FUNCTIONAL CHARACTERIZATION OF GENES INVOLVED IN SEX DETERMINATION OF *Meloidogyne incognita*

By

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To my dad mom family and friend, without whom I would have no one to complain to
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I would like to thank everyone that I’ve met along on this wonderful journey for helping me become the person that I am now. Also a special thank you to my graduate committee members: Dr. Tesfamariam Mekete Mengistu, Dr. Larry Duncan and Dr. William Crow for providing me the opportunity to conduct this research.
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Root-knot nematodes (*Meloidogyne* spp.) are among the most destructive and widespread plant-parasitic nematodes in the world, with extremely wide host and geographical ranges. Effective control measures like soil fumigation with chemicals such as methyl bromide are proven to be detrimental to the environment and human. So there is necessity of developing effective control strategies. Proper understanding of sex determination pathways could be an effective tool for developing sustainable management strategies against nematodes. In the bacterial feeding nematode *Caenorhabditis elegans*, the *sdc-1* gene has been shown to be the upstream regulator of the female sex determination cascade where as *tra-1* is the downstream regulator of the pathway. The orthologs of these genes have been identified in *M. incognita*. The aim of this study was to determine the possible function of the *Mi-tra-1* and *Mi-sdc-1* in sex determination of *M. incognita*. RNAi was performed by soaking secondary stage juveniles of *M. incognita* in a solution containing dsRNA of either *Mi-tra-1* or *Mi-sdc-1* for 24h. The levels of both *Mi-tra-1* and *Mi-sdc-1* mRNAs were significantly reduced in a
sequence-specific manner in nematodes soaked for 24h. The downregulation of both genes was observed 4 days after recovering the juveniles from the dsRNA treatment. Neither the treatment with dsRNA of Mi-tra-1 nor with the dsRNA of Mi-sdc-1 resulted in sex reversal to male nematodes. However, RNAi of Mi-sdc-1 significantly delayed the maturity of females, whereas the downregulation of Mi-tra-1 showed significant impact on female fecundity as compared to the treatment with dsRNA of GFP and untreated control.
CHAPTER 1
GENERAL INTRODUCTION

Nematodes

Nematodes are the most diverse and numerous metazoans in the soil and aquatic sediments. It is estimated that there are more than ten million species of nematodes (Abad et al., 2008). Nematodes are ubiquitous and can be found in every part of the ecosystem. Nematodes perform various roles as bacterivores, herbivores, parasites of animals and plants as well as consumers of dissolved and particulate organic matter. Nematodes perform a very important role in the flow of energy and cycling of nutrients in the ecosystem. Despite their huge economic and ecological impacts, nematodes are among least studied organisms. Among millions of estimated species, approximately 26,000 species have been described to date (Abebe et al., 2011). Nematodes have a simple body plan which is usually elongated, cylindrical and tapered on both ends (Decraemer and Hunt, 2006). The digestive system of nematodes is tube like structure within another tube (pseudocoelom). The pseudocoelom is filled with fluid, the hymolymph, and houses the reproductive system and other organs (Decraemer and Hunt, 2006).

Plant-Parasitic Nematodes

Plant-parasitic nematodes (PPN) causes huge damage in agriculture. PPN can alone or in combination with other pathogens like viruses, bacteria, fungi etc., can damage every part of plant like root, stem, leaves, fruits and seeds by direct feeding or by causing wounds (Agrios, 2005). The damage caused by PPN has been estimated at $80 billion per year (Nicol et al., 2011). This amount is probably underestimated as most growers, especially in developing countries, have a limited understanding about
nematodes. (Hooks et al., 2010, Anwar and McKenry, 2012). Yield losses due to PPN are higher in tropics as compared to temperate regions. In tropical climate nematodes have increased number of generation per year which has exacerbated the situation (Luc et al., 2005).

Most of the studies on plant-parasitic nematodes are focused on sedentary endoparasitic nematodes like *Meloidogyne* spp., *Heterodera* spp., and *Globodera* spp. as they are economically important PPN causing severe damages and losses in a wide variety of crops (Wyss, 1997; Rosso et al., 2005; Lilley et al., 2012).

Root-knot nematodes (RKN) are obligate plant parasites that are distributed worldwide. The genus consists of more than 100 species that parasitize different plant species (Holterman et al., 2009). The four most important species are the tropical species *M. incognita*, *M. javanica*, and *M. arenaria*, and the temperate species, *M. hapla*. Root-knot nematodes have a wide host range of about 250,000 flowering plants (Trudgill et al., 2001). Very few other nematodes like *Rotylechulus reniformis* and some mychorrhizal fungi have such a wide host range as that of *Meloidogyne* spp. Due to the wide host range, and sometimes association with other pathogens, these nematodes can cause huge loss in many plant species. RKN are very difficult to manage in the field because of wide host range, increased number of generation and high reproduction rate. Therefore, alternative strategies for the management of this pest are essential (Trudgill and Blok, 2001).

**Nematode Under Study: Meloidogyne incognita**

**Introduction**

*Meloidogyne* spp., also known as root-knot nematodes (RKN) are important PPN that causes huge economic damage globally in agricultural commodities (Bird and
Kaloshian, 2003). RKN are sedentary endo-parasites in which only the mobile second-stage (J2) juveniles have the ability to enter the host plants by invading the root elongation zone. The J2 first migrate to the root apex and later to the vascular cylinder and establish permanent feeding sites. After the establishment of a permanent feeding site, the J2 starts to uptake nutrients. Once the J2 starts feeding they become sedentary and undergo three molts to develop into third and fourth stage-juveniles (J3 and J4) and finally into adults (Atkinson et al., 1996). Meloidogyne incognita is the most widespread RKN species (Chan et al., 2010). Males of *M. incognita* have a vermiform shape and migrate out from the root and do not feed on plant roots whereas females remain immobile, have a saccate shape and produce egg masses (Williamson and Gleason, 2003).

**Taxonomical Position**

*Meloidogyne incognita* belongs to the order Rhabditida (class Chromadorea) (De Ley and Blaxter, 2002). Phylogenetic analysis of the 28s rRNA gene sequence revealed that *M. incognita* is closely related to the migratory ectoparasitic nematode *Pratylenchus* spp. (Subbotin et al., 2006) (Figure 1-1 and 1-2).
Figure 1-1. A phylogenetic tree (not drawn to scale) illustrating the taxonomic position of *Meloidogyne incognita*. *Meloidogyne incognita* shows sister relationship with *Pratylenchus coffeae* (De Ley and Blaxter, 2002).
Figure 1-2. Phylogeny of Meloidogynidae derived from SSU rDNA (Holterman et al., 2009)

**Morphological Characteristics**

Body size of the mature female ranges from 530 to 812 μm in length (Kaur and Attri, 2013). Generally, the female has a pear-shaped body 510 to 692 μm wide without a posterior protuberance. The stylet length varies from 16 to 27 μm with the stylet cone having its anterior half distinctly curved dorsally. Stylet knobs are rounded to transversely elongate and indented anteriorly. The esophagus has a large muscular median bulb 20 to 42 μm in diam. with a conspicuous valve plate. (Kaur and Attri, 2013). Males of *M. incognita* have a centrally concave prominent labial disc. Length of males varies from 600 to 2500 μm. The metacarpus of males is smaller than of females (Karssen et al., 2013).

**Biology**

A typical lifecycle of a RKN species is presented in Fig.1-3. The mature females lay eggs into a gelatinous mass composed of a glycoprotein matrix produced by their
rectal glands. This keeps the eggs together and protects them against environmental extremes and predation (Moens et al., 2009). The egg masses are usually found on the surface of galled roots or embedded within the gall tissue and can contain up to 1000 eggs (Jones et al., 2013). Within the egg, embryogenesis proceeds to the first-stage juvenile (J1), which molts into the J2. J2 hatch from the egg and, in general, hatching is dependent solely on suitable temperature and moisture conditions, with no stimulus from host plants being required (Moens et al., 2009; Jones et al., 2013). The emerged J2 are attracted to the roots of host plants by exudates emanating from the plant root and invasion of the roots takes place usually behind the root tip. J2 then move through the root to initiate and develop a permanent feeding site consisting of several giant cells. This feeding site serves as the only nutrient sink for the developing J2. The nematode growth and reproduction entirely depends on this established feeding site. Under favorable conditions, the J2 molts to the J3 after about 14 days, then to the J4, and finally to the adult stage (Moens et al., 2009). There is a tremendous variation exhibited in reproductive strategies of *Meloidogyne* species that ranges from amphimixis to obligatory mitotic parthenogenesis (Chitwood and Perry, 2009). Most species are parthenogenetic and males are only formed under adverse conditions. The life cycle of RKN takes three to six weeks to complete, depending on the species, the host plant and environmental conditions (Castagnone-Sereno et al., 2013). RKN populations can survive well in the presence of a suitable host and their population numbers usually build to a maximum as annual crops reach maturity. RKN have several generations in one cropping season, and more generations can be produced in tropical
conditions due to higher soil temperatures compared to temperate conditions (Figure 1-3).

Figure 1-3. Generalized life cycle of Southern root-knot nematode *Meloidogyne incognita*. (Abad et al., 2008).

**Damage Symptoms**

Southern root-knot nematode, *M. incognita*, can cause characteristic symptoms on plant roots and rapid yield losses. Once the J2 enters into a host plant root, it migrates to the developing vascular cylinder of root tissue to establish the giant cells. Giant cell formation is combined with expansion and proliferation of nearby pericycle and cortical cells which results in characteristic root galls (Bird et al., 2008). The amount of infection by *M. incognita* can be measured with the help of a root gall index (Greco and Di Vito, 2009). Heavy infestation by *M. incognita* on crops from which tap roots and tubers are harvested can cause the produce be worthless due to the resulting loss in
quality (Karssen et al., 2013). Above ground symptoms of infection are stunting, chlorosis of lower leaves or other abnormal coloration of foliage, patchy, stunted growth, thin or sparse foliage, and symptoms of drought stress such as wilting or leaf rolling. Die-back of perennial or woody plants with little or no new foliage can occur, along with reduced fruit and seed size resulting in low yields. Other aboveground symptoms of root-knot nematode infection are: failure to respond fertilizer application, tendency to react to drought stress more rapidly than healthy plants, and slow recovery from wilting, little or no new foliage development at the onset of a new growing season, severe weed problems (higher density of weeds) due to nematode-infected plant being less able to compete with weeds, and greater disease incidence because of suppressed plant resistance. Below-ground symptoms are root galls, shortened, stubby or abbreviated roots, root lesions, root or tuber necrosis, rotting or death, root or tuber cracking, deformed roots, and altered root architecture (Coyne et al., 2007).

Management Strategies

Owing to their wide host range, management of RKN is a difficult task. Different RKN management options such as chemical, regulatory, biological, cultural, resistance and physical have been tried worldwide. The application of nematicides has been the common and effective measure to manage RKN, but some of these nematicides have been withdrawn from the market because of their high costs, toxicity to humans, and associated serious environmental concerns. Since the phase out of methyl bromide began in 2005, several soil fumigants have been tested for nematode management. The alternatives that that have been tested include 1, 3-Dichloropropene (Telone II), 1, 3-Dichloropropene and Chloropicrin (Telone C-17), Chloropicrin and 1,3-Dichloropropene (Pic-clor 60), Sodium methylthiocarbamate (Vpam HL), Potassium N-
methylidithiocarbamate (KPam HL), Dimethyl disulfide (DMDS), and (Allyl isothiocyanate) AITC (Zasada et al., 2010). However, none of these fumigants provide comparable results to methyl bromide and may be phytotoxic to plants if there is no proper application schedule (Zasada et al., 2010). In addition, these fumigants also require a voluntary Good Agricultural Practices (GAPs) to reduce emissions and bystander exposures (Zasada et al., 2010). Moreover, the current trend indicates that the effective nematicides currently available to growers may not be available in the near future, or will have additional restrictions associated with their use following re-registration.

Cultural practices are the most environmentally sustainable and potentially successful methods. Since M. incognita has a wide host range, cultural practices require precise planning and economic investment. For example, M incognita can be easily managed on cotton by crop rotation with peanut. Similarly, use of cover crops like marigold can reduce the amount of nematode infection (Hooks et al., 2010). Nematode resistant plants have the capacity to hinder or reduce the development or multiplication of nematode inside the plant. Plant resistance may occur naturally in the plant or can be incorporated into the susceptible plant through the application of different breeding tools. Despite of the great scope of resistance breeding, it is successful in few crops due to the limited range of resistance and breaking down of resistance by virulent population of this nematode. Although plant resistance is important practice to control M. incognita, obligatory mitotic parthenogenetic mode of reproduction mechanism helps them to break the resistance very fast (Abad and Operman, 2009). Nematode management methods using antagonists have been developed in many parts of the
world but with some limitations (Vos et al., 2012). Developing effective biological control is hindered due to unavailability of large amount of biological material necessary for application in large area.

Several molecular techniques to engineer nematode resistant plants have been developed. Nematode management using RNAi refers to the natural defense phenomenon whereby the use of double stranded RNA triggers the reduction of homologous mRNA which ultimately leads to the depletion of encoded protein (Joseph et al., 2012). RNAi was first discovered on C. elegans (Fire et al., 1998). Due to research advancements RNAi has become a key technique to determine the function of different genes in number of parasitic nematodes. The successful application of this technique has been demonstrated on different plant-parasitic nematodes including sedentary endoparasitic nematodes such as Meloidogyne incognita, Globodera pallida, and Heterodera glycines as well as several migratory parasitic nematodes including Radopholus similis and Bursaphelenchus xylophilus.

**RNA Interference**

RNA interference is the highly conserved ancient self-defense mechanism of the eukaryotic cells, which is initially evolved to combat genomic parasites including viruses and transposable element that uses double stranded RNA in their life cycles (Bakhetia et al., 2005). In addition, it has been discovered that RNAi also perform various functions such as elimination of defective mRNA by degradation and regulation of protein levels in response to the environmental stimuli (Plasterk, 2002; McManus et al., 2002). RNAi was discovered in 2002 and won Nobel prize in 2006 (Fire et al., 1998). The discovery of RNAi it has opened the door for new research arena. The basic attributes of RNAi are: 1) It is induced by double stranded RNA, 2) The effect of RNAi is
systematic and 3) RNAi is heritable (Fire et al., 1998). The RNA molecule that induces RNAi belongs to two small RNA classes produced by different kind of genes i.e. micro RNAs (miRNAs) and small interfering RNAs (siRNAs) (Couzin, 2002). SiRNAs and miRNAs are considered to be the main component of RNAi. miRNA inhibit translation of RNA into protein are considered as vital that has evolutionary ancient components of regulator of different developmental genes in plant and animals (Williams, 2008; Lee et al., 2007; Moxon et al., 2008). The discovery of RNAi in C. elegans has played a pivotal role in understanding the basis of RNAi in other organisms (Fire et al., 1998).

*Caenorhabditis elegans* is regarded as the model organism due to high fecundity, complete genome sequence, ability to grow in laboratory condition and short life cycle. RNAi techniques are used mainly to clarify the function of certain gene by silencing or switching them off at the post-transcriptional level (Montgomery, 2004). In comparison to the time consuming, sophisticated and tedious traditional genetic manipulation techniques, RNAi has proven very handy and has great scope in determining the biological function of genes when it is used as reverse genetic tool (Montgomery, 2004). The discovery of RNAi has revolutionized the research studies of various organisms. It has been useful to understand the mechanism behind post-transcriptional gene silencing (PTGS) in plants and fungi (Baulcombe, 2004; Cogoni and Macino, 1997). Due to the rapid increase in availability of complete genome sequence of many species, it can be easily speculated that RNAi will aid to a more detailed understanding of complicated physiological process which are still unknown in the womb of nature. It can also alleviate the development of resistance against many harmful pathogens such as viruses, bacteria, insects and nematodes.
Basic Mechanisms of RNAi

The mechanism of RNAi was first discovered in a nematode *C. elegans* (Smardon *et al.*, 2000; Hannon, 2002; Philips *et al.*, 2012; Avgousti *et al.*, 2012). It was further studied on *Drosophila* (Clemens *et al.*, 2000; Lipardi and Paterson, 2009; Miyoshi *et al.*, 2010), Arabidopsis (Mourrain *et al.*, 2000; Filichkin *et al.*, 2010; Pontier *et al.*, 2012) and in mammals (Grimm *et al.*, 2010). Normally, RNAi is induced by a double stranded RNA source from outside the cells often termed as exogenous RNAi while RNAi is induced by dsRNA transcribed from coding or noncoding genomic sequences within the cell coined as endogenous RNAi (Grishok *et al.*, 2005; Grishok, 2012).

Double stranded RNA (dsRNA) in the cell can lead to the production of siRNA (small interfering RNA), shRNA (short hairpin RNA) and miRNA (micro RNA). Double stranded RNA (dsRNA) combine with dinucleotide 3’ overhang by an adenosine triphosphate (ATP) dependent enzyme named DICER and produce three type of small RNA. DICER is an ‘RNase III’ type of enzyme which specifically binds the double stranded RNA and breaks down the RNA to produce small RNA interfering particles. These three RNA interfering particles (siRNA, shRNA, micro RNA) are capable of silencing mRNA, which codes a specific protein. This type of silencing is termed as RNA mediated gene silencing (RNAi). To bring out this gene silencing process combination of many proteins is required. The combination of small RNA together with a protein complex is termed as RNA interfering silencing complex (RISC). RISC factor, which combines with siRNA, will ultimately silence the specific protein coding RNA or messenger RNA (mRNA). When mRNA is silenced protein synthesis will not be possible (Figure 1-4) and mRNA will not be able to produce protein inside the cell. The association of different molecules, which are named as SLICER in *C. elegans* and
argonaute commonly found in plant as well as in animals, are required to produce RISC during RNAi (Hamilton and Baulcombe, 1999; Hammond, 2005; Ghildiyal and Zamore, 2009; Zamore et al., 2000; Kaya and Doudna, 2012).

Recent studies revealed that there are two types of RNA pathways i.e. exogenous RNA pathways and endogenous RNA pathways. Endogenous RNA shares the same core component of RNAi pathway such as Dicer with exogenous RNA pathways. However, difference among pathways occurs in the requirement of specialized factors for the production of primary and secondary siRNAs such as argonaute protein bound with primary siRNAs and RNA dependent RNA polymerase (RdRps) required for the amplification of secondary siRNAs (Yigit et al., 2006; Vasale et al., 2010) (Figure 1-4).

Figure 1-4. Schematic demonstration of RNA interference in a mammalian cells. Step 1: Double stranded RNA is activated by the activity of Dicer, which results in cleavage of double stranded RNA into small fragments of 21-23 base pairs siRNA. Step 2: siRNA are loaded into multiprotein complex called RNA Induced Silencing Complex (RISC) and one side strand of the siRNA, which is also called as passenger strand is degraded, while the guiding strands remain attached with RISC as a template in the silencing reaction. Step 3: Guide strand, is assembled into a functional siRNA-RISC complex bound to Argonaute protein. The targeted mRNA are recognized and bounded by the siRNA-RISC complex. Step 4: mRNA degradation is induced as the target mRNA is degraded by siRNA. After the degradation of mRNA siRNA-RISC complex is released to process other mRNA targets. (Cuccato et al., 2011).
Application of RNAi in Nematodes

Application of RNAi as a Reverse Genetic Tools in Nematodes

RNAi has been widely used in the free-living nematode *C. elegans* where it has been used to carry out genome wide high throughput screens to identify the genes involved in various cellular and development process. Some progress has been achieved in using RNAi technique for plant-parasitic nematodes, while in animal parasitic nematode this technique has not been well utilized. Though some achievement has been observed in RNAi in PPN its diversity of genes with regards to sensitivity to RNAi has been observed.

Variation in RNAi between closely related species of *Caenorhabditis* regarding RNAi feeding suggests that differences in the RNAi mechanism exists even between closely related species (Lilley *et al.*, 2012). The first successful RNAi in a plant-parasitic nematode was discovered using the neuroactive compound octopamine to stimulate oral ingestion of dsRNA by pre parasitic J2 of pale cyst nematode *Globodera pallida* and soybean cyst nematode *Heterodera glycines* (Urwin *et al.*, 2002). The major breakdown of the RNAi was achieved after the development of successful techniques to enhance oral uptake of dsRNA from the soaking solution. RNAi in animal- and human-parasitic nematodes has yielded different levels of success. A human filarial parasite *Brugia malayi*, a rodent parasite *Nippostrongylus basiliensis* and entomopathogenic nematode *Heterorhabditis bacteriophora* were all found susceptible to RNAi (Hussein *et al.*, 2002; Aboobaker and Blaxter, 2003; Ciche and Sternburg, 2007; Ford *et al.*, 2009). The RNAi technique has been demonstrated to be a more feasible technique for plant-parasitic nematodes compared to human and animal parasitic nematodes (Urwin *et al.*, 2002). Since plant-parasitic nematodes do not contain specific mutant and
transformation systems, the RNAi approach can be used as an important tool to understand the function of gene involved in host-nematode interactions.

RNAi has been demonstrated in a wide range of sedentary endoparasitic nematodes such as *G. pallida*, *H. glycines*, and *M. incognito* (Rosso *et al.*, 2005) as well as in migratory parasitic nematodes like *P. coffeae*, *R. similis*, and *B. xylophilus* (Joseph *et al.*, 2012). Furthermore, siRNA have been used to enhance effectiveness of the RNAi. Some reports of using siRNA to induce efficient gene silencing in *G. pallida* and *M. incognito* have been published. Similarly, an experiment using siRNA concluded that siRNAs can enhance knock-down of the parasitism genes *Mi-CRT*, a calreticulin gene expressed in esophageal glands of *M. incognito* (Arguel *et al.*, 2012).

**Application of RNAi in Plant Parasitic Nematode Management**

Several research studies have proven that RNAi can be utilized as a valuable tool in nematode management by genetically engineering plants to express PPN-transcript-specific dsRNA. When the plant generates dsRNA or siRNA, nematode infecting these plants will take up the dsRNA or siRNA and induce the degradation of specific nematode genes. Meanwhile, the amplification of the silencing is triggered with the help of nematode RNA-dependent RNA polymerase (RDRP) (Shi *et al.*, 2013).

An ideal strategy for the silencing of genes that are essential to the parasites for parasitizing the plants and to characterize the function of nematode genes is host-delivered RNAi. Different research on PPN has confirmed the validity and effectiveness of the host-delivered RNAi for the management of nematodes. Host-delivered RNAi was first demonstrated on *M. incognito* by expressing the dsRNA of the two genes, which encodes integrase and a splicing factor in tobacco plants (Yadav *et al.*, 2006). Similarly, expression of the dsRNA of the parasitism gene 16D10 in *Arabidopsis thaliana* plants
was also obtained against four major RKN species (Huang et al., 2006). Though, different studies have demonstrated the phenotype for nematode parasitizing plant expressing dsRNA, few studies are able to characterize that presence of the phenotype is due to an RNAi effect (Joseph, 2013).

Some studies using RT-PCR analysis verifies that the generation of nematode resistance is the result of host-derived RNAi by significant down regulation of target nematode genes from nematode feeding on the transgenic roots (Sindhu et al., 2009). However, presence of the target gene in the host plant is found in few research reports. Some studies using RT-PCR revealed the presence of an unprocessed transcript by amplifying the intron or spacer region of the hairpin construct (Patel et al., 2010). To achieve effective host derived RNAi against the nematode it is crucial to have a large amount of dsRNAs or siRNAs at the delivery site between host and nematode (Charlton et al., 2010).

**Sex Determination in Nematodes**

The model nematode *C. elegans*, has two sexes i.e. male and hermaphrodite (self-fertile female). Both male and female have 5 pair of autosomal chromosomes. The Hermaphrodite at its fourth larval stage generates and stores the sperm inside the body to use in future for the fertilization with oocytes. Upon completion of spermatogenesis, oocytes are produced within the hermaphrodite gonads. The self-fertilized embryo is enclosed in eggs and further development occurs. Most of the sexual differences and earlier signs of sexual dimorphism starts from mid embryogenesis (Sulston and Horvitz, 1977; Sulston et al., 1983).

The female *C. elegans* contains one pair of X chromosome and five pair of autosomal chromosome while the male possesses a single X chromosome and five
pairs of autosomal chromosome. Since the male *C. elegans* contains heterogametic sex and it is denoted as ‘XO’ in contrast to ‘XX’ homogametic sex in hermaphrodite. The males and hermaphrodites are completely distinct organisms (Zanetti and Puoti, 2013). Hermaphrodites can reproduce by either cross-fertilization with a male or by self-fertilization. When a hermaphrodite reproduces by self-fertilization, it produces 98% hermaphrodites and only 2% males. In contrast, cross fertilization produces equal proportions of male and hermaphrodite progeny (Hodgkin, 1987) (Figure 1-5).

**XX Hermaphrodite**

- Self-fertilized

**XX Hermaphrodite**

- **XX Hermaphrodite**
  - 0-2% XO male
  - **XX Hermaphrodite**
    - 50% XO male
    - 50% XX hermaphrodite

Figure 1-5. Schematic diagram of the reproduction mechanisms of *Caenorhabditis elegans* showing both sexual and self-fertilization mode of offspring production. (Hodgkin, 1987).

*Meloidogyne incognita* can reproduce both by fusion of male and female gametes or by mitotic parthenogenesis (Moens *et al.*, 2009). However, sex determination is mostly regulated by the environmental factors, including temperature, availability of food and soil moisture. (Triantaphyllou, 1973). It was observed that when J2 enter the host roots, they are sexually undifferentiated. The determining factor for the sexual differentiation is controlled by environmental conditions during post infection development. If the situation is favorable for normal proliferation, the majority will
develop into females. In contrast, environmental stress such as over population, food shortage, and high temperature causes an increase in males. Sex reversal can occur when a juvenile that has begun differentiation into female then differentiates into a male in response to environmental stimuli. Such males, have two testes corresponding to the two gonads of females whereas normal males have single testes. (Triantaphyllou and Hirschmann, 1964).

Feeding duration of juveniles is one of the factors determining the sex in *M. incognita*. Juveniles which were extracted from roots of plants 10, 11 or 12 days after inoculation and consequently were unable to complete their normal feeding duration developed into males while juveniles which were extracted from plant 12 days after inoculation developed into females showing strong correlation between sex determination and feeding duration (Trudgill, 1972). Presence of stress in the host, which results in unavailability of food for developing nematode, directs towards the development of males. Similarly, high nematode inoculum density, the presence of resistant genes in the host plant, or damage to the host plant from pruning or herbicide has yielded increased numbers of males (Snyder *et al*., 2006). Sex differentiation was not affected in *M. incognita* across a wide range of temperatures, however, slight low temperature ≤ 15 °C caused slight increase in the number of males compared to females (Triantaphyllou, 1973). Nevertheless, there is lack of complete information about the genetic basis of sex determination. So, to further understand the process this should be studied in amphimictic or at least in facultative amphimictic species.

Application of molecular tools and understanding the mode of nematode parasitism of plants has increased recently. Genes that encode different cell wall-
degrading enzymes of different nematodes including \textit{M. incognita} (Huang \textit{et al.}, 2003) and \textit{H. glycines} (Alkharouf \textit{et al.}, 2006) have been identified and characterized. Availability of whole genome sequences and the functional characterization of various genes in response to the function played by these genes have been studied in various nematodes like \textit{C. elegans} (Waterston, 1998), \textit{C. briggsae} (Gupta and Sternberg, 2003), \textit{M. incognita} (Abad \textit{et al.}, 2008) and \textit{M. hapla} (Opperman \textit{et al.}, 2008).

The available primary sequence information and functional characterization of the genes involved in performing various functions and processes has been greatly studied by using RNA interference (Fire \textit{et al.}, 1998; Elbashir \textit{et al.}, 2001; Sioud 2011). RNAi techniques have been extensively used and recorded to provide the function of more than 20,000 genes and their phenotypic consequences by knocking down the gene effects in \textit{C. elegans} (Zhuang and Hunter, 2012). RNAi techniques have been implemented in plant-parasitic nematodes to strengthen the knowledge and understanding of developmental and parasitic genes (Joseph \textit{et al.}, 2012). The application of RNAi in plant-parasitic nematode can be a momentous novel method for plant-parasitic nematode management.

**Functional Characterization of Genes Involved in Sex Determination in \textit{Caenorhabditis elegans}**

Sex determination in \textit{C. elegans} is dependent on the ratio of sex chromosomes to autosomes. Male nematode have XO chromosomes and entirely produces sperm. Hermaphrodites have XX chromosomes and continuously produce both sperm and oocytes. The shift in developmental balance is shifted to female development as XX organism become hermaphrodite, which is able to reproduce by self-fertilization.
In *C. elegans* low quantities of X i.e. (XO) results in activation of male specific gene *xol-1*. Which is considered to be on the top of the sex determination pathway and stimulates male development. (Miller *et al.*, 1988). Mutation or loss of function of the X-linked gene *xol-1* results in shifting of sex determination pathways towards the hermaphrodite mode and to the death of XO animals. On other hand, an XX embryo is produced when *xol-1* is inactivated by the X-linked genes *sex-1* and *fox-1* (Carmi *et al.*, 1998). The inactivation of the *xol-1* gene induces the expression of the *sdc-2* gene in the female which functions together with *sdc-1* and *sdc-3*. It has been observed that mutation of *sdc-1* leads to variable sexual transformation of XX animals and *sdc-1* also helps to amplify the weakly masculinizing mutation in known sex determining genes (Villeneuve and Meyer, 1990). These *sdc* genes not only contribute to sex determination, but also help in equalization of gene expression between males and females (Lieb *et al.*, 2000) (Figure 1-6).

Figure 1-6. In XX animals high dose of X suppress the activity of *xol-1* at two levels i.e. *sex-1* and genes, which causes low *xol-1* transcript level. Similarly, *fox-1* and region 2 suppress *xol-1* post transcriptionally resulting low XOL-1 protein.
levels and hermaphrodite progeny along with doses compensation. In XO organisms low doses of X could not suppress the \textit{xol-1} in both levels which results in high transcript level and high XOL-1 protein level and male progeny (Carmi and Meyer, 1999).

It has been demonstrated that the pathway of sex determination consists of series of down-regulated interactions that occur alternatively either by activating or suppressing the activity of male or female specific genes. The SDC protein suppresses the activity of \textit{her-1} to prevent the development of males (Trent et al., 1991). Similarly, if HER-1 protein is absent TRA-2 protein blocks the action of FEM protein and results in the activation of \textit{TRA-1} which suppress the development of males and increases development of females. Furthermore, males have active HER-1, which repress the activity of TRA-2. Further down, the activation of FEM inactivates \textit{TRA-1}, which results in activation of FOG-1 and FOG-3 proteins and produces spermatogenesis. The activation of FOG-1 and FOG-3 can occur indirectly through the inactivation of \textit{TRA-1} or may occur directly through the FEM genes (Hodgkin, 1986) (Figure 1-7).
Figure 1-7. Basic mechanism of sex determination in Caenorhabditis elegans from a hermaphrodite germline. A double dose of X chromosome inactivates the activity of xol-1 genes, allowing SDC to repress the activity of her-1 and promote dose complementation. Tra-2 and fem-3 mRNA are responsible for the post-transcriptional regulation of momentary spermatogenesis where tra-2 is repressed by GLD-1 and FOG-2, which are essential for spermatogenesis. Similarly, FBF and MOG proteins repress FEM-3, switching to oogenesis. TRA-2 activates the TRA-1 and FEM proteins repressing its action. TRA-1 promotes oogenesis by repressing the activity of fog-1 and fog-3, thereby inhibiting spermatogenesis (Zanetti et al., 2013)
Sex Determination in *Meloidogyne incognita*

*Meloidogyne incognita* is an obligatory endoparasite that reproduces by mitotic parthenogenesis where there is neither reduction nor fusion of nuclei and eggs develop directly into embryos. Cytological analysis has shown that some isolates of *M. incognita* have chromosome numbers ranging from 30 to 39 while other isolates have chromosome numbers of 40 to 48. *Meloidogyne incognita* is considered to be diploid (2n) or hypotriploid (3n-x) (Castagnone-Sereno, 2006). *Meloidogyne incognita* is the most widespread nematode found both in tropical and temperate environment which has temperature up to $3^\circ$ C (Sasser *et al.*, 1983). The distribution of *M. incognita* in wide range of climatic conditions is due to its mitotic parthenogenesis mode of reproduction, which enables them to respond to overcome plant resistance genes (Castagnone-Sereno, 2002). *Meloidogyne incognita* reproduce by obligatory mitotic parthenogenesis where neither reduction nor fusion of nuclei occur and eggs directly develop into an embryo. If males are present they can inseminate females but sperm does not participate in fertilization as a degeneration of sperm nucleus occurs (Triantaphyllou, 1981). Sex determination in *M. incognita* is epigenetic under the influence of environmental conditions in which juvenile develops into females under favorable conditions while but develops into males under unfavorable conditions. Sex reversal has also been discovered in which females develop into males (Triantaphyllou, 1973) (Figure 1-8).
Genome analysis of the *M. incognita* and *C. elegans* revealed the presence of at least one of common homologues gene in every stage of the sex-determination pathway. Several studies suggest that presence of common *sdc-1* in the doses completion pathway, and *tra-1, tra-3* and *fem-2* in the sex determination pathway. Furthermore, studies suggests the presence of various downstream genes like *mog-1* which acts as inhibiter for male-promoting genes and *mab-23* which helps in regulating male differentiation and behavior (Abad *et al.*, 2009). Therefore, it can be concluded that *M. incognita* shares the same developmental pathway as *C. elegans* for the sex determination of its progeny. However, the difference arises between *M. incognita* and *C. elegans* based on the importance of environmental factors in determining male and female development (Abad *et al.*, 2009).

**Scope of the Study**

With the advancement in the molecular techniques and availability of the whole genome sequence of *M. incognita*, function of various genes involved in parasitizing host plants can be studied in detail. Since the development into either males or females is determined by both environmental factors and genetic factors, proper understanding of the genes involved in determining male and female development is necessary. This research work focuses on determining the feasibility of RNAi in the sex determination of *M. incognita*. 

Figure 1-8. Schematic representation of mitotic parthenogenesis in *Meloidogyne incognita*.
Hypothesis
Down regulation of sex determining genes that promotes oogenesis can arrest female development of *Meloidogyne incognita*.

Objectives

**Determine The Feasibility of RNAi In Down Regulating Genes Involved In The Sex Determination of *Meloidogyne Incognita*.**

Although most of the important physiological features in PPN are compared with the model organism, *C. elegans*, it should be noted that *C. elegans* does not represent the whole genomic diversity in the phylum. So, it is necessary to test the feasibility of RNAi in down regulating the genes involved in sex determination of *M. incognita* to fully understand the process involved in the sex determination. Studies have suggested the existence of RNAi pathway in different plant-parasitic nematodes illustrating target gene dependent variability in RNAi. While some genes are highly susceptible to RNAi others are unaffected by RNAi (Joseph *et al.*, 2012). In this study, we will determine the susceptibility of RNAi against different target genes that play key role in sex determination of *M. incognita*

**Provide A Functional Characterization of Genes Involved In Sex Determination.**

Advancement in molecular research and availability of genome sequence of major plant-parasitic nematodes has resulted in the successful silencing of the various genes in nematode parasitism. Despite the success in the silencing of different target genes, the application of RNAi in PPN is limited by different factors. Advanced research has been conducted on the different molecular aspects of *C. elegans*. RNAi research on plant-parasitic nematodes to identify gene function tries to correlate the gene function
with *C. elegans*. High throughput screens used to screen used to identify the function of genes in *C. elegans* seem to be irrelevant in the case of PPN (Lilley *et al.*, 2012).

Furthermore, dsRNA or siRNA triggered RNAi silencing effect have been successfully proven in plant-parasitic nematodes, but molecular details of pathways, including those of *M. incognita* have not been well described (Lilley *et al.*, 2012).
CHAPTER 2
FUNCTIONAL CHARACTERIZATION OF GENES INVOLVED IN SEX DETERMINATION OF *MELOIDOGYNE INCognita*

Introduction

Plant-parasitic nematodes (PPN) cause crop damage throughout the world estimated at $80 billion per year (Nicol *et al.*, 2011). However, this amount is probably underestimated as most growers, especially in developing countries, have limited understanding about nematodes (Hooks *et al.*, 2010; Anwar and Mckenry, 2012). Plant-parasitic nematodes can be classified based on their feeding type as ectoparasites, semi-endoparasites, migratory endoparasites, and sedentary endoparasites. Among these groups, economically important genera of sedentary groups include cyst nematodes (*Globodera* and *Heterodera*), root-knot nematodes (*Meloidogyne*) and other genera such as *Rotylenchulus*, *Tylenchulus* and *Nacobbus*. Sedentary nematodes modify the certain plant cells resulting in the re-differentiation of host cells into a specialized feeding site. The feeding sites of root-knot nematodes are called ‘giant cells’, which are formed by repeated cell division without division of cytoplasm.

Most studies on physiology and mechanisms of infection caused by plant-parasitic nematodes have focused on sedentary nematodes, and some success has been achieved in identifying RNAi interference approaches in sedentary and a few economically important migratory plant parasitic nematodes. Research on the use of RNAi on plant-parasitic nematodes relies on targeting the genes that cause parasitism or have good potential of increasing resistance to nematodes in the host plant (Charlton *et al.*, 2010, Joseph *et al.*, 2012).

To reduce losses caused by RKN different physical, biological and chemical strategies have been developed. One promising alternative to conventional nematode
management tactics is the use of RNA interference (RNAi) to silence genes of various plant-parasitic nematodes in order to induce plant resistance to infection (Lilley et al., 2007). Previously, RNAi was coined as post-transcriptional gene silencing in plants. RNAi was first demonstrated on C. elegans but since then silencing using ds RNA has been studied thoroughly (Jorgensen et al., 1996, Fire et al., 1998). The use of RNAi for the functional analysis of genes in plant-parasitic nematode was first reported in 2002. Lilley et al., 2007) reported the presence of various genes in different tissues and cell type using gene silencing in root-knot and cyst nematodes.

This research explores the possibility of using RNAi for the functional characterization of genes involved in sex determination of Meloidogyne incognita so, that it can be used as a management strategy. Availability of whole genome sequence of M. incognita and suitability of RNAi on this nematode has opened a new arena for the functional analysis of parasitic nematode genes. RNAi incorporation in this nematode can be achieved by feeding with double stranded RNA.

Sex determination in C. elegans is very closely related with doses compensation as it has an XX-XO determination pathway which is very different than M. incognita in which typically reproduces by mitotic parthenogenesis. Genetic contribution to the offspring is fully dependent on the female as the males have no contribution in reproduction. Sex determination is solely dependent on environmental factors (Triantaphyllou, 1973). Under suitable environmental condition and suitable host, the majority of offspring are females at maturity. In contrast, during unfavorable environmental conditions many of the offspring mature as males. In some cases, sex reversal of female to male and intersex phase also has been reported. Though there is
vast difference in sex determination pathway between these *C. elegans* and *M. incognita*, there is at least one homologous member in each step of the sex determination pathway such as *tra-1, tra-3* and *fem-2* including *sdc-1* from the doses compensation pathway, and some downstream genes like *mag-1* and *mab-23*. Therefore it is possible to identify various gene functions through comparative analysis between the genes of *C. elegans* and *M. incognita*. It is also possible there exists similarity between the sex determination pathways of *C. elegans* and *M. incognita*, with slight variation in male sex determination as in *M. incognita* it is controlled by environmental conditions.

Studies on different species of insects including flies, mosquitoes and beetles has revealed the possibility of producing one sex by manipulating the sex determination pathway (Pomerantz *et al.*, 2015). The regulation of sex determination in nematodes involves a cascade of genes, in which males and females are produced by suppression and repression gene activity. Among the different cascade genes some are involved in encoding RNA-binding protein which delimit the splicing of pre-messenger RNA into sex specific transcripts (Zanetti *et al.*, 2013). Some of the major genes of the sex determination pathway in *C. elegans* are *tra-1, tra-2, fem-2, her-1, mag-1, sdc-1, tra-3, fem-1, fem-2, fem-3* etc (Zarkower, 2006).

In *C. elegans* *tra-1* promotes the development of hermaphrodites and suppresses development of males. *Tra-1* expresses two mRNA which encode for zinc finger protein. The zinc finger domain of *tra-1* is highly similar to that of Drosophila Ci and other vertebrate GLI proteins. Furthermore, *tra-1* also controls the development of gonads, regulates the formation of somatic gonadal primordia and after hatching *tra-1*
controls gonadal cell proliferation (Zarkower, 2006). In somatic sex determination pathways \textit{tra-1} acts as a global regulator of sex-specific transcription (Haag, 2005). Similarly, SDC protein helps to connect sex determination and doses compensation. These proteins are large protein complexes that are confined to the X chromosome of the hermaphrodite (Zarkower, 2006). Gene \textit{sdc-1} is also a zinc finger encoding protein. Absence of \textit{sdc-1} in sex determination pathway of \textit{C. elegans} results in incomplete transformation of XX towards the male and its absence also increase the number of X-linked transcripts in the XX animal resulting in morphological defect without the causing XX-specific lethality (Villeneuve et al., 1990).

RNAi interference on \textit{tra-1} gene in \textit{C. elegans} resulted in transformation of the XX animal into a less fertile male. Contradictory to this, hyper activation of \textit{tra-1} transforms both XO and XX animal into a fertile female. Furthermore, reduction of \textit{tra-1} leads to incomplete masculinization of the hermaphrodite (Szabó et al., 2009). On other hand, RNAi of \textit{sdc-1} in \textit{C. elegans} leads to egg laying defects and variant in protein degradation.

Gene \textit{tra-1} is the only member of GLI transcription factor family in \textit{C. elegans}. This gene acts as a terminal regulator of the of the sex determination pathway and directs the hermaphrodite development. Furthermore, this gene is necessary for proper spermatogenesis in both sexes to regulate sex specific development and development of male somatic gonads. \textit{TRA-1} also functions by repressing the transcription of different genes like \textit{mab-3} present in intestine and \textit{egl-1} present in neurons. \textit{TRA-1} is essentially present in the nucleus and cytoplasm of hermaphrodites and in males. Different research has revealed that \textit{tra-1} gene has been highly conserved among
different species of nematodes for over 200 million years. Gene silencing of *tra-1* leads to the almost complete sex reversal of hermaphrodites to males in the soma region. Some mutants of *Cel-tra 1, Ppa-tra-1* in *C. elegans* also generated fewer oocytes indicating incomplete germ line transformation (Pires-daSilva *et al.*, 2004).

*Sdc -1* encodes a zinc-finger protein of C2H2 type. In *C. elegans* this gene affects the egg laying capacity and helps in development of the vulva. This gene is highly present in embryos and adults. It helps in the hermaphrodite sex determination. Gene *sdc-1* gene functions as a bridge between sex determination and doses compensation. This gene functions as embryonic transcription factor which regulate the downstream genes in the sex determination pathway, which facilitate and control both sex determination and doses compensation (Nonet and Meyer, 1991).

The objectives of this study are therefore to:

1. Test the feasibility of RNAi in down regulating genes involved in the sex determination of *Meloidogyne incognita*.

2. Provide a functional characterization of genes involved in sex determination.

**Materials and Methods**

**Meloidogyne incognita Cultures**

A homogeneous population of *M. incognita* was obtained from the University of Florida Nematode Assay Lab stock cultures that were maintained under greenhouse conditions on tomato (*Solanum lycopersicum* L. cv. Rutgers). The identity of the culture was confirmed using PCR. Briefly, DNA was extracted from eggs using Proteinase K method (Blin and Stafford, 1976) and PCR was done using *M. incognita* specific primers followed by gel electrophoresis. Eggs were extracted from galled tomato roots using NaOCl method and incubated at 28°C for 3 days in a hatching disc. Freshly hatched
juveniles were collected by a modified Baermann dish technique. The juvenile suspension was adjusted to 1000 J2/ml of water and used as inoculum for the experiments.

**Selection of Target Gene and Primer Design**

Sex determining genes of *C. elegans* were compared with whole genome sequence of *M. incognita* according to the functional information available for *C. elegans*. The main approach was to search for genes that are homologues between *C. elegans* and *M. incognita*. The second approach was to find genes that are essential for sex determination in *C. elegans* and are also present in *M. incognita*. Based on the result of Basic Local Alignment Search Tool (BLAST), we selected downstream (*tra-1*) and upstream (*sdc-1*) genes that are present both in *C. elegans* and *M. incognita*. Four primers (Mitra1F1, Mitra1R1, Misdc1F1, and Misdc1R1) were designed targeting the exon region of best hit nucleotide sequences (Table 2-1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitra1F1</td>
<td>5'- GCACCACATTTCTCTGTCG</td>
</tr>
<tr>
<td>Mitra1R2</td>
<td>TCCATCTGGCCTTATTCCAA-3'</td>
</tr>
<tr>
<td>Mitra1F1</td>
<td>5'- GCACCACATTTCTCTGTCG</td>
</tr>
<tr>
<td>Mitra1R1</td>
<td>GCAATGAGCACACTGATAAGG-3'</td>
</tr>
<tr>
<td>Mitra1T7F1</td>
<td>5'-TAATACGACTCACTATAGGGAGAGCACCACATTTCTTCTTCTGTCG</td>
</tr>
<tr>
<td>Mitra1T7R2</td>
<td>TAATACGACTCACTATAGGGAGATCCATCTGGCCCTTATTCCAA-3'</td>
</tr>
<tr>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Misdc1F1 5' - TGGTCAGTACAACCCCAACAA</td>
<td></td>
</tr>
<tr>
<td>Misdc1R1 CTCTACAAGTTCTTCTCAAATG-3'</td>
<td></td>
</tr>
<tr>
<td>Misdc1T7F1 5' - TAATACGACTCAGTTAGGGAGATGGTCAGTCAAACCCCAACAA</td>
<td></td>
</tr>
<tr>
<td>Misdc1T7R1 TAATACGACTCAGTTAGGGAGACTCTCAAAGTTCTTTCTCAAATG-3'</td>
<td></td>
</tr>
<tr>
<td>Miactin F 5' - GATGGGCTACAGCTGCTGGT</td>
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<tr>
<td>Miactin R GGACAGTGTGTTGCGTAAGG-3'</td>
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<tr>
<td>GFP F 5' - AGGTGATGCTACATACCGGAAG</td>
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<td>GFP R ACAGGTAATGGTTGTCTGGTAAA-3'</td>
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<tr>
<td>GFPPT7R TAATACGACTCAGTTAGGGAGACAGGTAATGCTGGTCTGGTAA-3'</td>
<td></td>
</tr>
</tbody>
</table>

**RNA Extraction and Cloning of *Mi-tra-1* And *Mi-sdc-1***

Total RNA was extracted from eggs and second stage juveniles of *M. incognita* using the Trizol method according to the manufactures protocol (Invitrogen, Thermo Fisher scientific- Carlsbad, CA, USA). The first strand cDNA was synthesized using 1000 ng of RNA, 20 mM dNTP, 100µM oligodT primer, 400 units of Reverse Transcriptase (SuperScript® IV RT, Invitrogen Thermo Fisher Scientific, Carlsbad CA, USA), 80 units of Ribonuclease inhibitor, and 0.2 M of DTT. After extraction, RNA was treated with DNase to avoid contamination. A 462-bp fragment of *Mitra-1* and 240-bp of *Misdc-1* was amplified from the cDNA using primers Mitra1F1 and Mitra1R2 for *tra-1* and Misdc1F1 and Misdc1R1 for *sdc-1*. The PCR conditions for *Mitra-1* and *Misdc-1* were: 95°C for 15 minutes followed by 40 cycles of 95°C for 1 minutes, 50°C for 45 second and 72°C for 1 minute, followed by incubation at 72°C for 7 minutes. Amplified
fragments were gel purified using Qiagen QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) following the manufactures protocol and were sent for Sanger sequencing. Sequencing result revealed high sequence similarity to *C. elegans* genome sequences.

The confirmed amplified fragment was inserted into a pDrive cloning vector and introduced into a Qiagen EZ competent cell followed by transformation of competent cell using manufacturer’s protocol (QIAGEN® PCR Cloning Handbook, Valencia CA, USA). The recombinant plasmid was then purified using Zyppy™ Plasmid Miniprep Kit (Zymo Research, CA, USA) using manufactures protocols. The expression of Mitra-1 and Misdc-1 were checked for all life stages of the nematode (egg, J2, J3, J4, early adult, and mature female). For the expression test, total RNA was extracted from all life stages using Tri-reagent. Briefly, about 25 nematodes were picked manually from the tomato roots 8, 12, 16, 23 and 30 days after inoculation and RNA extraction was done by using the Trizol method (Invitrogen, Thermo Fisher scientific Carlsbad, CA, USA) according to the manufacture protocol followed by cDNA synthesis as mentioned above. A PCR was performed on this cDNA for 35 cycles under the PCR conditions as described previously. A housekeeping actin gene was used as a control and amplified using Miactin F and Miactin R primers.

**Synthesis of Double Stranded RNA**

The desired templet of cDNA containing *Mitra-1* and *Misdc-1* genes from J2 was amplified by PCR to generate dsRNA of the respective genes. The standard condition for PCR was 95°C for 10 minutes followed by 35 cycle of 95°C for 1 minute, 50°C for 45 second and 72°C for 1 minute, which was followed by incubation at 72°C for 7 minutes. The respective primers with the T7 promotor sequence incorporated at 5’ end of either
the sense or antisense strand were used for PCR amplification (Table 1). The primers used to amplify sense strand of *Mitra-1* with promoter was Tra1T7F1 and Mitra1R1. Similarly, for promoters the attached antisense strand primers used were Mitra1 and Tra1T7R1. For *Misdc-1* gene with promoter on sense strand primer used was and Misdc1R1 followed by promoter on antisense strand primers were Misdc1F1 and SDC1T7R1. PCR products were transcribed and purified using Ambion MEGAscript® RNAi Kit (ZAGENO, Berlin, Germany) according to the manufacturer’s protocol. The double stranded RNA was made by incubating equimolar amounts of sense and antisense strands in boiling water for 5 minutes, followed by 1 hour at room temperature and subsequent treatment with DNAse to remove the template. The dsRNA was quantified spectrophotometrically and was checked on 1.5% agarose gel. To check dsRNA toxicity due to exogenous dsRNA supply, the dsRNA of Green Fluorescent Protein (GFP) was used as a control.

Green Fluorescent Protein was chosen as a dsRNA control because it does not have a direct impact on nematode sex determination in *M. incognita*. The use of GFP helps to assess the non-specific action of dsRNA on nematode sex determination. cDNA from J2 was used as a templet. The sense strand of GFP with its promotor region were amplified using primer GFPT7F and GFPR followed by antisense strand of GFP with promotor region was amplified by using GFPF and GFPT7R. The dsRNA from GFP was synthesized as mentioned above for *Mi-tra-1* and *Mi-sdc-1*.

For the second experiment all the treatments were the same as the first with additional combinatorial dsRNA treatment consisting of *Mi-tra-1* dsRNA and *Mi-sdc-1* dsRNA treatment. For the preparation of combinatorial dsRNA, 500 μg/μL concentration
of individual dsRNA was combined resulting final concentration of dsRNA to be 1000 µg/µL which is similar with individual dsRNA treatment.

**Plant Infection Assay in Pouches**

For the quantification of knock-down phenotype, cowpea (*Vigna unguiculata* cv. Whippoorwill) plants were grown in CYG seed growth pouches (Mega international, Minneapolis, MN, USA) and infected with dsRNA treated J2. Germinated seed were kept on the pouches and grown in a growth chamber at constant temperature of 28 ºC (Figure 2-1).

![Figure 2-1. 15-day old cowpea plant grown in CYG seed growth pouches and kept in a growth chamber at 28 ºC. Roots were well developed after 15 days.](image)

**In Vitro RNAi on *Meloidogyne incognita* and Analysis of MRNA Levels by Semi Quantitative RT-PCR.**

For the first trial, approximately 7000 J2 of freshly collected *M. incognita* were incubated for 24h in 200 µl of solution containing 100 µl of dsRNA, 50mM octapamine
(Sigma-Aldrich, St. Louis, MO, USA), 3mM spermidine (Sigma-Aldrich, St. Louis, MO, USA), 2μl of 5% gelatin (Sigma-Aldrich, St. Louis, MO, USA) and 80μl of distilled water with nematode for each of the three treatments i.e. Mitra-1, Misdc-1 and gfp dsRNA. For the negative control, nematodes were incubated in the same solution without dsRNA. The nematodes were incubated at 28°C for 24 hours in 1.5 ml micro-centrifuge Eppendorf tubes with agitation. For the second trial, all the treatments were same with an additional treatment containing combined dsRNA of Mitra-1 and Misdc-1. After incubation, the nematodes were washed thoroughly in distilled water by centrifugation at 4000 revolutions per minute for 1 minute and it was repeated 3 times. After 24 hours 400 nematodes were taken from each treatment for total RNA extraction using Trizol reagent (Invitrogen, Thermo Fisher Scientific Carlsbad, CA, USA) according to the manufacturer’s instructions. Equal amounts of RNA from each treatment were used in cDNA synthesis as described in the above section. The prepared RNA was treated with DNase to avoid contamination. After 48 hours of dsRNA soaking, 400 nematodes per treatment were taken and subjected to total RNA extraction and cDNA synthesis after 72 hour and 96 hour of treatment.

The cDNA was used as a PCR template with the primers and Mitra1R2 as the first step of nested PCR for 25 cycle and thus obtained PCR product was used as template for second PCR using the primer Mitra1F1 and for Mitra-1 dsRNA treated cDNA obtained after 24, 48,72 and 96 hours. Similarly, for Misdc-1 gene amplification Misdc1F1 and primer were used for cDNA of 24, 48, 72 and 96 hours. Furthermore, for GFP amplification primer used were GFPF and GFPR. For combinatorial RNAi both primers Mitra1F1 and MitraF2 and Misdc1F1 and Misdc-1R1 were used separately.
cDNA obtained from the nematode treated with combinatorial dsRNA was used as a template. PCR condition and number of cycle were the same as above. The PCR products after amplification were subjected to gel electrophoresis in 1.5 % agarose gel to visualize the effect of RNAi.

**Qualitative and Quantitative Characterization of Knock-Down Phenotype.**

For qualitative observation of the RNAi effect on sex determination, the treated nematodes were washed with sterile water and observed in a microscope to check for effects on mortality due to dsRNA toxicity on the nematodes. Cowpea seeds were sterilized using ethanol and bleach followed by incubation inside incubator for 24 hours at 28°C to enhance germination. Twenty-four hours after germination, seeds were transferred to pouches and kept inside a growth chamber at 28°C for 15 days to allow for proper root development. After this approximately 500 nematodes from each treatment were inoculated onto the 15 days old cowpea seedling. Nematodes were thoroughly distributed along the root of the plant. After inoculation plants were kept inside growth chamber at 28°C. Treated plant were distributed in random complete block design (RCBD) with ten replicates of each treatment. Fifteen days after inoculation, five pouches from each treatment were harvested and the roots were stained using the sodium-hypochlorite-acid-fuscin method (Byrd et al., 1983). Developmental stages of nematodes were observed and the number of nematodes in each root of each developmental stage were recorded. Thirty days after inoculation, the remaining 5 plants per treatment were harvested and stained as above and number of nematodes in each root were counted and of each developmental stage was recorded. Data analysis was done by one-way ANOVA with multiple mean comparison between treatments using Tukey’s test in R Studio statistical package (RStudio 0.99.473, ©
The number of nematodes from each developmental stage were standardized as a percentage of the total number of nematodes in the roots.

Experiments using dsRNA on different nematodes like in pale cyst nematode *Globodera pallida* and in southern root-knot nematode *M. incognita* has demonstrated that high concentrations of non-specific dsRNA cause profound phenotypic change in the infective juveniles (Dalzell et al., 2009). So, in order to confirm that the result of observed change in phenotype after RNAi is due to specific gene silencing rather than any toxicity or due to inhibitory action of dsRNA, GFP gene was chosen as additional control. GFP gene produces green fluorescent protein in jellyfish *Aequorea victoria*. This gene is not nematode specific so dsRNA treated with this gene will help any toxicity in nematode due to exogenous supply of dsRNA to the nematodes.

**Results**

**High Degree of Sequence Similarity Of *tra-1* and *sdc-1* among Different Nematodes**

Basic Local Alignment Search Tool (BLAST) was conducted on *Mi-tra-1* gene of *C. elegans* with whole genome sequence of *M. incognita* on the WormBase parasite database. Based on the BLAST results, the Minc 06243 contig region had the best hit with the *tra-1* gene of *C. elegans* resulting an alignment score of 451bp. Gene *tra-1* of *C. elegans* was 100% identical with that of *M. incognita*. Similarly, for the *Misdc-1* gene BLAST results for *C. elegans* and *M. incognita* revealed the best hit as the Minc10224 contig region with alignment score of 234bp and expected E value of 8.3E-129 with 99.6% identity with *M. incognita*.

Blastn analysis of *Mi-tra-1* and *Mi-sdc-1* amino acid sequences ([http://parasite.wormbase.org/Tools/Blast?db=core](http://parasite.wormbase.org/Tools/Blast?db=core)) showed high level of sequence
similarity among diverse groups of nematodes including plant-parasitic, animal and human parasitic, free living, and entomopathogenic nematodes (Figures 2-2 and 2-3). These sequences were highly nematode specific and have shown no similarity with plant and human sequences.

Figure 2-2. Gene tree showing the relationship of Minc06243 genes of *Meloidogyne incognita* with other root-knot nematodes and free living nematodes, including *Caenorhabditis elegans* and other animal homologs.
Figure 2-3. Gene tree showing the relationships of Minc10224 contig of *Meloidogyne incognita* with other root-knot nematodes and free living nematodes, including *Caenorhabditis elegans* and other animal homologs.
Mi-tra-1 And Mi-sdc-1 can be Efficiently Silenced by RNAi

Semi quantitative RT-PCR on cDNA was done on different life stages of *M. incognita* to check the presence of *Mi-tra-1*, *Mi-sdc-1* and the housekeeping gene actin (Figure 2-4).

![Amplification of Mi-tra-1 and Mi-sdc-1 mRNA from different life stages of M. incognita.](image)

**Figure 2-4.** Amplification of *Mi-tra-1* and *Mi-sdc-1* mRNA from different life stages of *M. incognita*. Different *M. incognita* life stages i.e. egg, second stage juvenile, third stage juvenile, fourth stage juvenile, early adult stage, and mature female. The PCR was performed for 35 cycle for *Mi-tra-1* and *Mi-sdc-1* and 30 cycle for Actin gene.

In non-RNAi control nematodes, we could easily visualize *Mi-tra-1* and *Mi-sdc-1* within 30 cycles of amplification. The transcript level of both genes was reduced in all nematodes that were incubated in the corresponding dsRNA-containing solution compared to the nematodes in control solution. Analysis of PCR revealed that both gene *Mi-tra-1* and *Mi-sdc-1* were silenced in all time periods (Figure 2-5).
Figure 2-5. Semi-quantitative RT-PCR of gene Mi-tra-1 and analysis of mRNA level, Mi-sdc-1 and housekeeping gene actin among different nematodes treated on dsRNA after 24, 48, 72 and 96 hours. Housekeeping genes were amplified for 30 cycles for all the three genes Mi-tra-1, Mi-sdc-1 and GFP whereas the genes Mi-tra-1 and Mi-sdc-1 were amplified for 35 cycles. Actin gene was transcribed equally among the different time periods. However, transcription of Mi-sdc-1 on dsRNA treated nematode was equal among all other nematode except those nematode treated with Mi-sdc-1 dsRNA. The intensity of transcription was highly reduced after 24 hours of treatment followed by gradual increase in transcription after 48 hours, 72 hours and 96 hours. Similarly, for gene Mi-tra-1, transcription of Mi-tra-1 on dsRNA treated nematodes was equal among all other nematodes except those nematodes treated with Mi-tra-1 dsRNA. The intensity of transcription was slightly reduced just after 24 hours of treatment followed by rapid reduction of transcription among 48, 72 and 96 hours.

In the first and second experiments, nematodes were soaked in a solution containing dsRNA of both genes Mi-tra-1 and Mi-sdc-1, and then subjected to investigation to check the durability of gene knock-down. After 24 hours soaking of nematode in dsRNA, the recovery of M. incognita was checked by soaking them in distilled water and then observing them under a microscope. The results showed that most of the nematodes that were soaked on dsRNA for 24 hours for all genes regained their normal sinusoidal movement. However, some of the nematodes in all the
treatments were either rigid with no movement, straight and still, or paralyzed with slower body movement (Figure 2-6).

![Image of nematodes](image)

**Figure 2-6.** RNAi phenotype observed under microscope after treating for 24 hours with dsRNA of *Mi-tra-1*, *Mi-sdc-1*, GFP and blank in the first experiment and additional dsRNA treatment of *Mi-tra-1* combined with *Mi-sdc-1* in the second experiment. A) Nematodes treated with blank treatment. B) Nematodes treated with GFP dsRNA, C) Nematodes treated with *Mi-sdc-1* dsRNA. D) Nematodes treated with *Mi-tra-1* dsRNA, and E) Nematode treated with combinatorial dsRNA of *Mi-tra-1* and *Mi-sdc-1*. All of the observation is taken in 10x magnification (AxioCam MRc 5, Zeiss).

No differences among treatments were observed in the sinusoidal movement of nematodes. This suggests that there was no exogenous toxicity to the nematode due to soaking and supply of dsRNA, and that observed changes in nematode phenotype is gene specific and is a result of gene silencing.
RNAi of *Mi-sdc-1* Results in Developmental Arrest of J3 and J4

Nematodes treated with *Mi-sdc-1* dsRNA resulted in 93% of population as third and fourth stage juveniles and 7% of population as early stage females, which was different from all other treatments (*P* ≤ 0.05). Nematodes treated with *Mi-tra-1* dsRNA resulted in 73% as third and fourth stage juveniles with 27% of nematodes as early adults. This result was not different from blank and GFP dsRNA treatments which had 68% third and fourth stage juveniles and 32% early adult, and 62% third and fourth stage juveniles and 38% of adults, respectively (Figure 2-7).

Figure 2-7. Effects of *in vitro* *Mi-tra-1* and *Mi-sdc-1* RNAi on the phenotype of *Meloidogyne incognita* in first experiment after 15 days. The number of nematodes in the plant roots at 15 days post infection are classified as dark grey and light blue. Dark grey represents the percentage of third and fourth stage juveniles and light blue indicates early stage females. Error bars indicate standard error.
RNAi of *Mi-tra-1* Results in Developmental Arrest of Mature Females

Thirty-five days after inoculation, roots were stained and different life stages of *M. incognita* were counted. The results showed that among the nematodes treated with *Mi-tra-1* dsRNA, 65% of females were in the early adult stage without eggs and 35% were females with eggs, different from other treatments (*P* ≤ 0.05). Nematodes treated with *Mi-sdc-1* dsRNA resulted 12% as females without eggs and 88% as females with eggs, which was not different from the dsRNA treated phenotype with GFP dsRNA and blank treatments which had 87% females with eggs and 13% females without eggs and 96% females with eggs and 3.8% of females without eggs, respectively (Figure 2-8).

![Figure 2-8](image-url)

Figure 2-8. Effects of *in vitro* Mi-tra-1 and Mi-sdc-1 RNAi on the phenotype of *Meloidogyne incognita* in first experiment after 35 days. Orange bars indicate the percentage of females without eggs. Error bars indicate standard error.
Combinatorial dsRNA treatment resulted in 84 % females without eggs, and 16 % females with eggs which is significantly different from GFP, *sdc-1*, *tra-1*, and blank treatments (*P* ≤ 0.05). Similarly, *Mi-tra-1* resulted in 54 % females without eggs and 46 % female with eggs which was significantly different from blank treatment (*P* ≤ 0.05). However, *Mi-sdc-1* resulted in 68 % females with eggs, which was not different from the dsRNA treated phenotype with GFP dsRNA and blank treatments (Figure 2-9).

Figure 2-9. Effects of in vitro *Mi-tra-1* and *Mi-sdc-1* RNAi on the phenotype of *Meloidogyne incognita* in second experiment after 35 days. Green bars indicate the percentage of females without eggs and blue bars indicate the percentage of females with eggs. Error bars indicate standard error.
CHAPTER 3  
DISCUSSION AND CONCLUSION  

Discussion  

This research was conducted to gain the initial understanding about the role of various orthologues of sex determining genes of *C. elegans* and *M. incognita*. Complementary DNA (cDNA) synthesis, and its predicted protein sequence, showed *Mi-tra-1* and *Mi-sdc-1* genes from *M. incognita* showed a high similarity with *tra-1* and *sdc-1* sequences from *C. elegans* suggesting these two genes have more or less common function. In addition, comparisons with homologous sequences from other parasitic nematodes affirms high degree of identity (≥88 %) with *M. floridensis* and *M. hapla* suggesting these genes can be potential target for different root-knot nematode species. 

Results of RNAi from our experiment has demonstrate that RNAi can be established in the sex determination pathway of *M. incognita*. Differences were seen among phenotypes soaked in different dsRNA solutions. Expression analysis of dsRNA of *Mi-tra-1* and *Mi-sdc-1* has indicated that the transcription level of these genes were drastically reduced in dsRNA treated nematodes, and that the reduction was gene specific. Similarly, the effect of silencing and specificity of RNAi was observed in phenotypes of the nematodes. 

Previous research results from *C. elegans* and *Pristionchus pacificus* have shown loss of function due to mutation or knockdown of *tra-1* that resulted in sex reversal from hermaphrodites to males (Pires-daSilva and Sommer, 2004). In our study, *M. incognita* sex reversal was not observed. However, delay in maturity of females and reduced number of females with egg masses was observed in comparison to nematodes in other treatments. This suggests that *tra-1* gene is important in the sex-
determination pathways of *M. incognita*. Nonetheless, the function of *Mi-tra-1* in *M. incognita* is less than observed in *C. elegans* though there is sequence similarity in *tra-1* gene between *M. incognita* and *C. elegans.

Gene *tra-1* in *M. incognita* is similar to C2H2 zinc finger motif–containing *tra-1* from *C. elegans*. On the other hand, there is vast differences in the sex determination pathway between *C. elegans* and *M. incognita* (Abad et al., 2008). In *M. incognita* the male sex determination pathway is also regulated by environmental factors. So, for better understanding of this pathway identification of other genes involved and detailed mechanism of sex determination are needed.

*Sdc-1* is a C2H2 type zinc finger protein encoding gene, and mutation of this gene causes defects in egg laying and vulval development. In *C. elegans* it is involved in hermaphrodite sex determination, which is basically expressed at a higher level in embryos and in adults. However, in *M. incognita* this gene is expressed throughout the lifecycle. Delay in transition of fourth stage nematode to female within the nematodes treated with *Mi-sdc-1* dsRNA suggests that this gene is necessary for the sex determination pathway. There were no differences in the phenotype after 35 days, which also indicates the presence of some other unknown genes or pathways which provide necessary signals for the development of females. In *C. elegans* SDC protein connects the sex determination and doses compensation, these protein link different gene-specific regulators controlling sex determination with the chromosome and wide process of doses compensation (Zarkower, 2006). However in *M. incognita* further studies needed to be carried out to understand the function of *Mi-sdc-1*.
Silencing effect of different genes after soaking is different in different nematodess and in different genes within the same nematodes. According to Urwin et al., (2002) durability of silencing effects on major sperm protein genes was recorded even after 14 days in cyst nematodes. Meanwhile, in the case of *Nippostrongylus brasiliensis* silencing lasted for 6 days in the acetylcholinesterase gene (Hussein et al., 2002). It is suggested that short recovery of the genes is a result of high transcriptional activity (Hussein et al., 2002; Urwin et al., 2002; Rosso et al., 2005). In our study the effect of silencing in the *Mi-tra-1* gene was prominent 4 days after silencing, while in case of *Mi-sdc-1* gene silencing was prominent for the first 3 days but it was relatively recovered on the fourth day. The reason behind difference in silencing between two genes may be the result of difference in transcriptional level and turnover rate.

Different RNAi studies in *M. incognita* and *G. pallida* have demonstrated that double stranded RNA derived from non-nematode double stranded RNAs induce profound phenotypic changes in nematodes (Dalzell et al., 2009). It has been reported that high doses of expressed short hairpin RNAs (shRNAs) cause mouse mortality in a dose dependent manner (Grimm et al., 2006). Additionally, sometimes there might be competition between endogenous RNAs and exogenous single interfering RNAs (siRNAs) and shRNAs for the RNAi machinery (Castanotto et al., 2007).

Optimal dose of RNAi is very crucial to effectively knockdown the target gene without causing non-specific inhibitory effect to the population (Lilley et al., 2012). In this study, no abnormal phenotype or difference in normal movement of nematodes was observed in nematodes incubated in dsRNA of *Mi-tra-1*, *Mi-sdc-1* and GFP for 24 hours.
at the concentration of 1µg/µl. Root-knot nematodes in all treatments showed similar sinusoidal movement as observed in the control incubated in water.

Several experiments of RNAi mechanism on *C. elegans* have indicated that some genes can be effectively silenced while others are resistant to RNAi. RNAi regulation is a complex process which involves multiple mechanisms. Different genes involved in regulation of RNAi in plant-parasitic nematodes has not been studied well. It can be anticipated that further enhancement of the efficacy of RNAi and persistence in plant-parasitic nematodes can only be achieved through detailed understanding of RNAi regulatory gene.

**Conclusion**

A better understanding of the RNAi regulatory pathway may aid in nematode management. Identifying the techniques and different components of RNAi mechanisms might enhance the efficacy and persistence of RNAi. An alternative solution to this can be achieved by feeding the nematodes with dsRNA produced by plants. Plants can be genetically modified to continuously produce and supply siRNA to the nematodes, which will result in long lasting RNAi effect in the nematodes. Basic local alignment search tool of nucleotide (BLASTn) sequence analysis has shown that *Mi-tra-1* and *Mi-sdc-1* is highly nematode specific, this minimizes the possibility of non-target effects on the plant or other organisms. Therefore, *Mi-tra-1* could be a potential target for RNAi mediated nematode control by reducing the number of females with eggs, or by increasing maturation time of females. This successful application of RNAi in *M. incognita* opens a new target for controlling different root-knot nematodes by targeting their sex determination pathway. To conclude, this study has demonstrated that *M. incognita* is readily susceptible to RNAi and the genes *Mi-tra-1* and *Mi-sdc-1* are evolutionary
conserved between the free living nematodes and the plant-parasitic *M. incognita*. In addition, RNAi effect on *M. incognita* is highly persistence.


Yadav, B. C., Veluthambi, K., and Subramaniam, K. 2006. Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Molecular and Biochemical Parasitology, 148(2), 219-222.


BIOGRAPHICAL SKETCH

Anil Babu Baniya was born in Gorkha, Nepal in 1989 to Laxmi Kumar Baniya and Hom Kumari Baniya. Mr. Baniya was home-schooled by his parents until beginning his undergraduate studies at the Institute of Agriculture and Animal Sciences (IAAS), Tribuvan University, Nepal in 2013. After which he worked at the Nepal Agriculture Research Council (NARC) as an Agronomist (Plant Breeder) until July 2015. In August of 2015 he was accepted into the graduate program at the University of Florida Entomology and Nematology Department to work on a master’s degree project in Nematology at the Nematode Assay Lab under the advising of Dr. Tesfa Mengistu.