapsae, with selected chemical pesticides used in turfgrass. Biocontrol Sci Technol 14:725–730

Bandyopadhyay-Ghosh, Sanchita, Subrata B. Ghosh, and Mohini Sain. "Synthesis of Soy-Polyol by Two Step Continuous Route and Development of Soy-Based Polyurethane Foam." *Journal of Polymers and the Environment* 18.3 (2010): 437-42. Web. 18 July 2012.

Barbara KA, Buss EA (2005) Integration of insect parasitic nematodes (Rhabditida: Steinernematidae) with insecticides for control of pest mole crickets

Barker, K.R., P.S. Lehman, and D. Huisingh. "Influence of Nitrogen and Rhizobium Japonicum On the Activity of Heteroidera Glycine 1)." *Nematologica* 17.3 (1971): 377-85. Web. 10 July 2012.

Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon. "Role for Bidentate Ribonuclease in the Initiation Step of RNA Interference." *Nature* 409 (2001): 363-66. Web. 31 Oct. 2012.

Bregitzer, P., L. S. Dahleen, S. Neate, P. Schwarz, and M. Manoharan. "A Single Backcross Effectively Eliminates Agronomic and Quality Alterations Caused by Somaclonal Variation in Transgenic Barley." *Crop Science* 48 (2008): 471-79. *National Agricultural Library*. Web. 6 July 2012. <http://naldc.nal.usda.gov/catalog/17571>.

Carter, Colin A. "Costs Uncertain: Methyl Bromide Phase-out Becomes Reality." *Californina Agriculture* 55.3 (2001): 2. Web. 27 Apr. 2013.

Concibido, Vergel C., Brian W. Diers, and Prakash R. Arelli. "A Decade of QTL Mapping for Cyst Nematode Resistance in Soybean." *Crop Science* 44.4 (2004): 1121. Web. 14 Oct. 2012.

Cullen, Bryan R. "Derivation and Function of SmallinterferingRNAs and MicroRNAs." *Virus Research* 102.1 (2004): 3-9. Web. 25 Oct. 2012.

Cuthbertson AGS, Head J, Walters KFA, Murray AWA (2003) The integrated use of chemical insecticides and the entomopathogenic nematodes, Steinernema feltiae, for the control of sweetpotato whitefly, Bemisia tabaci. Nematology 5:713–720

De Deyn, Gerlinde B., Jasper Van Ruijven, Ciska E. Raaijmakers, Peter C. De Ruiter, and Wim H. Van Der Putten. "Above- and Belowground Insect Herbivores

Differentially Affect Soil Nematode Communities in Species-rich Plant Communities." *Oikos* 116.6 (2007): 923-30. Web. 28 May 2013.

Faghihi, Jamal, and Virginia R. Ferris. "Purdue Pest Information | | Soybean Cyst Nematode." *Purdue Pest Information | | Soybean Cyst Nematode*. N.p., May 2012. Web. 08 June 2012. <http://extension.entm.purdue.edu/eseries3/view.php?article=articles/soybean_cyst_nematode.txt>.

Fire, Andrew, Donna Albertson, Susan W. Harrison, and Donald G. Moerman. "Production of Antisense RNA Leads to Effective and Specific Inhibition of Gene Expression in C. Elegans Muscle." *The Company of Biologists Limited* (1991): n. pag. Web. 11 June 2012.

Fire, Andrew, SiQun Xu, Mary Montgomery, Steven Kostas, Samuel Driver, and Craig Mellow. "Potent and Specific Genetic Interference by Double-strandedRNAin Caenorhabditis Elegans." *Nature* 391 (1998): 806-11. Web. 25 Mar. 2012.

Fitch, Maureen M. M., Richard M. Manshardt, Dennis Gonsalves, Jerry L. Slightom, and John C. Sanford. "Virus Resistant Papaya Plants Derived from Tissues Bombarded with the Coat Protein Gene of Papaya Ringspot Virus." *Bio/Technology* 10.11 (1992): 1466-472. Web. 11 June 2012.

Fuchs, Marc, and Dennis Gonsalves. "Safety of Virus-Resistant Transgenic Plants Two Decades After Their Introduction: Lessons from Realistic Field Risk Assessment

Studies." *Annual Review of Phytopathology* 45.1 (2007): 173-202. Web. 6 June 2012.

Gaugler, R. and Campbell, J. 1991. Selection for enhanced host-finding to scarab larvae (Coleoptera: Scarabaeidae) in an entomopathogenic nematode. Environ. Entomol. 20:700-706.

Gonsalves, Dennis. "CONTROL OF PAPAYA RINGSPOT VIRUS IN PAPAYA: A Case Study." *Annual Review of Phytopathology* 36.1 (1998): 415-37. Web. 10 July

Hamilton, Andrew, Olivier Voinnet, Louise Chappell, and David Baulcombe. "Two Classes of Short Interfering RNA in RNA Silencing." *The EMBO Journal* 21.17 (2002): 4671-679. *Http://www.nature.com/emboj/journal/v21/n17/full/7594680a.html*. Web. 8 Nov. 2012.

Hartman, Glen L., Ellen D. West, and Theresa K. Herman. "Crops That Feed the World 2. Soybean—worldwide Production, Use, and Constraints Caused by Pathogens and Pests." *Food Security* (2011): 5-17. 27 Jan. 2011. Web. 18 July 2012.

Head, J., Walters, K. F. A., and Langton, S. 2000. The compatibility of entomopathogenic nematode, Steinernema feltiae, and chemical insecticides for the control of the South American leafminer Liriomyza huidobrensis. BioControl 45:345–353

Huang, Guozhong, Rex Allen, Eric L. Davis, Thomas J. Baum, and Richard S. Hussey. "Engineering Broad Root-knot Resistance in Transgenic Plants by RNAi Silencing of a Conserved and Essential Root-knot Nematode Parasitism Gene." *Proceedings of the National Academy of Sciences* 103.39 (2006): 14302-4306. Web. 11 June 2012.

Inagaki, H., Tsutsumi, M. (1971) Survival of the soybean cyst nematode, *Heterodera glycines* Ichinohe (Tylenchida: Heteroderidae) under certain storage conditions. *Appl. Entomol. Zool.* **8**, 53-63.

Kamath, Ravi S., Andrew G. Fraser, Yan Dong, Gino Poulin, Richard Durbin, Monica Gotta, Alexander Kanapin, Nathalie Le Bot, Sergio Moreno, Marc Sohrmann, David P. Welchman, Peder Zipperlen, and Julie Ahringer. "Systematic Functional Analysis of the Caenorhabditis Elegans Genome Using RNAi." *Nature* 421.6920 (2003): 231-37. Web. 21 Oct. 2012.

Kaplan, Ian, Sandra Sardanelli, and Robert F. Denno. "Field Evidence for Indirect Interactions between Foliar-feeding Insect and Root-feeding Nematode Communities on." *Ecological Entomology* 34.2 (2009): 262-70. Web. 28 May 2013.

Koenning, S. R., and J. A. Wrather. "Suppression of Soybean Yield Potential in the Continental United States by Plant Diseases from 2006 to 2009." *Plant Health Progress* (2010): n. pag. Print.

Koenning, S. R., D. P. Schmitt, and K. R. Barker. "Effects of Cropping Systems on Population Density of Heterodera Glycine and Soybean Yield." *Plant Disease* 77 (1993): 780-86. Print.

Koppenhöfer AM, Brown IM, Gaugler R, Grewal PS, Kaya HK, Klein MG (2000a) Synergism of entomopathogenic nematodes and imidacloprid against white grubs: greenhouse and field evaluation. Biol Control 19:245–251

Koppenhöfer AM, Grewal PS, Kaya HK (2000b) Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism. Entomol Exp Appl 94:283–293

Koppenhöfer AM, Cowles RS, Cowles EA, Fuzy EM, Baumgartner L (2002) Comparison of neonicotinoid insecticides as synergists for entomopathogenic nematodes. Biol Control 24:90–97

Li, Jiarui, Timothy C. Todd, Tom R. Oakley, Junghoon Lee, and Harold N. Trick. "Host-derived Suppression of Nematode Reproductive and Fitness Genes Decreases Fecundity of Heterodera Glycine Ichinohe." *Planta* 232.3 (2010): 775-85. Web. 7 June 2012.

Lindbo, John A., and William G. Dougherty. "Plant Pathology and RNAi: A Brief History." *Annual Review of Phytopathology* 43.1 (2005): 191-204. Web. 11 June 2012.

Maeda, Ikuma, Yuji Kohara, Masayuki Yamamoto, and Asako Sugimoto. "Large-scale Analysis of Gene Function in Caenorhabditis Elegans by High-throughput RNAi." *Current Biology* 11.3 (2001): 171-76. Web. 8 Nov. 2012.

"NASS - National Agricultural Statistics Service." *NASS - National Agricultural Statistics Service*. N.p., n.d. Web. 07 June 2012. <http://www.nass.usda.gov/>.

Niblack, T. L., Tylka, G. L., Arelli, P., Bond, J., Diers, B., Donald, P., Faghihi, J., Ferris, V. R., Gallo, K., Heinz, R. D., Lopez-Nicora, H., Von Qualen, R., Welacky, T., and Wilcox, J. 2009. A standardized greenhouse method for assessing soybean cyst nematode resistance in soybean; SCE08 (standardized cyst evaluation 2008). Plant Health Progress doi:10.1094/PHP-2009-0513-01-RV.

Nicola-Negri, Elisa Di, Angela Brunetti, Mario Tavazza, and Vincenza Ilardi. "Hairpin RNA-Mediated Silencing of Plum Pox Virus P1 and HC-Pro Genes for Efficient and Predictable Resistance to the Virus." *Transgenic Research* 14.6 (2005): 989-94. Web. 30 June 2013.

Nobuyoshi Ishibashi and Shinji Takii (1993) Effects of Insecticides on Movement, Nictation, and Infectivity of *Steinernema carpocapsae*. J Nematol. 25(2): 204–213.

Oki, Delwyn S., and Thomas W. Giambelluca. "DBCP, EDB, and TCP Contamination of Ground Water in Hawaii." *Ground Water* 25.6 (1987): 693-702. Web. 13 June 2012.

Putter, I., J. G. Mac Connell, F. A. Preiser, A. A. Haidri, S. S. Ristich, and R. A. Dybas. "Avermectins: Novel Insecticides, Acaricides and Nematicides from a Soil Microorganism." *Experientia* 37.9 (1981): 963-64. Web. 20 May 2013.

Qiu, Shibin, Coen M. Adema, and Terran Lane. "A Computational Study of Off-target Effects of RNA Interference." *Nucleic Acids Research* 33.6 (2005): 1834-847. Web. 8 Dec. 2012.

Rand, T. A. "Biochemical Identification of Argonaute 2 as the Sole Protein Required for RNA-induced Silencing Complex Activity." *Proceedings of the National Academy of Sciences* 101.40 (2004): 14385-4389. Web. 25 Oct. 2012.

Sanford, J. C., and S. A. Johnston. "The Concept of Parasite-derived Resistance—Deriving Resistance Genes from the Parasite's Own Genome." *Journal of Theoretical Biology* 113.2 (1985): 395-405. Web. 12 June 2012.

Schmitt, D. P., Robert D. Riggs, and James Allen. Wrather. *Biology and Management of Soybean Cyst Nematode*. 2nd ed. Marceline, MO: Schmitt & Associates of Marceline, 2004. Print.

Shapiro-Ilan DI, Gouge DH, Piggott SJ, Fife JP (2006) Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol Control 38:124–133

Sindhu, A. S., T. R. Maier, M. G. Mitchum, R. S. Hussey, E. L. Davis, and T. J. Baum. "Effective and Specific in Planta RNAi in Cyst Nematodes: Expression

Interference of Four Parasitism Genes Reduces Parasitic Success." *Journal of Experimental Botany* 60.1 (2008): 315-24. Web. 30 Oct. 2012.

Singh, Guriqbal. *The Soybean: Botany, Production and Uses*. Wallingford, UK: CABI, 2010. Print.

Soriano PA, Del Pino FG (2000) Compatibilidad de los nematodos entomopatógenos (Rhabditida: Steinernematidae y Heterorhabditidae) con el oxamilo. Bol Sanid Veg Plagas 26:377–387

Steeves, Ryan M., Tim C. Todd, Juliane S. Essig, and Harold N. Trick. "Transgenic Soybeans Expressing SiRNAs Specific to a Major Sperm Protein Gene Suppress Heterodera Glycines Reproductio." *Functional Plant Biology* 33.11 (2006): 991-99. Print.

Stephens, David J., Nathalie Lin-Marq, and Alessandra Pagano. "COPI-coated ER-to-Golgi Transport Complexes Segregate from COPII in Close Proximity to ER Exit Sites." *Journal of Cell Science* 113 (2000): 2177-185. 25 May 2000. Web. 13 Sept. 2012.

United States of America. United States Department of Agriculture. Office of the Chief Economist. *World Agricultural Supply and Demand Estimates*. By United States Dept of Agriculture Economics and Statistics Service. U.S. Department of Agriculture, Economics and Statistics Service, 11 July 2012. Web. 15 July 2012.

United States. United States Department of Agriculture. Office of Global Analysis. *World Agricultural Production*. N.p., 11 July 2012. Web. 18 July 2012.

United States. USDA. Agricultural Research Service. *USDA National Nutrient Database for Standard Reference*. Beltsville, MD: USDA, Nutrient Data Laboratory, Agricultural Research Service, 2011. Web.

USA. USDA. National Agricultural Statistics Service. *Crop Values 2011 Summary (February 2012)*. N.p., Feb. 2012. Web. 19 July 2012.

Visarada, K. B. R. S., K. Meena, C. Aruna, S. Srujana, N. Saikishore, and N. Seetharama. "Transgenic Breeding: Perspectives and Prospects." *Crop Science* 49.5 (2009): 1555-563. Web. 10 July 2012.

Winstead, N. N., C. B. Skotland, and J. N. Sasser. "Soybean Cyst Nematode in North Carolina." *Plant Disease Reporter* 39.1 (1955): 9-11. Web. 13 June 2012.

"WormBase." *WormBase: Nematode Information Resource*. N.p., n.d. Web. 08 June 2012. <http://www.wormbase.org/>.

Wrather, J. A., and Steve Koenning. "MO Ag Experiment Station - Delta Research Center." *MO Ag Experiment Station - Delta Research Center*. N.p., n.d. Web. 18 May 2013.