Analysis of Root-Knot nematode Resistant and Susceptible Genes in Cotton (*Gossypium hirsutum L.*) roots and Effects of microRNA inhibition on nematode viability

by

Sarah Armstrong

December 2025

Director of Thesis: Xiaoping Pan, PhD

Major Department: Department of Biology

Plant parasitic nematodes (PPNs) are one of the most impactful pests on multiple species of plants that are important globally costing $100-150 billion annual revenue loss. One of the most impactful species of PPNs is *Meloidogyne incognita* (*M. incognita*). *M. incognita* are known as the root-knot nematode due to the nodules that are created on the roots that the nematodes spend their life cycle from their second juvenile stage. This is through the interaction of effector proteins that are excreted through the nematodes esophageal glands and stylet during the infection process. In response plants have genes that are both susceptible to and resistant to the PPN infection process. Susceptible genes are modulated by these effector proteins to create the optimal environment form the nematodes to inhabit. Resistant genes are those that are not manipulated by the effector proteins and have been noted to inhibit the infection of the root tissues. We have identified 6 anti-miRNAs that showed to have an impact on the viability of *C. elegans* and are also known to be present within *M. incognita*. These anti-miRNAs all showed decreases in the viability of the *C. elegans* within a 72-hour period. We also identified 25 possible susceptible and resistant genes that are present within the Cotton (*Gossypium hirsutum L.*) genome. These genes were all shown to be expressed in the genome as a baseline for the expression of these genes.

Analysis of Root-Knot nematode Resistant and Susceptible Genes in Cotton (*Gossypium hirsutum L.*) roots and Effects of microRNA inhibition on nematode viability

A Thesis

Presented To

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfilment of the Requirements for the Degree

Master of Science in Biology

By:

Sarah Armstrong

December 2025

Director of Thesis: Xiaoping Pan, PhD

Thesis Committee Members:

Myon-Hee Lee, PhD

Yumin Li, PhD

Baohong Zhang, PhD

©Copyright by Sarah Armstrong

All Rights Reserved

Acknowledgements

I would like to thank my thesis advisor, Dr. Xiaoping Pan, for all her help with the guidance and support she provided to me during my graduate study here at East Carolina University. I would like to thank her for allowing me to continue this research of her lab, for providing me a lab space and resources to complete this thesis project, and for giving me this opportunity to learn research skills and obtain lab experience. Her assistance in giving me feedback, suggestions, and thesis writing edits can never be appreciated enough. I would like to thank Dr. Baohong Zhang for his help with learning how to successfully run RNA extraction and how to measure gene expression via qPCR. I would like to thank Dr. Myon-Hee Lee and Dr. Yumin Li for being on my committee and giving me their time through this whole degree. I would also like to thank my friends and family for their wonderful and kind support through this whole program. They helped to keep me going on some of the hardest days I had and will continue to inspire me to be my best self-possible in all regards.

Table of Contents

Title……………………………………………………………………………………………i

Copyright…………………………………………………………………………………ii

Acknowledgements………………………………………………………………………iii

List of Tables.......…………………………………………………………………...v

List of Figures…………………………………………………………………….....vi

Chapter 1: Introduction..…………………………………………………………......1

Hypothesis…………………………………………………………………….......8

Objectives…………………………………………………………………….......8

Chapter 2: Materials and Methods……..………………………………………………...11

Plant Growth Protocol…………….……………………………………………...11

Plant collection Protocol…………………………………………………………11

RNA extraction from plant tissues………………………………………………11

Primer Design Protocol..………………………………………………................12

RT-PCR Protocol ……………………………………………………...............13

qRT-PCR Protocol.…………………………………………………….........14

C. elegans Soaking Protocol….……………………………………………….....15

Chapter 3: Results…..……………………………………………….........................17

Chapter 4: Discussion…..………………………………………………......................33

Chapter 5: Conclusion……………………………………………………………………39

References……………..……………………………………………….......................40

List of Tables

Table 1. Susceptible genes in plant species with cotton homolog..6

Table 2. Resistant genes in plant species with cotton homolog..7

Table 3. Results from RNA extraction (n=4 for each kit) using mirVana, mirPremier, EASYspin, and RNeasy Plant Mini isolation kits on extraction from *Gossypium hirsutum* root tissue……..18

Table 4. Susceptible genes with cotton homologs primers..19

Table 5. Resistant genes with cotton homologs primers………..19

Table 6. Anti-miRNA inhibitor sequences...25

Table 7. Fold changes calculated for each inhibitor using each treatment's respective control and typical method of fold change calculation………………………………………………………25

List of Figures

Figure 1. Life Cycle of *Meloidogyne incognita*…………………………………2
Figure 2. Average CT Values of susceptible homolog genes from uninfected unmodified Cotton plants at day 15 post germination (n=3)……………………………21
Figure 3. Average CT Values of resistant homolog genes from uninfected unmodified Cotton plants at day 15 post germination (n=3)……………………………22
Figure 4. Average CT Values of susceptible homolog genes from uninfected unmodified Cotton plants at day 30 post germination (n=3)……………………………23
Figure 5. Average CT Values of resistant homolog genes from uninfected unmodified Cotton plants at day 30 post germination (n=3)……………………………24
Figure 6. Inhibition levels of anti-miR-1, anti-miR-49, anti-miR-71, anti-miR-72, anti-miR-92, anti-miR-100a, anti-miR-124, and anti-miR-228……………………26
Figure 7. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-1 treatments over a 72-hour period……………………………27
Figure 8. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-49 treatments over a 72-hour period……………………………28
Figure 9. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-72 treatments over a 72-hour period……………………………29
Figure 10. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-92 treatments over a 72-hour period……………………………30
Figure 11. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-100a treatments over a 72-hour period……………………………31
Figure 12. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-228 treatments over a 72-hour period……………………………32

Chapter 1: Introduction

Technological advances in agriculture have increased efficiency, ultimately increasing crop yields. However, climate change and pest control practices threaten food insecurity worldwide [27]. For example, long-term use of chemical pesticides and insecticides can lose efficacy and have lasting non-target environmental and health effects [2]. Plant Parasitic Nematodes (PPNs) are one of the types of pests that are most impacting crop yields.

PPNs can be classified as either sedentary or migratory based on the mode of parasitism used. Sedentary nematodes are those that remain in the root for their adult life while migratory nematodes are those that will move freely through the soil or plant tissues. They are further divided into endoparasitic, semi-parasitic, or ectoparasitic. Ectoparasitic nematodes feed on plant tissues from outside of the plant while endoparasitic nematodes feed from within the plant tissues, as shown in Figure 1 [17]. *Meloidogyne incognita* are classified as sedentary endoparasitic nematodes as the adult females will remain within the feeding site they create within the root system while stage 4 Juvenile (J4) males will migrate from the root system into the soil. *M. incognita* reproduce via mitotic parthenogenesis with favorable conditions resulting in the development of female juveniles while less favorable conditions will result in juvenile males being produced. These males play a role in the population even without adding to the genetic diversity of the species, as these males will migrate from the root tissue resulting in more resources being available to the females. Females will remain in the root system and produce large egg masses that are extruded in a gelatinous matrix out of the root [10].

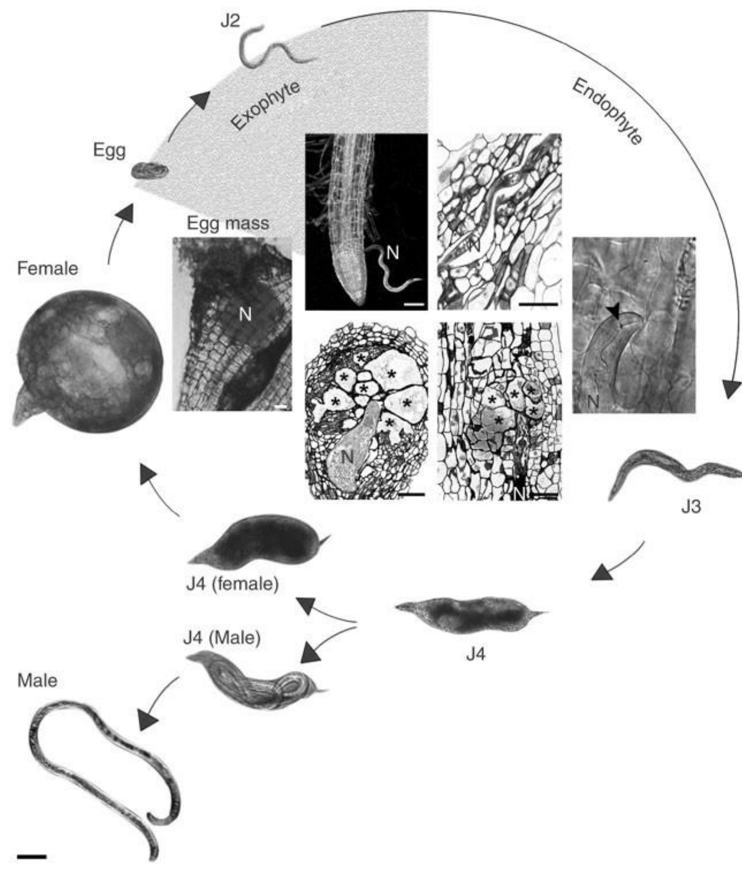

Figure 1. Life Cycle of *Meloidogyne incognita.* This figure shows a representation of the life cycle of the RKN *M. incognita* within a host plant root system. Stage one (1) is the egg which was laid in a gel matrix outside of the plants root system that will develop into a J1 (Juvenile stage 1) within the egg (2), from this point the J2 will hatch from the egg and begin migrating and penetrating the host plants root where it will move through the tissues until it reaches the vascular cylinder and becomes sedentary within the root, they will then enter J3 and J4 where there may be some males produced (3). Males will exit the roots while females will remain in the roots and will release eggs in a gel matrix on the surface of the root. Image Adapted [17].

The feeder cells that these nematodes reside in are created through a process of continuous nuclear division without the formation of a cell wall. This nuclear division is stimulated through the release of effector proteins from the dorsal gland of the nematode [57].

Some of the effectors that have been studied have been noted as being secreted in the apoplast during intercellular migration to the root while others are directly interacting with specific proteins in the cytoplasm or the nucleus of the host cell [46]. Along with the effector proteins released during the infection process, calreticulin (CRT) is released as well. CRTs are highly conserved within multicellular eukaryotes and have shown to play a significant role in the suppression of plants' immune system. This means the RKN effector act by suppressing plant's immune response. Jaouannet et al. used RNAi to knock down of *Mi-CRT* in nematode result in impaired the nematodes infectivity to the host plant [29]

Current research into PPNs has shown that some plants have resistant (R) and susceptible (S) genes to PPN infections. This occurs when the plant immune system recognizes effector proteins that are released by PPN during the infection process [17]. In the case of R genes, they code for avirulence proteins (Avr) and once R protein recognizes the corresponding effectors, it can lead to effector-triggered immunity (ETI) to against nematode infection. While S genes are plant compatibility factors that subjected to PPN effectors manipulation [17]. For example, the *AtSWEET11* gene is involved in the regulation of glucose uptake and efflux and have been noted to be upregulated by *M. incognita* infection [37][66], thus considered a S gene. There are more S genes identified within many plant species as compared to R genes, with this in mind it would be likely beneficial to knock down the S genes to prevent the facilitation of PPN infections. There are very few cotton cultivars that are resistant to PPN infections. One of the few cultivars that have been identified is the "Auburn 623 RNR", with the RNR denoting Resistance to Nematode Root, which was derived from mating two moderately resistant parents (Wild Mexican Jack Jones and Clevewilt 623) to create an RKN-resistant line [33][54]. Though this has been noted to be an RKN-resistant cultivar it is unknown as to what gives it this resistance, this resistance has

been correlated to a reduction of eggs and root galls in the plants [33]. Research has been moving towards not only breeding cultivars together to create these resistant cultivars by chance but to also identify resistant genes within cultivars that can be knocked in or susceptible genes that can be knocked out to lead to resistance.

With this knowledge 25 S and R genes have been selected that have homologs within upland cotton (*G. hirsutum)*. *FTRc* is a key element within the *Arabidopsis thaliana* antioxidant network and results in enhanced ROS-scavenging activity involving an iron-sulfur protein. This is believed to be targeted by *M. javanica* which exploit the ROS-scavenging system within the plant via the effector *MjTTL5* to suppress ROS burst thus suppress the immune response to nematode infection [36]. bHLH, found in *Arabidopsis,* is a basic helix-loop-helix transcription factor that positively influences *Heterodera schachtii*, a cyst nematode, parasitism, with two of these factors being known to be able to dimerize *in planta* with overexpression increasing the parasitism [30]. *HIPP27,* a heavy metal -associated isoprenylated plant protein, is a susceptible gene candidate in *Arabidopsis* supporting *Heterodera schachtii* infection. When *HIPP27* is knocked down susceptibility is reduced without affecting the plant phenotype [18][50]. *HVA22* is associated with the abscisic acid (ABA) signaling pathway which functions as a growth inhibitor and promotes cell differentiation and is believed to play a role in the hypersensitive cell death response [55]. *MAPK9* is known to be involved in plant-pathogen interactions and are downregulated following infections of *M. incognita* [39]. *MAP65* is essential for giant cell ontogenesis, associated with a novel kind of cell plate that separates daughter nuclei. In the absence of *MAP65* giant cells developed but did not fully differentiate and were consequently destroyed [9]. As mentioned above, *AtSWEET11* is involved in the regulation of glucose uptake and efflux and has been noted to be upregulated by *M. incognita* infection [37] [66]. *SmD1* is

modulated by *MiEFF18* which facilitates giant cell formation [44]. *SmD1* is an essential component of the spliceosome, a complex involved in pre-mRNA splicing and alternative splicing. *SlWRKY45* is known to enhance susceptibility to nematodes, believing to be manipulated by invading nematodes [13].

GmEXPA1, which facilitates 'cell wall loosening' allowing for turgor-driven cell expansion, was found to be upregulated in a nematode-resistant genotype within soybean (*Glycine max)* [3]. *GH3.6,* part of the *GH3* gene family, was found to be related to indole-3-acetic acid-amino synthetase (IAA) with low expression and little change being noted after *M. incognita* infection [39]. *GH3* is known to be involved in hormone homeostasis and is known to be involved in the stress response which can trigger the immune response within the plant. *Glyma10g017100* encodes for a protein with two domains one that is similar to a pectinesterase (PEC) and a pectin methylesterase inhibitor (PMI) and shows expression in young leaves, seeds, and nodules [47]. *H1* confers resistance to *Globodera rostochiensis,* known as the yellow potato cyst nematode which are also sedentary endoparasitic nematodes, and is still effective against these nematodes even though it was first discovered in 1952 with a gene-for-gene interaction being genetically supported [22] [28]. *HsfA1a,* a heat shock factor gene is required for both basal defense in a susceptible genotype as well as for *Mi-1.2* mediated RKN resistance [65]. *Ma* confers complete-spectrum resistance against the *Meloidogyne* species [14]. *Me1* was identified in one of two known resistant pepper cultivars, specifically PM217 and is resistant to *M. arenaria, M. incognita* and *M. javanica* [11]. *Mi-1* is a commercially available source of resistance and is induced as early as 2 weeks after germination in the leaves and roots of tomato plants [19]. *Mi-3*, one of the R genes that was identified was originally identified as a R gene in *Lycopersicon peruvianum,* tomato endemic to Peru, that is believed to be a dominant gene [62].

qMi-C11 is a previously identified resistance locus on chromosome 11 withing the *G. hirsutm,* Upland cotton, genome [31]. *Rhg1* is a resistance locus within soybeans that is resistant to *Heterodera glycines,* known as the soybean cyst nematode and is also a sedentary endoparasitic nematode, and is the most popular source of resistance against these cyst nematodes [8]. *Rhg4* is a gene that is a known polymorph with confirmed resistance in soybean, with two separate homologs noted within cotton [38] [63]. *Rk* has been bred into several cowpea varieties and is effective against *M. incognita* though there are known populations of *M. incognita* that have the *Rk* gene [48]. *Rma1* is a resistance gene that was identified in peanuts which was originally been suggested as a single dominant gene but a second gene was found [7]. *TGA21* is a gene from soybeans that is involved in the regulation of growth, development, and responses to pathogens and abiotic stress [34].

Table 1. Susceptible genes in plant species with cotton homolog

Original Plant gene	Original Plant	Cotton Homolog	Source
FTRc	*Arabidopsis thaliana*	*FTR*	[25]
bHLH	*Arabidopsis thaliana*	*FBOX*	[30]
HIPP27	*Arabidopsis thaliana*	*HmP27*	[18]
HVA22	*Solanum lycopersicum*	*HVA*	[55]
MAPK9	*Cucumis sativus*	*UNCH*	[39]
MAP65	*Arabidopsis thaliana*	*65M*	[9]
AtSWEET11	*Arabidopsis thaliana*	*BR1*	[60]
SmD1	*Arabidopsis thaliana*	*GAPCP*	[44]
SlWRKY45	*Solanum lycopersicum*	*WRKY*	[13]

Table 2. Resistant genes in plant species with cotton homolog.

Original Plant gene	Original Plant	Cotton Homolog	Source
GmEXPA1	*Glycine max*	*EXPA1*	[3]
GH3.6	*Cucumis sativus*	*IAA*	[39]
Glyma10g017100	*Glycine max*	*PECT*	[47]
H1	*Solanum tuberosum*	*17KD*	[51]
HsfA1a	*Solanum lycopersicum*	*V6Z11*	[65]
Ma	*Prunus cerasifera*	*RPV*	[53]
Me1	*Capsicum annuum*	*V6Z11*	[11]
Mi-1	*Solanum lycopersicum*	*DISRP*	[19]
Mi-3	*Lycopersicon peruvianum*	*ERO*	[11]
qMi-C11	*Gossypium hirsutum*	*HSPRO*	[16]
Rhg1	*Glycine max*	*IMK*	[8]
Rhg4	*Glycine max*	*TMK*	[38]
Rhg4	*Glycine max*	*HSPRO*	[63]
Rk	*Vigna unguiculata*	*RK*	[48]
Rma1	*Arachis hypogaea L.*	*V6Z12*	[7]
TGA21	*Nicotiana tobacum*	*TGAL*	[6]

However, these S and R gene expressions in cotton remains largely unknown. Understanding the baseline expression of these genes will help the target identification for genetic engineering of cotton plants using RNAi or the emerging CRISPR/Cas biotechnology.

MicroRNAs (MiRNAs) are noncoding RNAs, first identified in *C. elegans* that interact with argonaute (Ago) proteins to modulate gene expression, they are roughly 17-27bp in length [52]. Within plants these miRNAs play a role in development, growth, response to the environment and abiotic factors [58]. Previous research completed within the Pan lab identified a total of 254 miRNAs that could impact nematode viability. These were identified across 161

families with a total of 35 novel miRNAs from 31 families with no known homologs in other nematode species. Of the miRNAs found there were eight major families that contained four or

Table 4 The most abundant 16 *Meloidogyne incognita* miRNAs that have >5000 miRNA reads

miRNA name	miRNA read count	% of total miRNA read
min-miR-100a	1,925,637	37.0
min-miR-124	938,674	18.1
min-miR-71a	796,156	15.3
min-miR-1	386,875	7.4
min-miR-228	351,680	6.8
min-miR-92	219,504	4.2
min-miR-72	150,410	2.9
min-miR-49b	92,110	1.8
min-miR-58	83,715	1.6
min-miR-252	34,942	0.7
min-miR-lin-4	23,089	0.4
min-miR-87	14,978	0.3
min-miR-2a	14,658	0.3
min-miR-34a	9164	0.2
min-miR-50a	7893	0.2
min-miR-279a	6754	0.1

more members; miR-35, miR-130, miR-7880, miR-8202, miR-223, miR-580, and miR-335. From these families the most abundant 16 were identified with each having >5000 miRNA read sequences, listed in the table below [64]. These miRNAs are all conserved within nematode species with miR-100a being the most abundant miRNA with 37% of total reads.

Given miRNAs are important gene regulators, we asked could miRNAs be good target for developing nematode management strategy? To answer this question, we test if inhibition of these miRNAs will result in nematode mortality.

Objectives

Aim 1- Vetting of RNA Extraction Kits

Extraction of high-quality nucleic acids such as RNA and DNA from plant tissue, specifically cotton tissues, has been known to pose some issues. This is due to secondary metabolites that are present within the tissues, for example polysaccharides and polyphenolics [41]. To deal with this issue, optimization of the extraction method was completed, this was done across four commercially available kits. The kits selected were the mirVana miRNA Isolation Kit by ThermoFischer Scientific with modified procedures to increase the possibility of higher quality and quantity RNA, Rneasy Plant Mini Kit for RNA Extraction by Qiagen, miRPremier microRNA Isolation Kit by Sigma-Aldrich, and the EASYspin Plus Plant RNA Kit from Aid Lab.

Aim 2- Gene expression analysis of Resistant and Susceptible genes in Upland Cotton (*Gossypium hirsutum)*.

Nematode resistant and susceptible genes were selected mainly based upon literature research, these genes were identified across a number of papers which have been reviewed in the introduction. The selected resistant genes were *Mi-3* identified in *Lycopersicon peruvianum; Rhg1, Rhg4, GmEXPA1*, and *Glyma10g017100* identified in *Glycine max; Rk* identified in *Vigna unguiculata; Me1* identified in *Capsicum annuum; Rma1* identified in *Arachis hypogaea L.; qMi-C11* identified in *Gossypium hirsutum; GH3.6* identified in *Cucumis sativus; TGA21* identified in *Nicotiana tobacum; Ma* identified in *Prunus cerasifera; H1* identified in *Solanum tuberosum;* and *Mi-1*and *HsfA1a* identified in *solanum lycopersicum.*

The susceptible genes selected are *HIPP27, MAP65-3, AtSWEET1, SmD1, FTRc* and *bHLH* identified in *Arabidopsis thaliana; SlWRKY45* and *HVA22* identified in *Solanum lycopersicum;* and *MAPK9* identified in *Cucumis sativus.* The homologs of these genes were then identified within *Gossypium hirsutum* via a BLAST search using the original genome. From

the homologs primers were made using primer-BLAST which were used in RT-PCR and qRT-PCR.

Aim 3- Effect of microRNA inhibition on nematodes survival

With the use of microRNA inhibitors we performed a loss-of-function studies of conserved *M. incognita* miRNAs in *C. elegans.* These genes have been chosen based on previous findings from the Pan lab and literature, with a selection of 8 microRNAs subjected to the inhibition study. This selection includes the most abundantly expressed in *M. incognita* miRNAs the Pan lab has previously identified and verified [64]. These miRNAs were identified by using a previously developed program called miRDeepFinder which combines stem-loop structure, minimum free folding energy, minimum free folding energy index, location of miRNAs, and other characteristics to identify conserved and novel miRNAs in both model and non-model species [61].

The soaking procedure we used in our preliminary work on *C. elegans* was completed across 72-hours to give ample time to see the impacts of miRNA inhibitions on the *C. elegans*. The worms were then imaged via WormLab software in a mix of pictures and videos that were used to collect movement data to visually confirm viability.

Chapter 2: Materials and Methods

Plant Growth Protocol

Plants were grown in the ECU Greenhouse, 3 *Gossypium hirsutum* seeds were first potted within small plastic pots in regular potting soil with fertilizer added. Plants were watered twice a day and growth was checked to note when germination occurred to be able to collect samples at 15- and 30-days post germination. Plants were labeled with the common plant name, date, and growers initials.

Plant collection Protocol

Plants were collected at two different time points for RNA extraction, 15 days and 30 days post germination. All plants heights were measured and the roots weighed after they had been gently cleaned and dried with paper towel.

RNA extraction from plant tissues

Before beginning ensure that all tools and work spaces are clean and that there is ice and liquid nitrogen ready to be used. Place a clean mortar and pestle into a -80ºC freezer for a minimum of 10 minutes prior to use. Prepare a 2mL centrifuge tube for each sample and place this on ice before adding 400uL of lysis buffer. Plant tissue should be collected, cleaned if there is soil or other contaminants present, wrapped in aluminum foil and flash frozen in liquid nitrogen to ensure that there is minimal degradation of RNA. When ready to begin working with the sample the mortar and pestle should be removed from the -80ºC freezer, the tissue sample collected from the liquid nitrogen and ground while adding liquid nitrogen to the mortar to prevent the sample from thawing. Once the tissue is ground to a fine powder transfer the powder to the 2mL centrifuge tube with the lysis buffer. Sonicate the tube on ice for 15-20 seconds on ice

before adding 40uL of miRNA homogenate additive to the lysate and mix by gently inverting the tube. The tube should be left on ice for 10 minutes before 400uL of Acid-Phenol:Chloroform is added and mixed through inverting the tube. Centrifuge the mixture for 5 minutes at max speed to separate the aqueous and organic phases, if needed centrifuging can be repeated a second time. After centrifuging collect the aqueous phase using a clean pipette, typically 300uL is collected and placed to a fresh tube where 1.25 volumes of room temperature ethanol is added and mixed via inverting. This mixture is then pipetted to a filter cartridge that has been placed in a new tube and centrifuged for 15 seconds at 10,000rpm. Flow-through is discarded before 700uL of miRNA wash solution 1 is applied to the filter and centrifuged for 10 seconds. Again discard the flow-through before applying 500 uL of wash solution 2/3 and centrifuging for 10 seconds and discarding the flow-through, this step is repeated a second time. Once the flow-through from the final wash is discarded centrifuge the tube for 1 minute to dry the filter before transferring the filter to a clean new tube. Once in the new tube 50 uL of pre-heated 95ºC nuclease free water is pipetted to the center of the filter and centrifuged for 30 seconds at maximum speed. The tube is gently mixed and lightly tapped on the bench top to ensure all RNA is collected. This sample is then measured for concentration and quality on the Nanodrop before being used for RT-PCR or being stored at -20ºC or below.

Primer Design Protocol

The primers used for these experiments were designed in lab using the primerBLAST site. Each of the primers is designed to be in length between 20-30 base pairs long with a GC content between 40-60% and having melting temperatures between 65ºC to 75 ºC. For the R/S primers the original genome was input into BLAST and searched against the *Gossypium hirsutum* genome with homologs being used to create the primers.

RT-PCR Protocol

Each RNA sample will have 1ug of total RNA used per 15uL reaction with the volumes of water and RNA sample being calculated based off of the concentration of RNA in the sample. For each tube you must include the following:

Component	**Volume (uL)**
Nuclease-free water	Calculate: $15 - (3.84 + RNA\ Sample) = ?$
RNase inhibitor, 20U/uL	0.19
100mM dNTPs (with dTTP)	0.15
10X Reverse Transcription Buffer	1.50
Primer	1.00
Multiscribe™ Reverse Transcriptase, 50U/uL	1.00
RNA Sample	Calculate: $Concentration \div 1000 = ?$
Total	**15**

Gently mix the tube after adding all components before centrifuging for 10 seconds at 2000rpm before incubating the tubes on ice for 5 minutes and keeping on ice until ready to load into the thermal cycler. When ready to perform reverse transcription ensure that the settings are as follows.

Temperature ºC	Time (minutes)
16	30
42	30
85	5

4	Hold

Once RT-PCR is completed add 80uL of DNase free water to each tube and mix before either performing qRT-PCR or storing the tube at -20 ºC or below.

qRT-PCR Protocol

For each reaction that will be run there should be three (3) 0.2 mL sterilized tubes following the amounts below:

Component	**Volume (uL) per tube**
Nuclease-free Water	6
2X SyberGreen PCR Master Mix	10
Diluted Product from RT-PCR reaction	2
Primer	2
Total	**20**

Ensure that the components are pipetted to the bottom of the tube due to how small the amounts are and centrifuge after adding all components to ensure that all reagents are in the 20uL. The reaction will be pipetted from the tubes they are located into a qRT-PCR plate with a written record of placement in the tube as there can be no markings made on the plate. Centrifuge the plate to ensure that all reactions are located at the bottom of each well before loading the plate into the machine and following the protocol listed below.

Step	**Enzyme Activation**	**PCR**

		Cycle (45 cycles)	
		Denature	Anneal/Extend
Time	10 minutes	15 Seconds	60 Seconds
Temp ºC	95	95	60

After running qRT-PCR wrap the plate in aluminum foil and store the plate at -20 ºC or below.

***C. elegans* Soaking Protocol**

This is done by creating a transfection solution on the first day of the experiment, typically referred to as Day 0 or 0-Hours. This solution is made up of 9.5g of 50 mM Octopamine Hydrochloride, 0.0153g of 3 mM Spermadine, and 0.01g of 0.05% Gelatine mixed within 20ml of M9 to create a stock solution. This stock solution is then used in tandem with the inhibitors within the soaking procedure, this is because the transfection solution allows for uptake of the inhibitors. To set up the wells individually calculations are made to determine how much of the transfection solution, anti-miRNA, and *C. elegans* are needed. For my experiments I was able to keep it consistent with the control group having 80 ul of transfection solution, 0 ul of anti-miRNA, and 20 ul of *C. elegans,* the 100ul treatment has 76 ul of transfection solution, 4 ul of anti-miRNA, and 20 ul of *C. elegans,* treatment 200 ul has 72 ul of transfection solution, 8 ul of anti-miRNA, and 20 ul of *C elegans,* treatment 400 ul has 64 ul of transfection solution, 16 ul of anti-miRNA, and 20 ul of *C. elegans,* and finally treatment 800 ul has 48 ul of transfection solution, 32 ul of anti-miRNA, and 20 ul of *C. elegans.* Each well contained 40-60 worms to give ample population size. These worms are kept in an incubator that is set to 27℃ to attempt to minimize environmental impacts on survival. The worms are checked at the same time each day

for 24, 48, and 72-hour measurements when the worms are counted, and the living worms are recorded based off their movement within the well.

Chapter 3: Results

RNA extraction within *Gossypium hirsutum* tissue and *C. elegans* and method optimization

Within the Pan lab we have optimized the process of extracting RNA from *C. elegans* and have found that the mirVana miRNA Isolation Kit by ThermoFischer Scientific is the best kit for use within our lab. However, with the amount of time spent on RNA extraction it was decided to find the optimized procedure for *Gossypium hirsutum* tissue as our previous work was resulting in poor quantity and quality of RNA using the kit we normally used within the Pan Lab. We began by selecting four commercially available RNA extraction kits to find the best kit to use for *Gossypium hirsutum* tissue, specifically root tissue, with samples being collected across three separate pots of cotton plants.The kits selected were the mirVana miRNA Isolation Kit by ThermoFischer Scientific with modified procedures to increase the possibility of higher quality and quantity RNA, Rneasy Plant Mini Kit for RNA Extraction by Qiagen, miRPremier microRNA Isolation Kit by Sigma-Aldrich, and the EASYspin Plus Plant RNA Kit from Aid Lab.

To minimize unintended impacts all kits were tested across the variables of increased grinding time testing 5 minutes, 10 minutes, and 15 minutes as well as sample size testing samples from about 100 mg to 150 mg. These changes had similar impacts on the quantity and quality of the RNA from the mirVana and mirPremier kits while the EASYspin kit showed an increase in both quantity and quality (Table 3). Within mirVana we also increased the amount of lysis buffer used and this resulted in a small yield and low-quality RNA. We decided the best kit to use for *Gossypium hirsutum* tissue RNA extraction was the EASYspin kit from Aid Labs. The difference between this kit and the other two kits tested was the inclusion of ‘plantaid’ which is included within the kit to aid in removal of polysaccharides, polyphenols and pigments from the

plant tissue which aids in removal of contaminants, similar to the use of the Acid:Phenol Chloroform during washing steps in the mirVana kit.

Table 3 shows the average results of each kit across a sample size of 16 samples from 4 different plants. The kit that performed the best overall was the RNeasy Plant Mini kit followed by the EASY Spin kit. These both are kits that are designed specifically for use within plant tissue samples which means that they have additional reagents that will help to remove the polysaccharides and other secondary metabolites that will be within the sample that may impact quality. In addition to this these kits have a higher concentration than the others likely due to the fact that they are designed for the plant tissue and have a lysis buffer that may lyse plant cells better than kits that are more commonly used for mammalian cell lines. Given these results we chose to continue all further extraction protocols with the RNeasy Plant Mini kit given that it had the least amount of contamination from other proteins and salts while also having the highest concentration.

Table 3. Results from RNA extraction (n=4 for each kit) using mirVana, mirPremier, EASYspin, and RNeasy Plant Mini isolation kits on extraction from *Gossypium hirsutum* root tissue.

RNA Extraction Kit	**Concentration (ng/µL)**	**260/280**	**260/230**	**Plant Weight (g)**
mirVana	39.5±23.8	1.69±0.27	0.69±0.55	0.1280±0.0296
mirPremier	91.4±61.8	2.28±1.54	1.33±0.64	0.1233±0.0312
EASYspin	98.7±31.5	2.05±0.09	2.57±1.81	0.1157±0.0454
RNeasy Plant Mini	338.6±245.2	2.06±0.03	1.70±0.57	0.1646±0.0589

Cotton Gene Expression

Tables 4 and 5 contain the primers for the susceptible and resistant cotton gene homologs that were used for all gene expression experiments. Figures 2-5 show the CT values for the susceptible and resistant genes at both 15 and 30 days post germination.

Table 4. Susceptible genes with cotton homologs primers

Gene	Cotton homolog	Forward primer	Reverse primer	GC%	TempºC
FTRc	*FTR*	TCCTGCTGCAACTCAGAACC	TCCTCTGGAATGATGCTGCC	55	60.25/59.82
bHLH	*FBOX*	GAGCGAGACGAGTGTCAAGG	CAAGCCTCCTCTACTCTGGC	60	60.46/59.54
HIPP27	*HmP27*	GATCAAGTTTAGACTCC	AACCTGGGAGCTTCTGG	50	58.81/58.26
HVA22	*HVA*	CTTGGGAGAGCCAAACCTC	GAGGATCTGATTGGTGGTG	55/52.38	59.30/59.79
MAPK9	*UNCH*	TCCTGTGGAAGAGACCCGTA	CGTCTCTCCATGTCTCGACTC	55/52.38	59.59/59.60
MAP65	*65M*	GGAGGCAAGGATCTTCAGGG	GTCCAACGCGTTCTGTTGTT	60/50	59.82/59.90
AtSWEET11	*BR1*	CTAAGCCCATTCCCAACTG	GGGCATGGAAATGACG	52.38/55	59.72/60.39
SmD1	*GAPCP*	GCTAAGGCTGCAAAGGGTGC	TCTGCTAACTGTCCTTACGG	55/50	60.61/60.68
SWRKY45	*WRKY*	CCAATGTCATGCCTCCCAC	TAAGGCAGCTCTGGAAGCTC	55/52.38	60.32/60.07

Table 5. Resistant genes with cotton homologs primer

Gene	Cotton homolog	Forward primer	Reverse primer	GC%	TempºC
GmEXPA1	*EXPA1*	ACAGCTCCATGAGCTTCAGAC	GGGTCACCTAAAGTGGGATGG	52.38/57.14	60.07/60.06
GH3.6	*IAA*	TTGCTGCCATTCGGTGC	GGTCCGGTGATACACAACA	58.82/50	58.62/58.94
Glyma10g017100	*PECT*	GCAGCGGAAATGTTAGGAGC	GGACCTATACCTGCGCACTT	55/55	59.62/59.90
H1	*17KD*	GCAATGGCAGCATTTGCCTCG	CTCTCTGGCACCAAGGGATCA	55	60.80/60.55
HsfA1a	*V6Z11*	AGGGATTGGCGGAGC	AGGGATTGGCGGAGC	55/50	60.18
Ma	*RPV*	ACCCATTCTCCATCTCGCTTTC	TCACCACAGGCCTTGATTTCTC	50	60.42/60.55

Me1	*V6Z11*	CACGTGGTCACAAGGTCATC	CTATGCACCACACTCTCCAC	50/53.38	59.24/59.72
Mi-1	*DISRP*	GATGGAGGATGTAGGCGAAGAAG	CTCCGCTCTGAAATGTCCCTG	50/57.14	59.63/60.14
Mi-3	*ERO*	ATGAAGGAGGTTGCTGAGGC	TTTAGTCCAGCAATGGCCC	55	60.03/60.32
qMi-C11	*HSPRO*	CTTGGGAGAGCCAAACCTC	GAGGATCTGATTGGTGGTG	55/52.38	59.30/59.79
Rhg1	*IMK*	CAACCTCTCTGGTCCTGTTCC	CACTCAGTAGAGGTACGGGAAG	57.14/54.55	60/60.74
Rhg4	*TMK*	ATGTTCACTGGTCAGATCCC	GAACTGGACCGGTCAATAAG	50/52.38	59.27/58.37
Rhg4	*HSPRO*	CTTATTGCCTCGGCTGGTG	TGGCTCTCCCAAGCCTAAC	55	60.11/59.67
Rk	*RK*	ACCGCTTGCACCCCC	GGAGGAGGAAGAAGGGAGTGA	73.33/57.14	59.07/60.27
Rma1	*V6Z12*	CTAAGCCCATTCCCAACTG	GGGCATGGAAATGACG	52.38/55	59.72/60.39
TGA21	*TGAL*	TGGAGGCTTCCGTTCATCTG	TCAGCCTGTTGGGATGACTG	55	59.75/59.67

All the genes were observed to have expression in both 15- and 30-day samples. For day 15 *HmP27, 17KD, ERO, HSPRO, IAA, RK,* and *TGAL* all showed higher CT values when compared to the housekeeping gene *PTB,* which corresponds to less expression. The gene that showed the highest expression for the susceptible and resistant genes are *HVA* and *IMK* with CT values of 16.32 and 10.50 respectively. For day 30 samples *65M, BR1, FBOX, FTR, GAPCP, HmP27, HVA, WRKY, 17KD, ERO, EXPA, HSPRO, IAA, RK, RPV, TGAL, TMK, V6Z11, V6Z11,* and *V6Z12* all had lower expression the housekeeping gene *PTB*. For day 30 the genes that had the highest expression when compared to the housekeeping gene were *DISRP, HSPRO,* and *IMK* with CT values of 24.65, 25.45, and 22.19 respectively. Further it was observed that the CT values for the same gene between sample days did show a shift with all the genes showing an increase in CT value in the older root samples. The genes that showed the biggest change in expression level were *FTR* and *IAA* with CT values at 15 days being 16.67 and 26.66 respectively and increasing to 35.90 and 32.91 respectively.

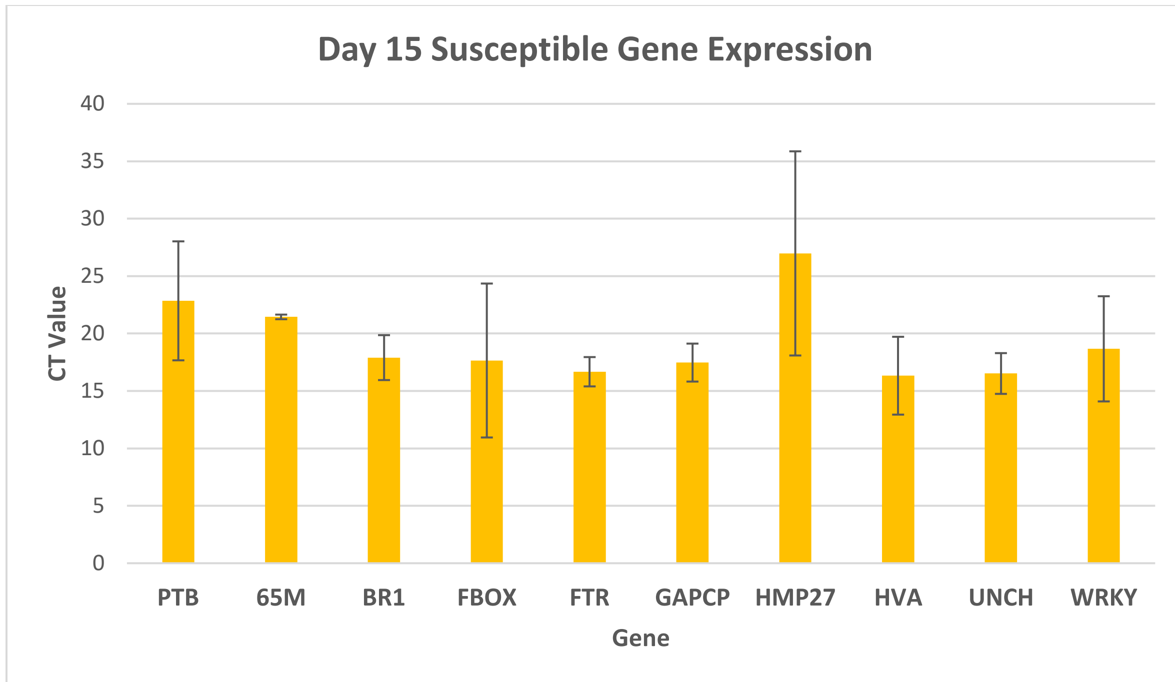

Figure 2. Average CT Values of susceptible homolog genes from uninfected unmodified Cotton plants at day 15 post germination (n=3). The gene PTB is the housekeeping gene used while all others correspond to the genes of interest that are known to be susceptible to *M. incognita* infection in other plant species.

Figure 3. Average CT Values of resistant homolog genes from uninfected unmodified Cotton plants at day 15 post germination (n=3). The gene PTB is the housekeeping gene used while all others correspond to the genes of interest that are known to be resistant to *M. incognita* infection in other plant species.

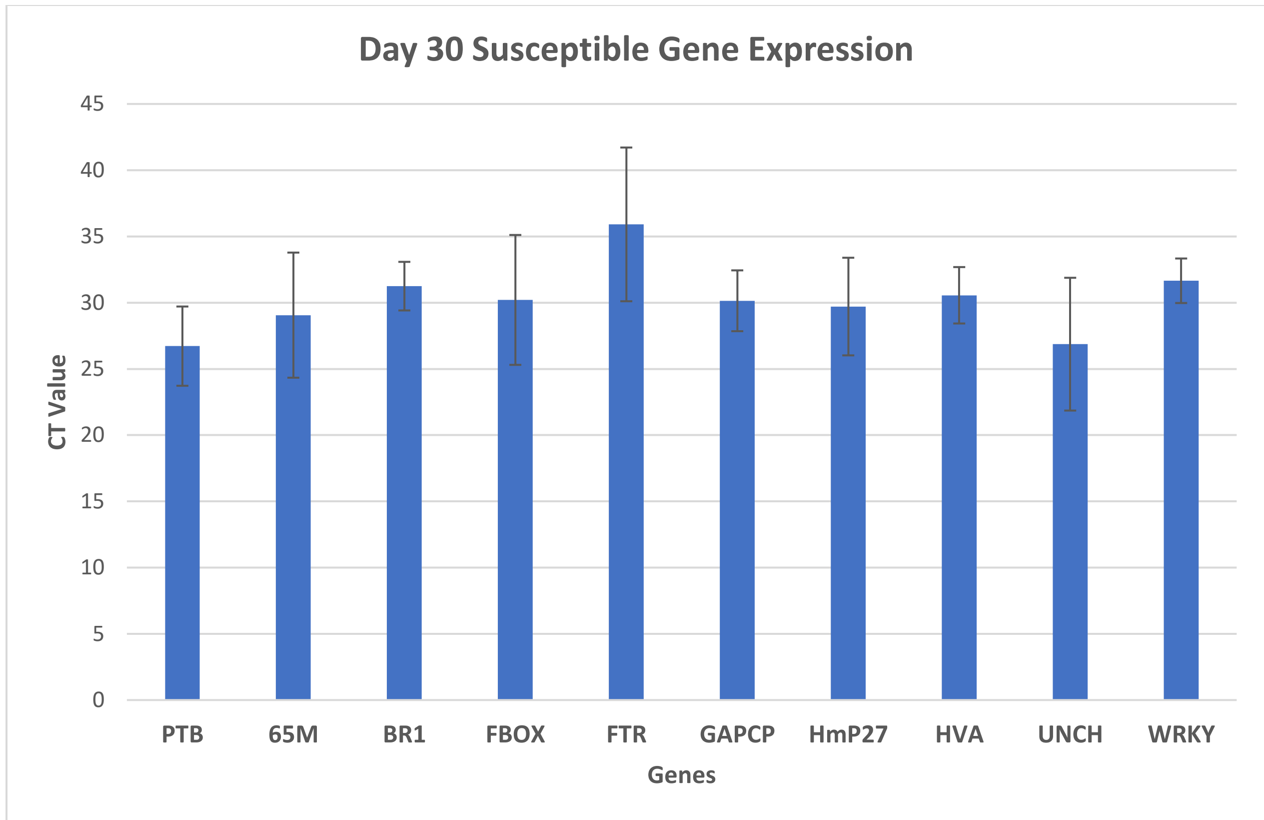

Figure 4. Average CT Values of susceptible homolog genes from uninfected unmodified Cotton plants at day 30 post germination (n=3). The gene PTB is the housekeeping gene used while all others correspond to the genes of interest that are known to be susceptible to *M. incognita* infection in other plant species.

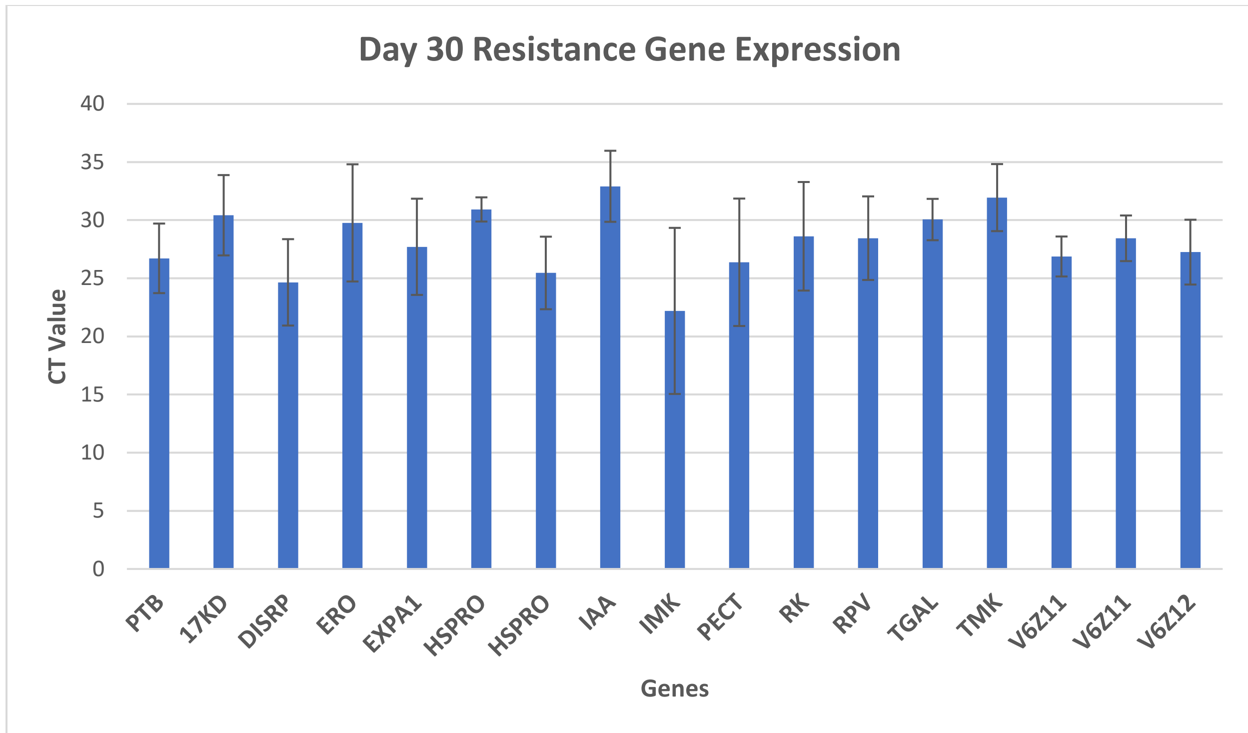

Figure 5. Average CT Values of resistant homolog genes from uninfected unmodified Cotton plants at day 30 post germination (n=3). The gene PTB is the housekeeping gene used while all others correspond to the genes of interest that are known to be resistant to *M. incognita* infection in other plant species.

Inhibition levels

Following RNA extraction RT-PCR and qRT-PCR are run with the samples collected and fold change values were calculated. From the fold change values we can determine the inhibition effects being seen by the inhibitors within the *C. elegans* that have been exposed following the soaking procedure. The data indicated down regulation after being treated with anti-miRNA treatments, found by subtracting each expression level from one. The average miRNA gene expression inhibitions were found to be 64.1%, >99.9%, 98.8%, 99.8%, 98.8%, >99.9%, >99.9%, and >99.9% for anti-miR-1, anti-miR-49, anti-miR-71, anti-miR-72, anti-

miR-92, anti-miR-100a, anti-miR-124 and anti-miR-228 respectively (Figure15). These results are consistent to those that were found from previous research in the Pan lab showing further promising targets for future work with *Meloidogyne incognita.*

Table 6. Anti-miRNA inhibitor sequences

Anti-miRNA Inhibitor	Sequence
Min-miR-1	AUACUUCUUUACAUUCCA
Min-miR-49b	AUCUUCAACAUGGUGCUU
Min-miR-72	AAUGCCAACAUCUUGCCU
Min-miR-92	AGGCCGAAACGAGUGCAAUA
Min-miR-100a	AGACUAGUUCGGAUCUACGGGUU
Min-miR-228	CCGUGAAUUCAUUUGGUGCCAUU

Table 7. Fold changes calculated for each inhibitor using each treatment's respective control and typical method of fold change calculation.

Fold Change	**Anti-miR-1**	**Anti-miR-49**	**Anti-miR-71a**	**Anti-miR-72**	**Anti-miR-92**	**Anti-miR-100a**	**Anti-miR-228**
Replicate 1	0.8461586	0.000168146	0.00146793	0.222698951	0.115943994	0.000229058	6.291918572
Replicate 2	0.017051	0.000196934	0.00507984	0.196713335	0.227588569	3.79905E-05	18.87624635
Replicate 3	0.21493912	0.000176995	0.02683017	0.170420012	0.053050126	6.51411E-06	-3.24338436
Replicate 4		4.51786E-05		0.001043876	0.002187502		-3199.21004
Replicate 5		8.25141E-05		0.001915805	0.021160243		-422.702841
Replicate 6		6.06556E-05		0.000798271	0.000929988		-2723.97925
Average	0.359382307	-4.4501E-05	0.01112598	-0.00140576	-0.01137982	0.01399124	-0.00094877

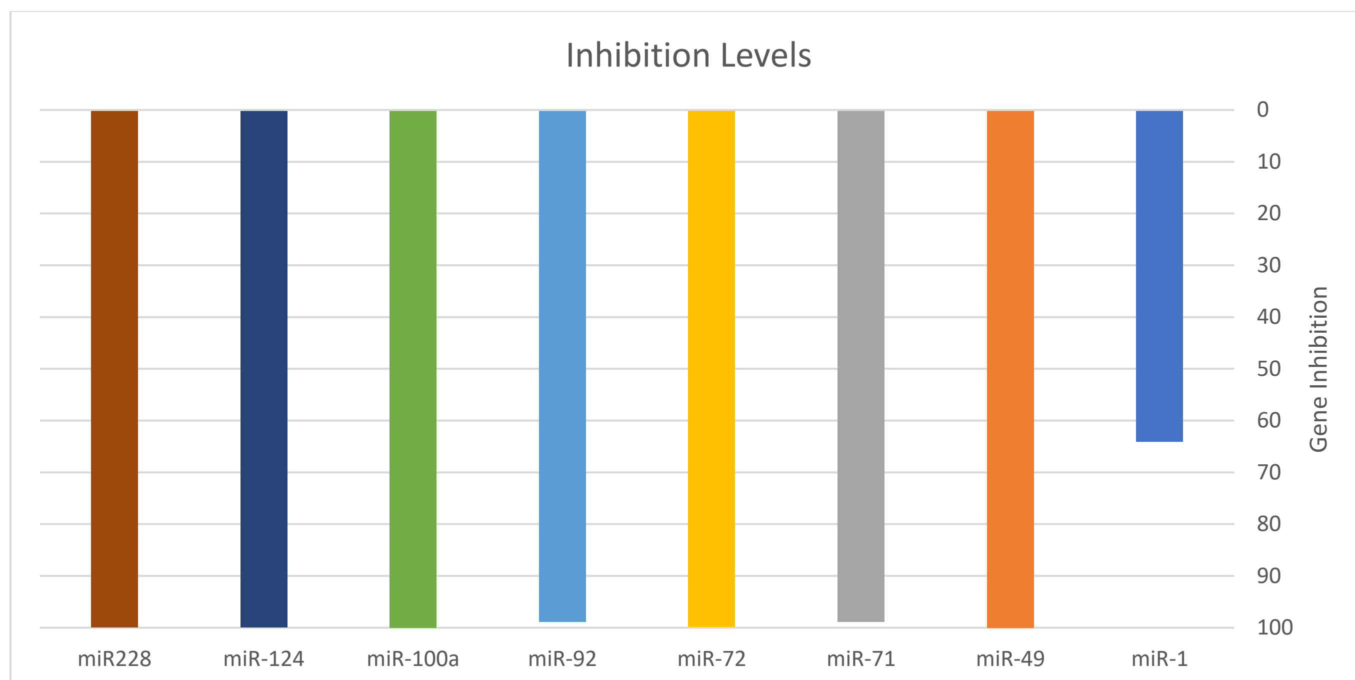

Figure 6. Inhibition levels of anti-miR-1, anti-miR-49, anti-miR-71, anti-miR-72, anti-miR-92, anti-miR-100a, anti-miR-124, and anti-miR-228.

Treatment effects on viability

All data was collected across a 72-hour period and calculated using a one-way ANOVA with comparisons to the controls for the respective time day and time control. P-values less than 0.05 were noted as significant.

Anti-miRNA-1 effects on viability

For the 24 hour treatments 100 nM had a decrease of 41.81% when compared to the vehicle control with a p-value of <0.001, 200 nM had a decrease of 51.61% with a p-value of <0.001, 400 nM had a decrease of 43.06% with a p-value of <0.001and the 800 nM treatment decreased by 22.05% with a p-value of 0.009. After 72 hours the 100and 200 nM treatments had

decreases of 21.85% (p-value 0.009) and 21.71% (p-value 0.010) respectively when compared to the control at 72hours.

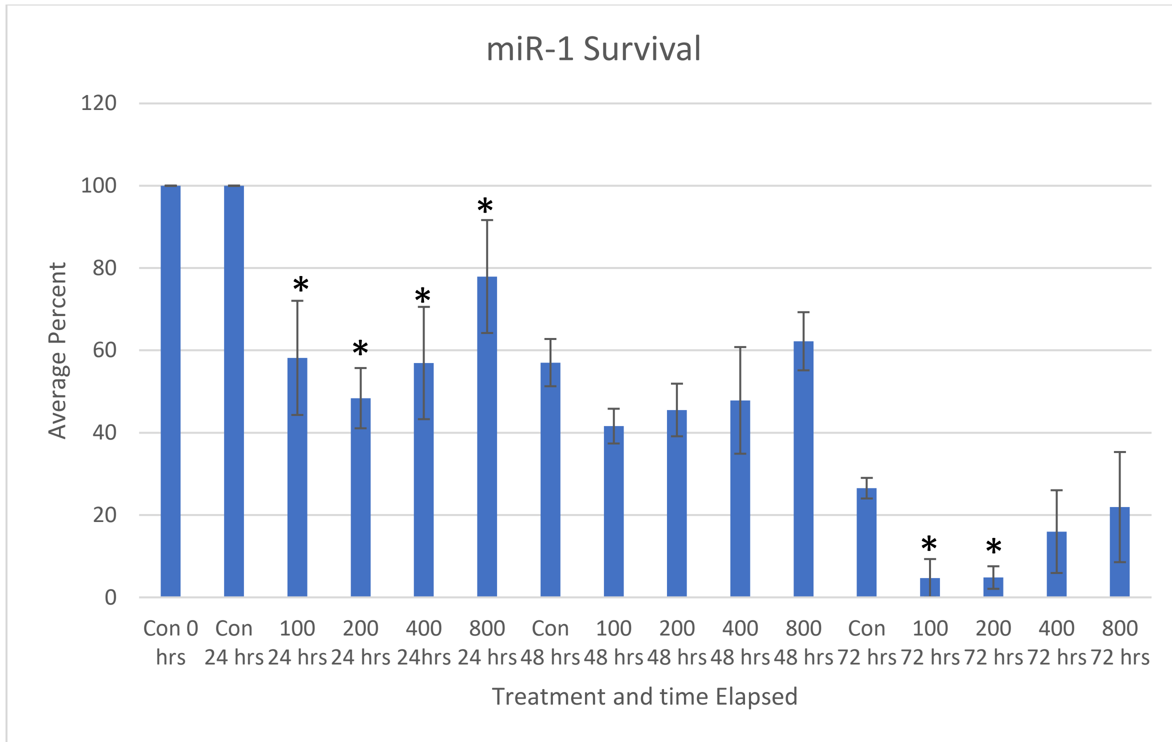

Figure 7. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-1 treatments over a 72-hour period. Significant differences (*) were determined using p-values that were less than 0.05 gathered via one-way ANOVA with the control at the same time points.

Anti-miRNA-49b effects on viability

In the 72-hour time point the 200 and 800 nM treatments showed decreases of 7.77% (p-value 0.008) and 9.42% (p-value 0.002) respectively.

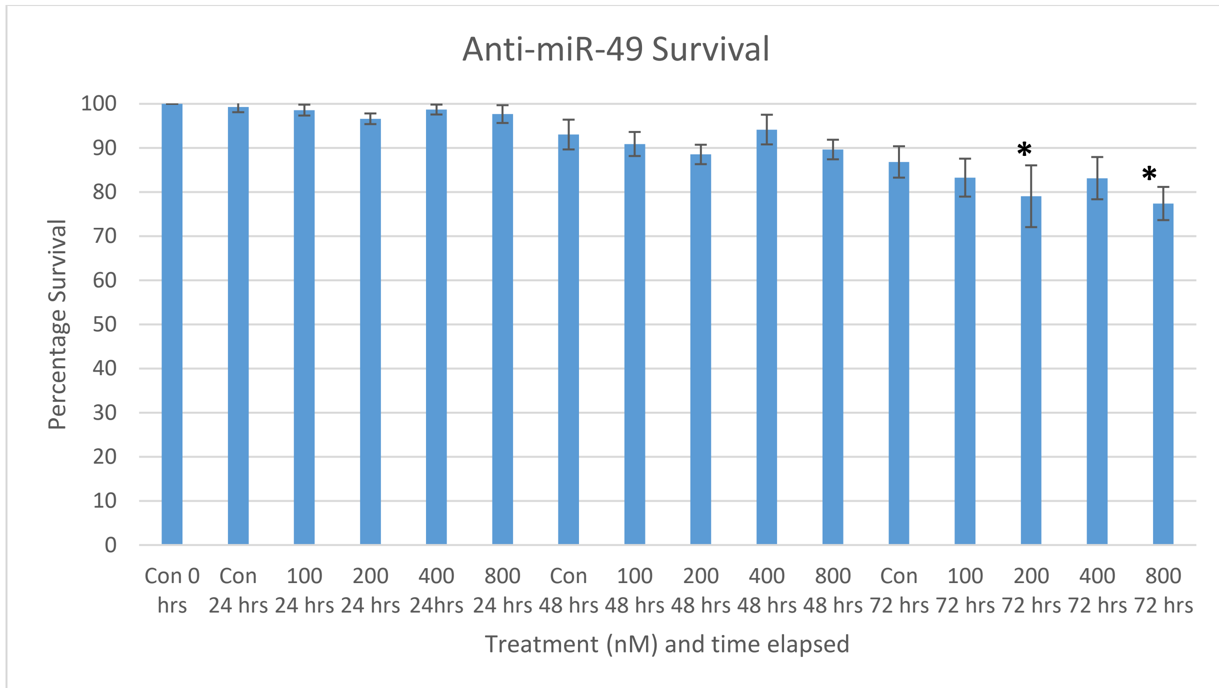

Figure 8. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-49 treatments over a 72-hour period. Significant differences (*) were determined using p-values that were less than 0.05 gathered via one-way ANOVA with the control at the same time points.

Anti-miRNA-72 effects on viability

In the 72-hour time point the the 400 and 800 nM treatments showed decreases of 12.73% (p-value 0.028) and 20.19% (p-value <0.001) respectively.

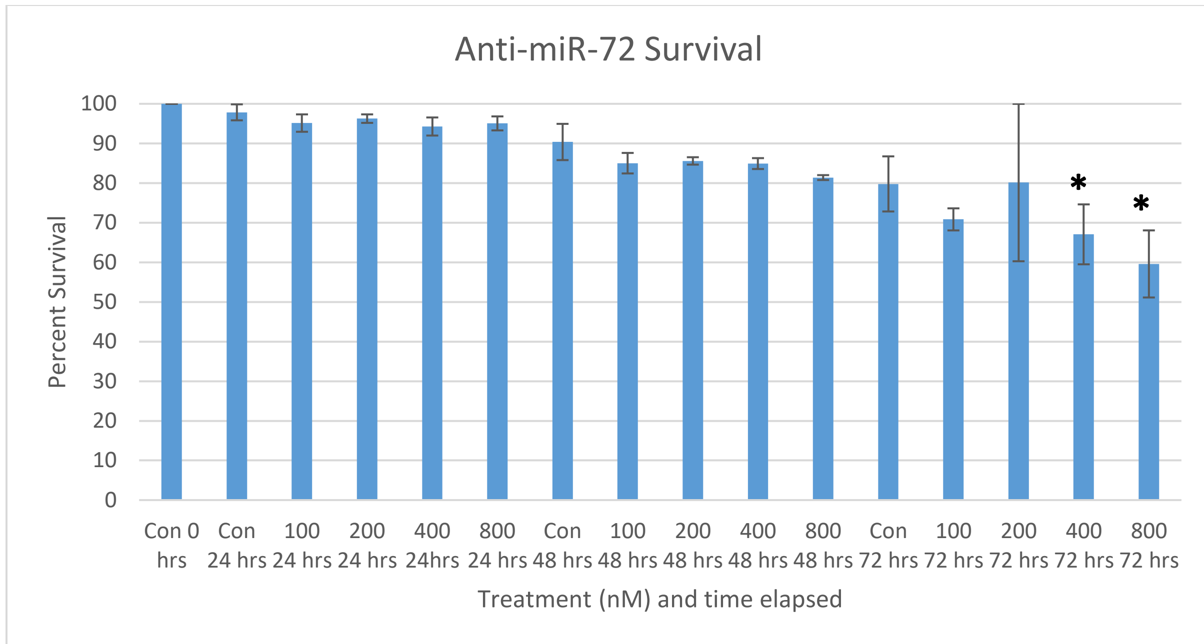

Figure 9. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-72 treatments over a 72-hour period. Significant differences (*) were found using one-way ANOVA with a p-value less than 0.05 when compared to the control at the same time point.

Anti-miRNA-92 effects on viability

In the 24 hour treatment 800nM showed a decrease of 10.37% with a p-value of 0.046. This decrease is continued to be observed at the 48 hour mark with the 800nM treatment having a decrease of 12.72% with a p-value of 0.011. At 72 hours treatments 400 and 800 nM show significant decreases of 10.92% (p-value 0.027) and 18.27% (p-value <0.001) respectively.

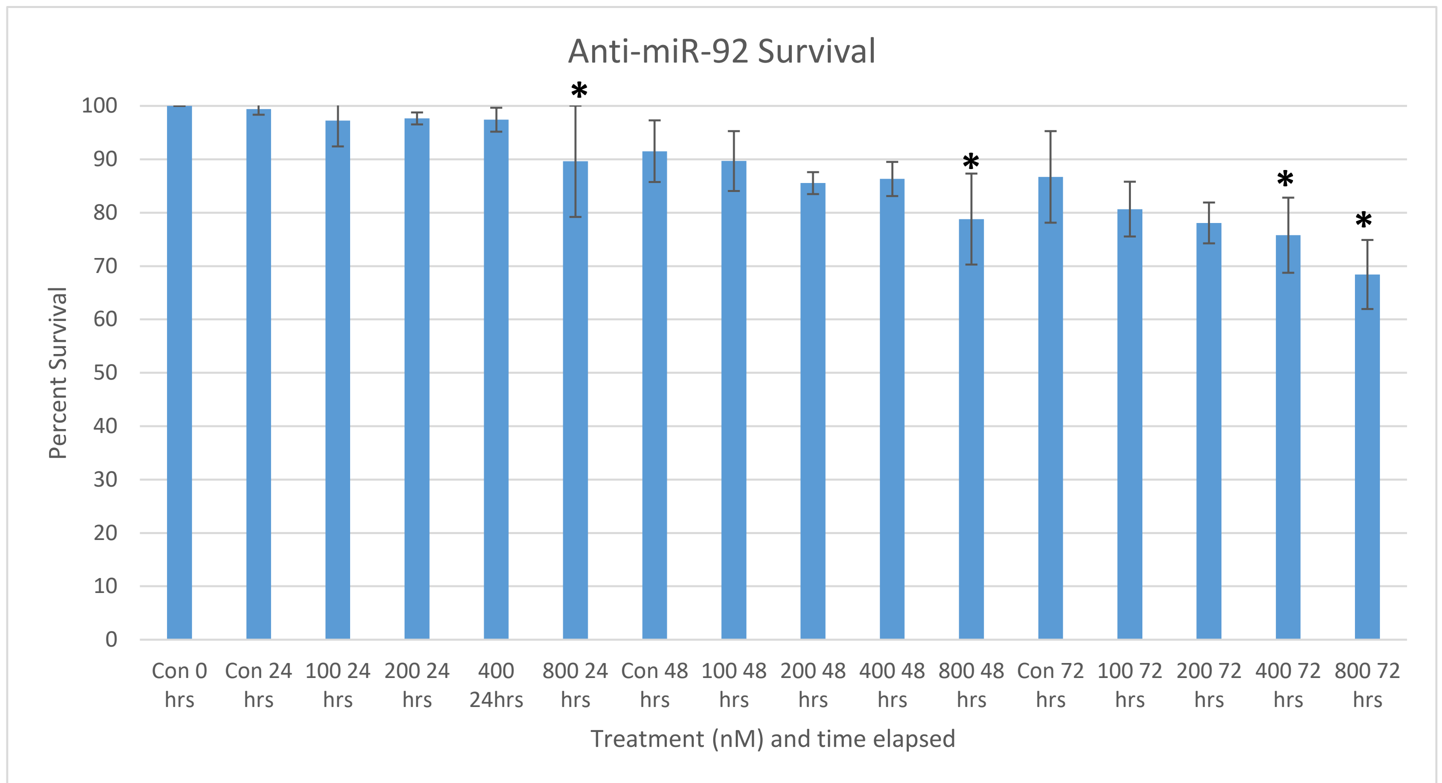

Figure 10. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-92 treatments over a 72-hour period. Significant differences (*) were determined using p-values that were less than 0.05 gathered via one-way ANOVA with the control at the same time points.

Anti-miRNA-100a effects on viability

In the 24 hour treatment the 200, 400 and 800 nM treatments showed decreases of 46.82% (p-value <0.001), 48.29% (p-value <0.001) and 29.75% (p-value 0.010) respectively. At 72 hours treatments 100 and 200 nM showed decreases of 27.13% (p-value 0.018) and 22.63% (p-value 0.045) respectively.

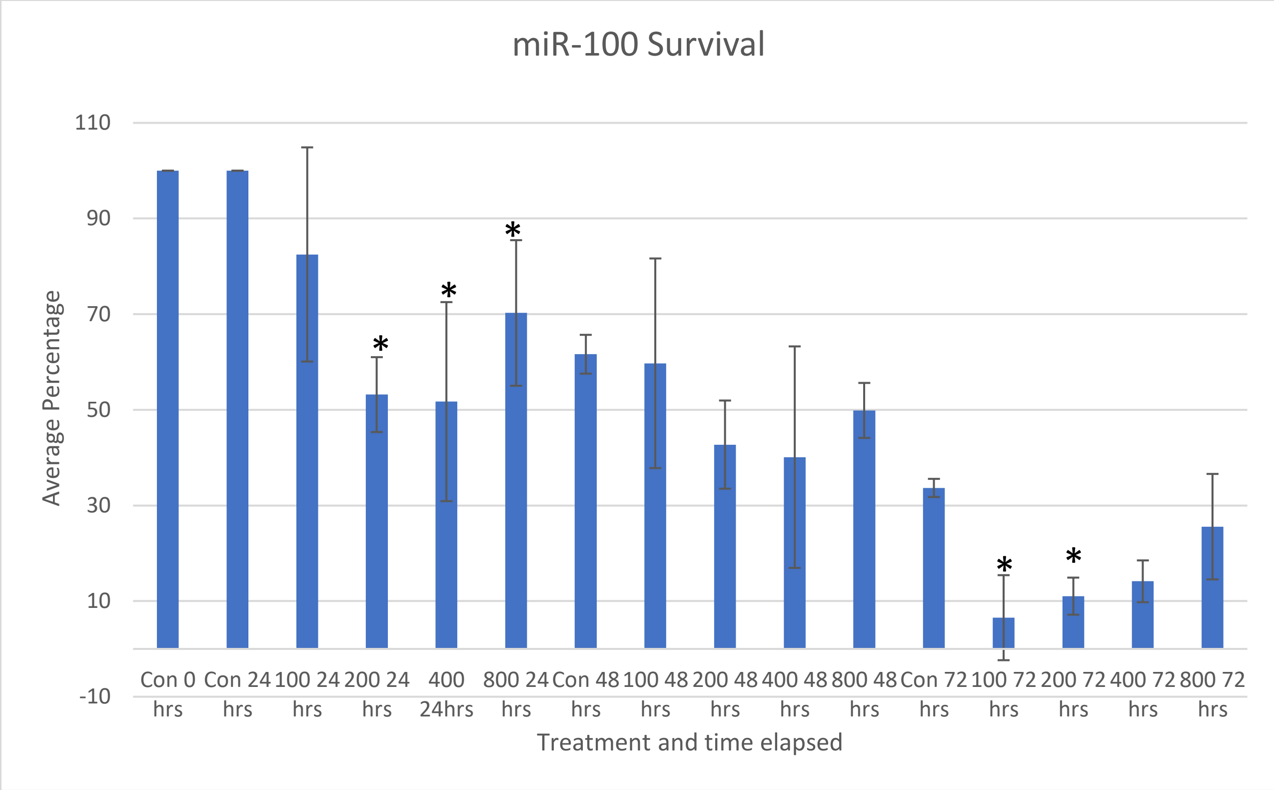

Figure 11. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-100a treatments over a 72-hour period. Significant differences (*) were found using one-way ANOVA with a p-value less than 0.05 when compared to the control at the same time point.

Anti-miRNA-228 effects on viability

At 72 hours treatments 100, 200 and 400 nM showed decreases of 14.35% (p-value 0.009), 19.31% (p-value <0.001) and 19.31% (p-value <0.001) respectively.

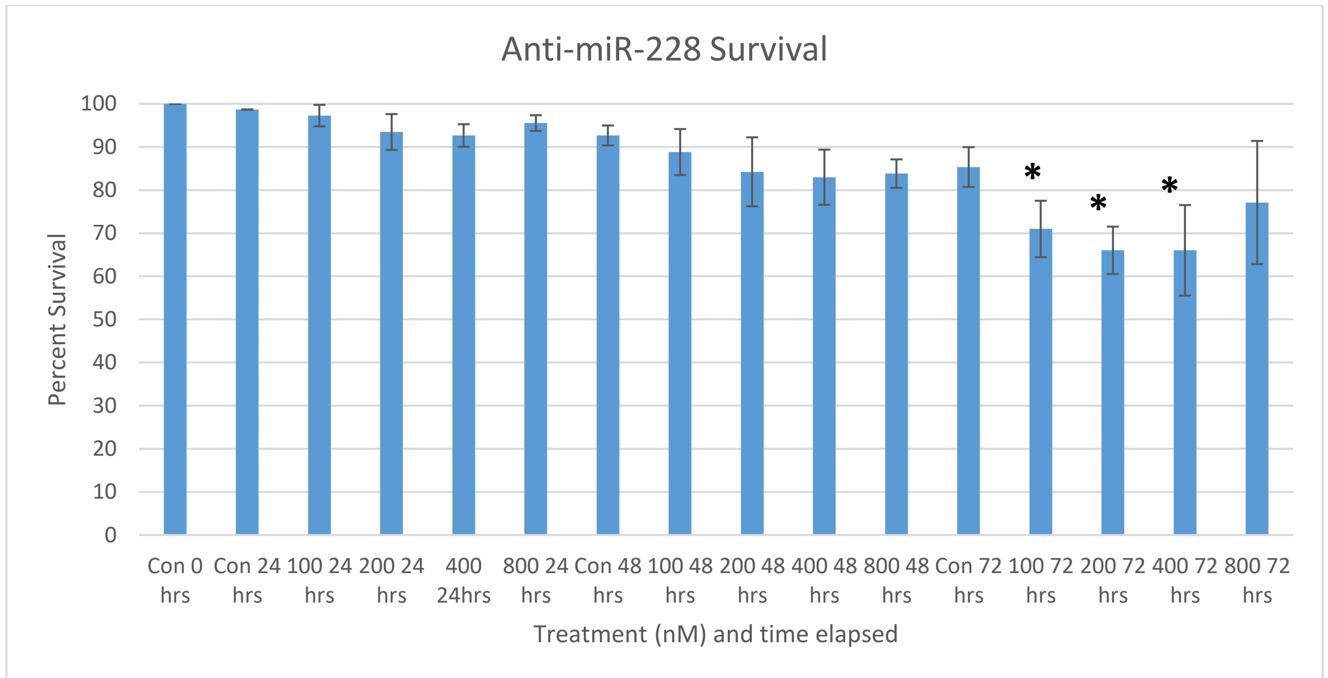

Figure 12. Percent survival/viability of *C. elegans* under different concentrations of anti-miR-228 treatments over a 72-hour period. Significant differences (*) were found using one-way ANOVA with a p-value less than 0.05 when compared to the control at the same time point.

Chapter 4: Discussion

Optimization of RNA extraction from cotton root tissue was completed before determining genes of interest. This was done so that the best commercially available kit could be used to maximize quality and quantity while minimizing possible future replication errors. Additional difficulties come from the collection of the root tissue, if the plants have not grown enough to develop enough of a root system there is not a large enough sample to collect. Alternatively, if the root system develops for too long it becomes tough to break down and extract RNA without damaging the nucleic acid.

Figures 2-5 show the CT values of the selected susceptible and resistant genes within cotton root samples collected and tested 15 and 30 days after germination, respectively. While working with these genes housekeeping genes were run alongside the selected targets, for these PTB was used. All genes that were selected show almost complete or complete expression in both time points. The housekeeping gene PTB, polypyrimidine tract-binding, are RNA binding proteins in eukaryotes which bind to select target RNAs to facilitate metabolic transport processes including mRNA polyadenylation, splicing repression in pre-mRNAs, RNA transport, mRNA stability/decay, and translational [32].

Ct values between 15 and 35 are a typical and reasonable range any Ct values above 35 are not significantly expressed. Apart from *EXPA1* and *IMK* the CT Values collected for day 15 all showing higher levels of expression. *EXPA1* and *IMK* were noted to have much lower CT values that could point to issues with the loading of the samples or other issues. All the genes showed a decrease in expression of day 30 samples. All Day 15 genes showed lower CT values as compared to Day 30, with all being normalized by the housekeeping gene PTB. The two genes that showed the most change within Day 15 to Day 30 are *ERO* and *HmP27*. *ERO* has been

studied previously being regulated by the reduction of intramolecular disulfide bonds which is a regulatory mechanism that is essential for effective oxidative protein folding. It has been proposed that regulation of *ERO1* is a safety mechanism to preserve the integrity of oxidative protein folding processes [43]. *HmP27,* a heavy metal-associated isoprenylated plant protein 27, this family of genes have been connected to plant tolerance to heavy metals, plant development, growth and stress responses. These are thought to be membrane-associated factors in the plant metal homeostasis and stress responses though many of the specific genes have yet to be characterized functionally [5].

These genes show promise as potential targets for future projects given that they have been identified as being susceptible or resistant to *M. incognita* infection in other plant species. With these homologs in cotton showing expression is a promising start to identify if these genes are susceptible or resistant to infections as well. This could be completed by using CRISPR to knock down susceptible genes that have been identified here and infecting the cotton plants with *M. incognita* and observing the galling, growth and gene expression of the plants following the infection. Ideally these genes can then be used to create transgenic cotton that will have resistance to *M. incognita* which would aid the cotton industry greatly.

MiR-1 is a highly conserved miRNA that has homologs in parasitic nematodes including *A. cantonensis*. The expression of miR-1 increases from infective larvae to young adults and is the highest expressed miRNA in the young adults of *A. cantonensis* [12]. Within the Pan lab a previous study showed that miR-1 represents 7.4% of the total miRNA's within *M. incognita* [64]. The findings indicated that exposure to anti-miR-1 (Figure 7) affects *C. elegans* viability significant decreases were seen at the 24- and 72-hour marks. At 24 hours there is a significant decrease in all the treatment samples with decreases of 41.81%, 51.61%, 43.06% and 22.05%

respectively. This may be because of the inhibitor taking effect quickly and beginning to target the regulation of neuromuscular junction function as it has been observed to play a role in this through both the pre- and post-synaptic properties [56]. This decrease continues and is significant again at the 72-hour mark, specifically within treatments 100 and 200 nM with decreases of 21.85% and 21.71%, this is a further 19.96% and 29.90% decrease from the observed decreases at 24 hours. This may be due to the further impact that the inhibitor is playing within the regulation of the muscular function though it may also be due to the nematodes not having access to food which would cause them to die. It was also found that anti-miR-1treatment (Figure 6) resulted in down regulation within *C. elegans* following exposure with a decrease in expression of 64.1%.

Within *C. elegans* miR-49b is seen to help with locomotion as it is linked to a GABA receptor that only requires all three subunits of miR-49 to be present [4]. miR-49 is also found within *Ascaris suum* which is a parasitic nematode found within the small intestine of animals and adapts to its lifestyle by possessing a large reproductive capacity with a female laying as many as 25,000,000 eggs. Within *Ascaris sum* it is not fully known the function of miR-49 though it is expressed within embryogenesis leading to the believe that it could be playing a role in the fertility of the nematodes [59]. Within the study of anti-miR-49b (Figure 8) has a significant decrease in viability in the 72-hour treatments of 200 and 800 nM with decreases of 7.77% and 9.42% this may be due to the inhibitor as the same samples at the 24- and 48-hour point follow a similar trend of decreasing though not significantly. It may be that this inhibitor takes a full 72 hours to become fully integrated or to have full effect in the system of the nematode. It is also noted that the gene expression shows a >99.9% downregulation (Figure 6) of the gene associated with anti-miR-49b, with this level of inhibition within *C. elegans* it is

likely that this gene is a good candidate for a target gene for preventing *M. incognita* infections in plants.

Li, Z et al. found that miR-72 shows more than 1000 reads in both female and male *A. cantonensis* [40]. It was also found that out of 88 of the *C. elegans* miRNAs that Lim et al. studied miR-72 was one of the six that did not have a homolog of at least 75% of the sequence identity linked to a *C. briggsae* ortholog [35]. At 72-hours anti-miR-72 (Figure 9) shows a significant decrease in treatments 400 and 800 nM with decreases of 32.93% and 40.40% respectively. These decreases follow a trend that begins to develop after the first 24 hours but is not found to be significant until the 72-hour time point. Within *C. elegans* miR-72 is known to play a role in the regulation of oocyte development but has been observed to not be essential for embryonic development [45]. Given that this is likely not impacting the further development of nematodes being counted in this study it is likely that this decrease is due to the lack of food. Gene expression results show that anti-miR-72 was downregulated 99.8% (Figure 6) which would result in significant knockdown effects within *Meloidogyne incognita* which is what we anticipate will be the response due to the similarities between *C. elegans* and *M. incognita*.

The miR-92 is found within a family of miRNA clusters that are composed of three related, highly conserved, polycistronic miRNA genes that encode for a total of 15 miRNAs. This miR has homologs that have been identified in both *D. melanogaster* and *C. elegans,* with the homolog in *C. elegans* being miR-235which plays a role in regulating development, stress response and cellular inactivity and a study that removed miR-235 within *C. elegans* that showed increased levels of adult lethality with an increase of 80% adult lethality [42]. The miRNA family has been shown to regulate heart development and cell death [15]. miR-92 is also found within the infectious nematode *Brugia malayi* [49]. Inhibition of anti-miR-92 on survival

(Figure 10) showed significant decreases across the full 72 hours the study was conducted. In the first 24-hours treatment 800nM decreased by 10.82% and this continued at 48 hours with a decrease of 12.72%, this is a further decrease of 1.89% from the 24-hour decrease. This trend continues to the 72-hour time point with significant decreases observed at both 400 and 800 nM treatments with decreases of 10.92% and 18.27% respectively. The decrease in the 800 nM treatment at 72-hours is a further 5.55% decrease from the previous point. Based off of this I believe that the inhibition of anti-miR-92 was possibly increasing the cellular inactivity which may result in nematodes not getting the necessary metabolites. There was significant downregulation noted in the gene expression with 98.8% down regulation (Figure 6) being noted within the gene expression results. We anticipate that similar results should be found within the gene expression of *M. incognita* and will ideally result in lessened infection rates in plant tissues.

Within *M. incognita* the most abundantly expressed miRNA is miR-100a, this represents 37% of the total sequence reads [64]. miR-100a has been identified within *Haemonchus contortus* which is a parasitic nematode found within livestock and recently within *Trichuris muris* which is a parasitic nematode that infects mice [23]. Within *C. elegans* miR-100 is represented by the miR-51 family, which is essential for development with complete deletion resulting in embryonic lethality and has also been observed to be involved in cell signaling [20]. Inhibition of anti-miR-100a (Figure 11) showed significant decreases in viability at 24- and 72-hours. At 24-hour treatments 200, 400 and 800 nM decreased by 46.82%, 48.29% and 29.75% respectively. The decreases in treatments, 100 and 200 nM continues to be observed at 72-hours with decreases of 27.13% and 22.63% respectively. These decreases are a further 9.62% and 24.19% respectively. The decrease observed may be due to the impact on cell signaling that could be occurring through the inhibition of anti-miR-100a. It was also found that anti-miR-100a

resulted in down regulation within *C. elegans* following exposure when fold change was calculated (Figure 6) with >99.9% downregulation being noted from previous Pan lab data. Given this preliminary data within *C. elegans* and the knowledge of abundant expression within *M. incognita* anti-miR-100 is a great candidate for use in CRISPR within cotton tissue to create a transgenic resistant plant.

In Marks et al. [42] study it was suggested that miR-228 might have an inhibitory effect on larval development that can be observed *in vitro* based on their experiment with *Haemonchus contortus*. Epistasis studies conducted on *C. elegans daf-2* mutants showed that miR-228 and miR-235 synergize with the FOXO transcription factor *DAF*-16 within the insulin signaling pathway suggesting that they might suppress the metabolic and transcription factor activity that is required for development [42]. Inhibition of anti-miR-228 (Figure 12) showed a significant decrease at the 72 hour time point in treatments 100, 200 and 400 nM with decreases of 14.35%, 19.31% and 19.31% respectively. This decrease may be related to the suppression of metabolic and transcription activity that this inhibitor is known to play a role in. There is an observable trend in the decrease of these treatments starting at 48 hours, though it is not a significant trend it may be that the impacts on the metabolic and transcription activity may take a full 72 hours to build up in the nematode. Gene expression (Figure 6) showed that the inhibition of this gene was roughly >99.9% downregulated, this is a promising result within our preliminary research as we anticipate similar responses within the *Meloidogyne incognita*.

Chapter 5: Conclusion

Inhibition of anti-miR-1, anti-miR-49, anti-miR-72, anti-miR-92, anti-miR-100a and anti-miR-228 were observed to have an impact on the viability of *C. elegans*. The most promising of these being anti-miR-49, anti-miR-72, and anti-miR-92 as they showed the most consistent downward trend without major impacts being observed on the control samples which increases the chances that these decreases could be from the inhibitor's effects and not due to lack of food in the experimental set up. These inhibitors also showed a decrease of >99.9%, >99.9% and 98.8% when gene expression was run on nematodes that had been exposed to the inhibitor to determine inhibition levels within *C. elegans.* Following the optimization of RNA extraction and the identification of possible *M. incognita* susceptible and resistant cotton homologs there is a good base for building on this knowledge. Knowing that these genes are present in the cotton genome we can use CRISPR Cas or other biotechnological processes to determine if the susceptibility or resistance observed in other plant species carries to the cotton genome. Once this information is learned and understood it can be used again with CRISPR Cas or other gene-editing technologies to generate transgenic plants that carry some form of resistance to *M. incognita* infections.

References

1. Abad, P., Gouzy, J., Aury, JM. *et al.* Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat Biotechnol* **26**, 909–915 (2008). https://doi.org/10.1038/nbt.1482
2. Aktar, M. W., Sengupta, D. & Chowdhury, A. Impact of pesticides use in agriculture: their benefits and hazards. *Interdiscip Toxicol* **2**, 1–12 (2009).
3. Basso, M. F. *et al.* Overexpression of the GmEXPA1 gene reduces plant susceptibility to Meloidogyne incognita. *Plant Cell Rep* **42**, 137–152 (2023).
4. Bamber, Bruce A.; Beg, Asim A.; Twyman, Roy E.; and Jorgensen, Erik M. The *Caenorhabditis elegans unc-49* Locus Encodes Multiple Subunits of a Heteromultimeric GABA Receptor. The Journal of Neuroscience 19(13):p 5348-5359, Jul 1 1999. DOI: 10.1523/JNEUROSCI.19-13-05348.1999
5. Barr, Z. K., Werner, T., & Tilsner, J. (2023). Heavy Metal-Associated Isoprenylated Plant Proteins (HIPPs) at Plasmodesmata: Exploring the Link between Localization and Function. *Plants*, *12*(16), 3015. https://doi.org/10.3390/plants12163015
6. Binhui Guo, Yi Dai, Lin Chen, Zhenzhi Pan, Li Song, Genome-wide analysis of the soybean root transcriptome reveals the impact of nitrate on alternative splicing, *G3 Genes|Genomes|Genetics*, Volume 11, Issue 7, July 2021, jkab162, https://doi.org/10.1093/g3journal/jkab162
7. Branch, W. D., Brenneman, T. B. & Noe, J. P. Evidence for a Second RKN Resistance Gene in Peanut. *Peanut Science* **43**, 49–51 (2016).

8. Butler, K. J., Chen, S., Smith, J. M., Wang, X. & Bent, A. F. Soybean Resistance Locus Rhg1 Confers Resistance to Multiple Cyst Nematodes in Diverse Plant Species. *Phytopathology®* **109**, 2107–2115 (2019).
9. Caillaud, M.C. et al. MAP65–3 Microtubule-associated protein is essential for nematode-induced giant cell ontogenesis in Arabidopsis. Plant Cell 20,423–437 (2008)
10. Castagnone-Sereno, P. Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. *Heredity* **96**, 282–289 (2006). https://doi.org/10.1038/sj.hdy.6800794
11. Castagnone-Sereno, P., Bongiovanni, M., Palloix, A. & Dalmasso, A. Selection forMeloidogyne incognita virulence against resistance genes from tomato and pepper and specificity of the virulence/resistance determinants. *Eur J Plant Pathol* **102**, 585–590 (1996).
12. Chang, S.-H., Tang, P., Lai, C.-H., Kuo, M.-L. & Wang, L.-C. Identification and characterisation of microRNAs in young adults of *Angiostrongylus cantonensis* via a deep-sequencing approach. *Mem. Inst. Oswaldo Cruz* **108**, 699–706 (2013).
13. Chinnapandi, B. *et al.* Tomato SlWRKY3 acts as a positive regulator for resistance against the root-knot nematode Meloidogyne javanica by activating lipids and hormone-mediated defense-signaling pathways. *Plant Signaling and Behavior* **14**, (2019).
14. Claverie M, Dirlewanger E, Bosselut N, Van Ghelder C, Voisin R, Kleinhentz M, Lafargue B, Abad P, Rosso MN, Chalhoub B, Esmenjaud D. The Ma gene for complete-spectrum resistance to Meloidogyne species in Prunus is a TNL with a huge repeated C-terminal post-LRR region. Plant Physiol. 2011 Jun;156(2):779-92. doi: 10.1104/pp.111.176230. Epub 2011 Apr 11. PMID: 21482634; PMCID: PMC3177275.

15. Concepcion, Carla P.; Bonetti, Ciro; Ventura, Andrea. The MicroRNA-17-92 Family of MicroRNA Clusters in Development and Disease. The Cancer Journal 18(3):p 262-267, May/June 2012. | DOI: 10.1097/PPO.0b013e318258b60a
16. Da Silva M.B., Davis R.F., Kumar P., Nichols R.L., Chee P.W. Resistance Quantitative Trait Loci *qMi-C11* and *qMi-C14* in Cotton Have Different Effects on the Development of *Meloidogyne incognita*, the Southern Root-Knot Nematode. Plant Dis. 2019 May;103(5):853-858. doi: 10.1094/PDIS-06-18-1050-RE. Epub 2019 Mar 12. PMID: 30864940.
17. Dutta, T. K., Ray, S. & Phani, V. The status of the CRISPR/Cas9 research in plant–nematode interactions. *Planta* **258**, 103 (2023).
18. Dutta, T. K. *et al.* Functional analysis of a susceptibility gene (HIPP27) in the Arabidopsis thaliana-Meloidogyne incognita pathosystem by using a genome editing strategy. *BMC Plant Biol* **23**, 390 (2023).
19. El-Sappah, A. H. *et al.* Tomato Natural Resistance Genes in Controlling the Root-Knot Nematode. *Genes (Basel)* **10**, 925 (2019).
20. Emilio M. Santillan *et al.,* An ancient and essential miRNA family controls cellular interaction pathways in *C. elegans.Sci Adv.* **11**, eadz1934 (2025). DOI: 10.1126/sciadv.adz1934
21. Feinbaum, R. & Ambros, V. The timing of lin-4 RNA accumulation controls the tinming of postembryonic developmental events in Caenorhabditis elegans. *Developmental Biology* 87–95 (1999) doi:dbio.1999.9272.
22. Finkers-Tomczak A, Bakker E, de Boer J, van der Vossen E, Achenbach U, Golas T, Suryaningrat S, Smant G, Bakker J, Goverse A. Comparative sequence analysis of the

potato cyst nematode resistance locus H1 reveals a major lack of co-linearity between three haplotypes in potato (Solanum tuberosum ssp.). Theor Appl Genet. 2011 Feb;122(3):595-608. doi: 10.1007/s00122-010-1472-9. Epub 2010 Nov 4. PMID: 21049265; PMCID: PMC3026667.

23. Gu, H. Y. *et al.* Conservation of a microrna cluster in parasitic nematodes and profiling of mirnas in excretory-secretory products and microvesicles of Haemonchus contortus. (2017).
24. Hsieh Y-W, Chang C, Chuang C-F (2012) The MicroRNA *mir-71* Inhibits Calcium Signaling by Targeting the TIR-1/Sarm1 Adaptor Protein to Control Stochastic L/R Neuronal Asymmetry in *C. elegans*. PLoS Genet 8(8): e1002864. https://doi.org/10.1371/journal.pgen.1002864
25. Huang, Q. *et al.* CRISPR/Cas9-mediated mutagenesis of the susceptibility gene OsHPP04 in rice confers enhanced resistance to rice root-knot nematode. *Frontiers in Plant Science* **14**, (2023).
26. Hussey, R. S. & Boerma, H. R. A Greenhouse Screening Procedure for Root-Knot Nematode Resistance in Soybeans1. *Crop Science* **21**, cropsci1981.0011183X002100050041x (1981).
27. Italy, U. S. Mission. How climate change affects the food crisis. *U.S. Embassy & Consulates in Italy* https://it.usembassy.gov/how-climate-change-affects-the-food-crisis/
28. Janssen R, Bakker J, Gommers FJ. Mendelian proof for a gene-for-gene relationship between virulence of Globodera rostochiensis and the H1 resistance gene in Solanum tuberosum ssp. andigena CPC 1673. Rev Nematol. 1991;14:213–219.

29. Jaouannet, Maëlle; Magliano, Marc; Arguel, Marie-Jeanne; Gourgues, Mathieu; Evangelisti, Edouard; Abad, Pierre; Rosso, Marie-Noëlle. The Root-Knot Nematode Calreticulin Mi-CRT Is a Key Effector in Plant Defense Suppression. Molecular plant-microbe interactions: MPMI. 26. (2012). 10.1094/MPMI-05-12-0130-R.

30. Jin, J., Hewezi, T. and Baum, T.J. 'The arabidopsis BHLH25 and BHLH27 transcription factors contribute to susceptibility to the cyst nematode *heterodera schachtii*', *The Plant Journal*, 65(2), pp. 319–328. (2011) doi:10.1111/j.1365-313x.2010.04424.x.

31. Kumar P, Khanal S, Da Silva M, Singh R, Davis RF, et al. Transcriptome analysis of a nematode resistant and susceptible upland cotton line at two critical stages of *Meloidogyne incognita* infection and development. PLOS ONE 14(9): e0221328(2019) . https://doi.org/10.1371/journal.pone.0221328

32. Kondhare, K.R., Kumar, A., Hannapel, D.J. *et al.* Conservation of polypyrimidine tract binding proteins and their putative target RNAs in several storage root crops. *BMC Genomics* **19**, 124 (2018). https://doi.org/10.1186/s12864-018-4502-7

33. Lahlali, R., Banu, G., Mokrini, F., Ibrahim, D. S. S. & Belabess, Z. Chapter 22 - Nematode problems in cotton and their sustainable management. in *Nematode Diseases of Crops and their Sustainable Management* (eds. Rahman Khan, M. & Quintanilla, M.) 531–564 (Academic Press, 2023). doi:10.1016/B978-0-323-91226-6.00004-3.

34. Li, B. *et al.*'Genome-wide characterization and expression analysis of soybean TGA transcription factors identified a novel TGA gene involved in drought and salt tolerance', *Frontiers in Plant Science*, 10. (2019) doi:10.3389/fpls.2019.00549.

35. Lim, L. P. *et al.* The microRNAs of Caenorhabditis elegans. *Genes Dev.* **17**, 991–1008 (2003).

36. Lin, B. *et al.* 'A novel nematode effector suppresses plant immunity by activating host reactive oxygen species-scavenging system', *New Phytologist*, 209(3), pp. 1159–1173. (2015) doi:10.1111/nph.13701.

37. Li-Qing Chen et al., Sucrose Efflux Mediated by SWEET Proteins as a Key Step for Phloem Transport.Science335,207-211(2012).DOI:10.1126/science.1213351

38. Liu, Shiming et al. "A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens." *Nature* vol. 492,7428 (2012): 256-60. doi:10.1038/nature11651

39. Li, X. *et al.* Transcriptomic and Histological Analysis of the Response of Susceptible and Resistant Cucumber to Meloidogyne incognita Infection Revealing Complex Resistance via Multiple Signaling Pathways. *Front. Plant Sci.* **12**, (2021).

40. Li, Z. *et al.* MicroRNA expression profile in the third- and fourth-stage larvae of Angiostrongylus cantonensis. *Parasitol Res* **113**, 1883–1896 (2014).

41. Mandaliya VB, Bhatt P, Thaker VS. High quality RNA extraction protocol for polyphenolics-rich Cotton tissue. Anal Biochem. 2024 Nov;694:115604. doi: 10.1016/j.ab.2024.115604. Epub 2024 Jul 8. PMID: 38986795.

42. Marks, N. D. *et al.* Profiling microRNAs through development of the parasitic nematode Haemonchus identifies nematode-specific miRNAs that suppress larval development. *Sci Rep* **9**, 17594 (2019).

43. Matsusaki M, Okuda A, Matsuo K, Gekko K, Masuda T, Naruo Y, Hirose A, Kono K, Tsuchi Y, Urade R. Regulation of plant ER oxidoreductin 1 (ERO1) activity for efficient oxidative protein folding. J Biol Chem;294(49):18820-18835. (2019) doi:

10.1074/jbc.RA119.010917. Epub 2019 Nov 4. PMID: 31685660; PMCID: PMC6901294.

44. Mejias, J. *et al.* The root-knot nematode effector MiEFF18 interacts with the plant core spliceosomal protein SmD1 required for giant cell formation. *New Phytologist* **229**, 3408–3423 (2021).

45. Minogue, Amanda L et al. "Functional genomic analysis identifies miRNA repertoire regulating C. elegans oocyte development." *Nature communications* vol. 9,1 5318. 14 Dec. 2018, doi:10.1038/s41467-018-07791-w

46. Mitchum, M. G. *et al.* Nematode effector proteins: an emerging paradigm of parasitism. *New Phytologist* **199**, 879–894 (2013).

47. Passianotto, A. L. de L. *et al.* Genome-wide association study for resistance to the southern root-knot nematode (Meloidogyne incognita) in soybean. *Mol Breeding* **37**, 148 (2017).

48. Petrillo, M. D., Matthews, W. C. & Roberts, P. A. Dynamics of Meloidogyne incognita Virulence to Resistance Genes Rk and Rk2 in Cowpea. *J Nematol* **38**, 90–96 (2006).

49. Poole, Catherine B.; Davis, Paul J.; Jin, Jingmin; and McReynolds, Larry A. Cloning and bioinformatic identification of small RNAs in the filarial nematode, *Brugia malayi*. Molecular and Biochemical Parasitology 169(2):p 87-94, Oct 16 2009. DOI: https://doi.org/10.1016/j.molbiopara.2009.10.004

50. Radakovic ZS, et al. *Arabidopsis* HIPP27 is a host susceptibility gene for the beet cyst nematode *Heterodera schachtii*. Mol Plant Pathol.;19:1917–28. (2018)

51. Rice, S., L., Leadbeater, B.S.C., Stone, A.R., Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes. I. Potatoes with resistance gene

H1 derived from *Solanum tuberosum spp. andigena, Physiological Plant Pathology* 27(2), 219-234 (1985). https://doi.org/10.1016/0048-4059(85)90069-4.

52. Robertson, B. *et al.* Specificity and functionality of microrna inhibitors. *Silence* (2010). Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC2864222/.
53. Saucet, S. B., Van Ghelder, C., Abad, P., Duval, H. & Esmenjaud, D. Resistance to root-knot nematodes Meloidogyne spp. in woody plants. *New Phytologist* **211**, 41–56 (2016).
54. Shen, X., Van Becelaere, G., Kumar, P. *et al.* QTL mapping for resistance to root-knot nematodes in the M-120 RNR Upland cotton line (*Gossypium hirsutum* L.) of the Auburn 623 RNR source. *Theor Appl Genet* **113**, 1539–1549 (2006). https://doi.org/10.1007/s00122-006-0401-4
55. Shukla, N. *et al.* Transcriptome analysis of root-knot nematode (Meloidogyne incognita)-infected tomato (Solanum lycopersicum) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. *Molecular Plant Pathology* **19**, 615–633 (2018).
56. Simon, David J et al. "The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions." *Cell* vol. 133,5: 903-15. (2008) doi:10.1016/j.cell.2008.04.035
57. Thakur, S., Rana, A., Sharma, A. *et al.* Plant Nematode Interaction and Omics: A Focus On *Meloidogyne incognita*. *Journal of Crop Health* **76**, 1281–1291 (2024). https://doi.org/10.1007/s10343-024-01025-4
58. Wang, J., Mei, J. & Ren, G. Plant micrornas: Biogenesis, homeostasis, and degradation. *Frontiers* (2025). Available at: https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.00360/full.

59. Wang, J. *et al.* Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental profiles. *Genome Res* **21**, 1462–1477 (2011).

60. Warmerdam, S. *et al.* Genome-wide association mapping of the architecture of susceptibility to the root-knot nematode Meloidogyne incognita in Arabidopsis thaliana. *New Phytologist* **218**, 724–737 (2018).

61. Xie, Fuliang et al. "miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs." *Plant molecular biology*, 31 Jan. 2012, doi:10.1007/s11103-012-9885-2

62. Yaghoobi, J & Yates, J & Williamson, Valerie. Fine mapping of the nematode resistance gene Mi-3 in Solanum peruvianum and construction of a S. lycopersicum DNA contig spanning the locus. Molecular genetics and genomics : MGG. 274. 60-9. (2005) 10.1007/s00438-005-1149-2.

63. Yuan, CP., Li, YH., Liu, ZX. *et al.* DNA sequence polymorphism of the *Rhg4* candidate gene conferring resistance to soybean cyst nematode in Chinese domesticated and wild soybeans. *Mol Breeding* **30**, 1155–1162 (2012). https://doi.org/10.1007/s11032-012-9703-1

64. Zhang, Y. *et al.* Identification and characterization of microRNAs in the plant parasitic root-knot nematode Meloidogyne incognita using deep sequencing. *Funct Integr Genomics* **16**, 127–142 (2016).

65. Zhou, J. *et al.* Heat Shock Factor HsfA1a Is Essential for R Gene-Mediated Nematode Resistance and Triggers H2O2 Production. *Plant Physiology* **176**, 2456–2471 (2018).

66. Zhou Y, Zhao D, Duan Y, Chen L, Fan H, Wang Y, Liu X, Chen LQ, Xuan Y, Zhu X. AtSWEET1 negatively regulates plant susceptibility to root-knot nematode disease. Front

Plant Sci.;14:1010348. (2023) doi: 10.3389/fpls.2023.1010348. PMID: 36824200; PMCID: PMC9941640.