Promotors

Prof. dr. Godelieve Gheysen
Department of Molecular Biotechnology
Faculty of Bioscience Engineering
Ghent University

Prof. dr. ir. Rony. Swennen
Divisionof Crop Biotechnics and Laboratory of Tropical Crop Improvement
Department of Biosystems
Faculty of Bioscience Engineering
KULeuven

Prof. dr. K. Subramaniam
Department of Biological sciences and Bioengineering,
Indian Institute of Technology, Kanpur, India

Dr. ir. Serge Remy
Divisionof Crop Biotechnics and Laboratory of Tropical Crop Improvement
Department of Biosystems
Faculty of Bioscience Engineering
KULeuven

Dean:

Prof. dr. ir. Guido Van Huylenbroeck (Ghent University)

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Rector:

Prof. dr. Paul Van Cauwenberge (Ghent University)

Prof. dr. Mark Waer (KULeuven)

Analysis of RNAi strategies against migratory parasitic nematodes of banana

Soumi Joseph

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Analyse van RNAi-strategieen tegen migratorische plantenparasitaire nematoden van banaan

Cover illustration:

A DNA helix-the storehouse of genetic information (*lejeuneusa.org*) and a graphical illustration of *in vitro* RNAi technique used in this thesis to generate knock down phenotype in *Pratylenchus coffeae.*

Printing

DCL Signs, Zelzate

Joseph, S. (2013). Analysis of RNAi strategies against migratory parasitic nematodes of banana

ISBN: 978-90-5989-625-3

This research was supported by VLIR-UOS ICP-PhD grant from the Flemish Interuniversity Council (VLIR).

Examination committee

Prof. dr. ir. Koen Dewettinck (**chairman**)
Department of Food Safety and Quality
Faculty of Bioscience Engineering
Ghent University

Prof. dr. ir. Guy Smagghe (**secretary**)
Laboratory of Agrozoology
Department of Crop Protection
Faculty of Bioscience Engineering
Ghent University

Prof. dr. Godelieve Gheysen (**Promotor**)
Department of Molecular Biotechnology
Faculty of Bioscience Engineering
Ghent University

Prof. dr. ir. Rony Swennen (**Promotor**)
Division of Crop Biotechnics and Laboratory of Tropical Crop Improvement
Faculty of Bioscience Engineering
Katholieke Universiteit Leuven

Dr. ir. Serge Remy (**Promotor**)
Division of Crop Biotechnics and Laboratory of Tropical Crop Improvement
Faculty of Bioscience Engineering
Katholieke Universiteit Leuven

Prof. dr. K. Subramaniam (**Promotor**)
Department of Biological Sciences and Bioengineering
Indian Institute of Technology,
Kanpur, India

Prof dr. John Jones
The James Hutton Institute, Invergowrie
Scotland UK

Prof. dr. ir. Bartel Vanholme
VIB Departement Planten Systeembiologie,
Ghent University

Dr. ir. Tina Kyndt
Dept. Molecular biotechnology
Faculty of Bioscience Engineering,
Ghent University

Dr. ir. Christine Vos
Centre of Microbial and Plant Genetics
Faculty of Bioscience Engineering
Katholieke Universiteit Leuven

Acknowledgements

*Always giving thanks to God the Father for everything, in the name of our Lord Jesus Christ (**Ephesians 5:20)**.*

I would like to express my deep and sincere gratitude to my promoter Prof. Godelieve Gheysen for her support throughout the period of my Ph.D. I am grateful for the patience, motivation and immense knowledge that she has bestowed on me throughout my study. I am also grateful to my co-promoters Prof. Rony Swennen and Dr. Serge Remy of Division of Crop Biotechnics and Laboratory of Tropical Crop Improvement, KU Leuven and Prof. K. Subramaniam of Indian Institute of Technology, India for their criticisms, stimulating suggestions and encouragement.

My warmest thanks goes to Drs. Annelies Haegeman, Tina Kyndt and Joachim Jacob for their immense support and assistance throughout this study. My appreciation also goes to the staff of Department of Molecular Biotechnology at Gent, Laboratory of Tropical Crop Improvement at Leuven and also Department of Biological sciences and Bio-engineering at IIT, India for their assistance in my laboratory and glasshouse experiments.

To my beloved husband, my lovely daughter and my dearest parents, I say thank you for your love and support. I also thank my dear friends in Gent and Leuven especially Shalet, Jijo, Leo proot, Joice and Nithya who have been a great support for me during my stay in Belgium. Finally I wish to thank VLIR-UOS for giving me the PhD student fellowship to pursue this study and the Indian Council of Agricultural Research for supporting plant nematode research at the K.S. laboratory through the NAIP scheme, Ghent University (BOF 01B00308) for financially supporting this project.

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List of Abbreviations

AGO	argonaute
ANOVA	analysis of variance
APN	animal parasitic nematode
AS	acetosyringone
ATP	adenosine triphosphate
BAP	benzylaminopurine
CBM	carbohydrate binding module
CCD	charge-coupled device
cDNA	complementary DNA
CTAB	cetyltrimethylammoniumbromide
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
dsRNA	double stranded RNA
DTT	dithiothreitol
ECS	embryonic cell suspension
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
FLN	free-living nematode
F-primer	forward primer
GFP	green fluorescent protein
GO	gene ontology
H	hour
HAC	hospital antiseptic solution
HGT	horizontal gene transfer
ID	identifier
KAAS	KEGG Automated Annotation Server
KEGG	kyoto encyclopaedia of genes and genomes
M	relative molecular mass
min	minutes
miRNA	micro RNA
mRNA	messenger ribonucleic acid

MS	murashige and skoog
MSR	modified strullu-romand
NCBI	national center for biotechnology information
NR	non redundant peptides
OD	optical density
PCN	potato cyst nematode
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPN	plant parasitic nematode
PROL	proliferation
PTS	post transcriptional silencing
RdRP	RNA dependent RNA polymerase
REG	regeneration
RISC	RNA induced silencing complex
RKN	root knot nematode
RNA	ribonucleic acid
RNAi	RNA interference
R-primer	reverse primer
RT-PCR	reverse transcription polymerase chain reaction
S	seconds
SCV	settled cell volume
siRNA	small interfering RNA
SRA	sequence read archive
SSC	saline-sodium citrate
SV	somaclonal variation
TAE	tris –acetate- EDTA
TRI	trizol
TS	transcriptional silencing
U	unit
USDA	United states department of agriculrure
YEP	yeast extract peptone
YM	yeast mold

CHAPTER ONE

General introduction

1.1 Nematodes

Nematodes, the most abundant Metazoa on earth have simple body plans which are usually (in approximately 99% of cases) elongate, cylindrical, and tapered at both ends (Decraemer and Hunt, 2006). The body is made up of a tube (the digestive tract) within another tube (the body wall) with the space between (the pseudocoelom) filled with a fluid, the hymolymph, that houses the reproductive system and other organs (Decraemer and Hunt, 2006). Despite their simple morphology, nematodes have been successful in colonising a wide range of environments. With simple changes in their body plan, nematodes have adapted to different habitats including the soil environment, oceans and freshwater lakes as well as rivers (Luc *et al*., 2005). They have also been found within animals (including other nematodes), algae, fungi and higher plants (Luc *et al*., 2005). The phylum Nematoda comprises >25,000 described species including free-living and parasitic species of plants and animals. Free-living species, which feed on bacteria and fungi, are found in soil, fresh water and marine habitats. The free-living species are considered as beneficial organisms since they are involved in nutrient turn over and are used as indicator species for pollution monitoring (Yeates *et al*., 2009) while the parasitic species affect human life by infecting a wide range of crops, livestock and by causing various human diseases.

1.2 Plant-parasitic nematodes (PPN)

A number of nematodes are highly damaging to plants including agronomic and vegetable crops, fruit and nut trees, turfgrass, and forest trees. This parasitic way of life according to Holterman *et al*. (2008) is thought to have evolved in an attempt to move away from an unpredictable environment for more stable conditions.

Some PPN have become very specialized and infect plants using a variety of strategies together with adaptation to feed on a variety of plant organs such as seeds and leaves although the majority of PPN species are restricted to plant roots (Bird and Koltai, 2000; Mbega and Nzogela, 2012). To successfully invade the host, PPN are equipped with stylets that are repeatedly thrusted into the host cell thus allowing feeding (Baldwin *et al*., 2004). Feeding by nematodes on the cytoplasm of the ruptured cells leads to a loss in turgor pressure and, where the nematode moves to another cell, this results in cell death and necrosis along the feeding path of the nematode (Wyss, 1997; De Waele and Elsen, 2002; Wyss, 2002). As an adaptation to plant parasitism, PPN have two sets of pharyngeal glands, dorsal and subventral glands, which are important for the production of a cocktail of proteins that are secreted through the stylet into the plant cells helping the nematode to establish within the host plant (Vanholme *et al*., 2004).

PPN are classified into groups based on their feeding strategies. Nematodes such as *Xiphinema americanum* that draw their nourishment from plant roots without physically entering the roots are referred to as ectoparasites. These nematodes have the ability to feed on different plants while but are more vulnerable to predators and unfavourable soil conditions (Decraemer and Hunt, 2006). Nematodes that actively invade the root system in order to feed are endoparasites. This group of nematodes can be further subdivided into three groups namely semi-endoparasites, migratory endoparasites and sedentary endoparasites (Chitwood and Chitwood, 1974; Decraemer and Hunt, 2006). Semi-endoparasites (*e.g*. *Rotylenchulus reniformis*) usually penetrate the plant roots with their head and form a permanent feeding site.

Sedentary nematodes such as the cyst nematodes (*Globodera* and *Heterodera*) and the root-knot nematodes (*Meloidogyne*) form elaborate nematode-specific (Sijmons *et al*., 1994) feeding structures consisting of modified root cells.

The infective second stage juveniles (J2) invade the root and inject secretions into selected cells of the host root to stimulate the formation of feeding sites (Wyss, 1997). The adult females remain sedentary and grow obese while males become vermiform again before maturity (Wyss, 2002).

Contrary to sedentary endo-parasitic nematodes, migratory endoparasitic nematodes are infective at all stages after hatching. They migrate inter- or intracellularly within the root sucking out the cytoplasmic content of the root cells, corms or tubers causing severe cell death (Wyss, 1997; 2002). The extensive wounds caused by these nematodes predispose their hosts to secondary infection by bacteria and fungi that further damage the root system (Zunke, 1991). Although these nematodes feed and reproduce primarily within the plant tissue, they are also able to move into the soil in search of new roots to invade. Examples of migratory endo-parasitic nematodes include *Pratylenchus* (lesion nematode), *Radopholus* (burrowing nematodes) and *Hirschmanniella* (rice root nematode).

1.2.1 Economic importance of PPN

PPN reduce crop yield by directly damaging plant cells through their feeding, by transferring viruses that damage plants or by indirectly providing access to the plant for other pathogens to invade through the wounds they create (Agrios, 2005). The total yield loss caused by PPN in agriculture on a global scale is estimated to be in excess of US $100 billion annually (Hooks *et al*., 2010).

There is, however, a disparity between the losses caused by PPN in the developing and the developed world with higher losses being reported in the developing world (Anwar and McKenry, 2012).

Yield losses due to PPN according to Hooks *et al.* (2010) may be higher in the tropics as opposed to temperate regions as a result of shorter nematode lifecycles and increased number of generations per year (Luc *et al*., 2005) and the lack of resources to combat infections (De Waele and Elsen, 2007).

Most of the economically important PPN are sedentary endo-parasitic nematodes (Wyss, 1997; 2002). Root-knot nematodes such as *Meloidogyne incognita* have a wide host range including over 2,000 plant species and they cause an estimated annual crop loss of 5% worldwide (Agrios, 1997; Hussey and Janssen, 2002). Yield loss to potato production as a result of *Globodera* spp is also estimated to be in excess of €300 million annually in Europe (Deliopoulos *et al*., 2007).

Most research on PPN have focused on the sedentary endoparasitic nematodes especially *Globodera spp*, *Meloidogyne spp* and *Heterodera spp* due to their prevalence in developed western countries as well as their economic importance in agriculture (Rosso *et al*., 2005; Lilley *et al*., 2012). This thesis, however, focuses on the migratory endo-parasitic nematodes, *Radopholus similis* (Cobb, 1893) Thorne, (1949) and *Pratylenchus coffeae* (Zimmerman, 1898) Filipjev & Schuurmans Stekhoven, (1941). These nematodes are widespread in the tropics and cause considerable losses to banana, a staple food for about 400 million people (Sundararaju, 2005).

1.3 Nematodes under study: *Radopholus similis*

1.3.1 Introduction

R. similis (Cobb) also known as the burrowing nematode is one of the most important PPN in the tropics (Haegeman *et al*., 2010). This nematode is known to cause extensive damage in banana (especially the Cavendish group) in the tropics (Jones, 2009) although it also parasitizes over 250 other plant species including many weeds,

black pepper, coconut, tea, tuber crops, fruit trees and ornamentals (Haegeman *et al*., 2010). It also causes pepper yellows of black pepper a disease that caused severe losses to the cultivation of black pepper on an Indonesian island in the early 1930s (Ramana and Eapen, 2000; Thorne, 1961). *R. similis* is native to Australasia and is currently found in tropical and subtropical regions around the world (Tan *et al*., 2010). It is believed to have spread through Africa, Asia, Australia, North and South America, and the Caribbean through the import of infested planting materials (O'Bannon, 1977, Trinh *et al*., 2004; Gowen *et al*., 2005).

1.3.2 Taxonomical position

R. similis is classified as belonging to Tylenchida (class Chromadorea) (Subbotin *et al*., 2006; Haegeman *et al*., 2010) with the genus *Radopholus* being classified under the family Pratylenchidae within the Tylenchoidea superfamily of the Rhabditid order of the phylum Nematoda (De Ley and Blaxter, 2002; Trinh *et al*., 2009). However, phylogenetic studies mainly based on rDNA sequences or the internal transcribed spacers (Bert *et al*., 2008; Holterman *et al*., 2009; Subbotin *et al*., 2006) have proven that *R. similis* is closely related to ectoparasitic and cyst-forming endoparasitic nematodes (Hoplolaimidae and Heteroderidae) (Figure 1.1)

Intraspecific variability of *R. similis* identified two physiological races based on host plant and cytogenetic differences; *R. citrophilus* which is responsible for the disease "spreading decline of citrus", and *R. similis* (the banana race), cosmopolite and polyphagous (Huettel *et al*., 1984). There is however gene flow between these two races, therefore, *R. similis* was reinstated as the valid species with two pathotypes, races or subspecies, *R. similis similis* and *R. similis citrophilus* (Elbadri *et al*., 2002; Kaplan and Oppermann, 1997; Kaplan *et al*., 1997, 2000; Valette *et al*., 1998).

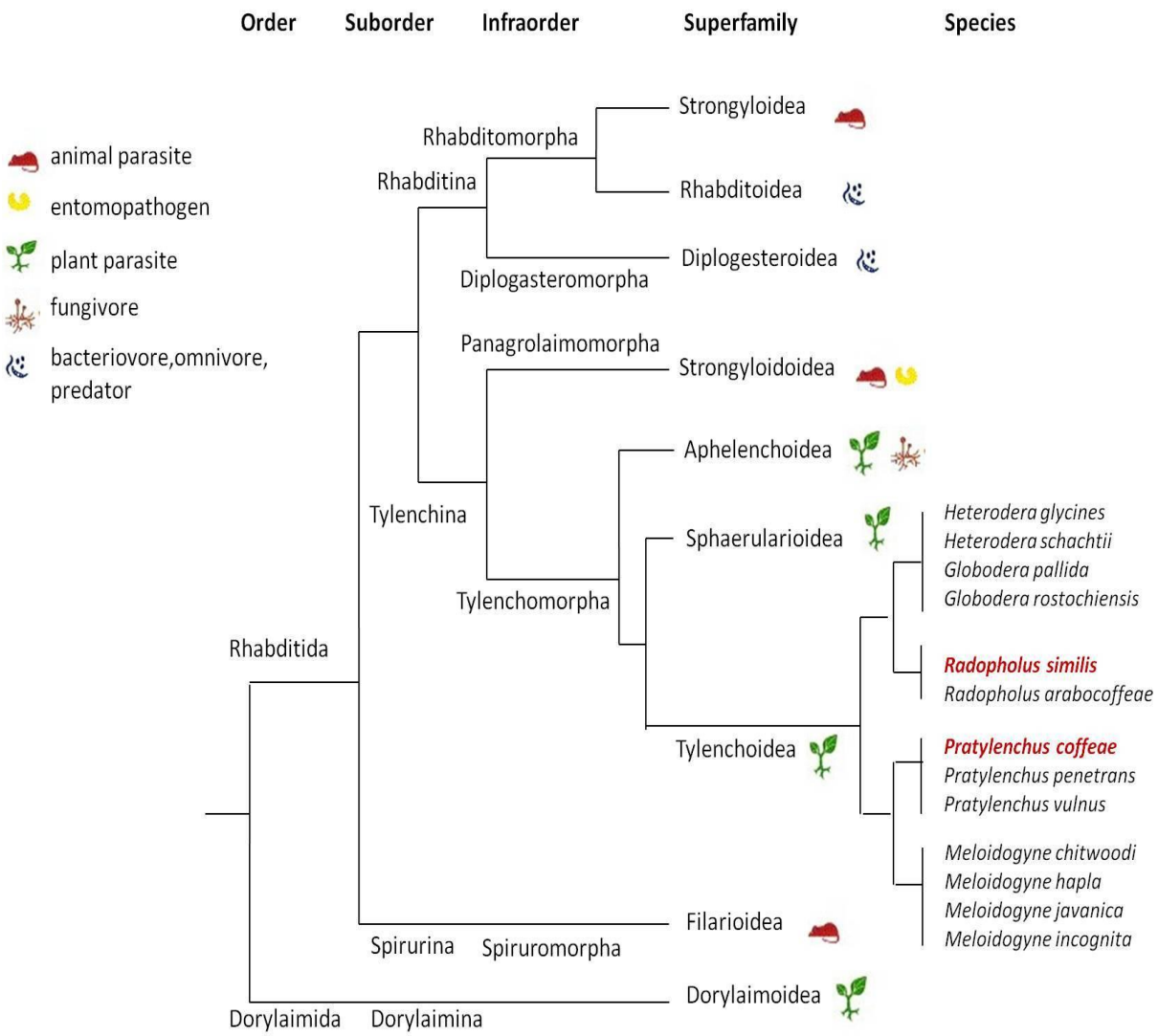

Figure 1.1. A phylogenetic tree (not drawn to scale) illustrating the taxonomical position of the two nematode species under study as inferred from Bert *et al*. (2008), De Ley and Blaxter (2002) and Holterman *et al*. (2009). While *R. similis* shows a close relationship with cyst-forming nematodes, *P. coffeae* shows a sister relationship with root-knot nematodes.

1.3.3 Morphological characteristics

The female nematode of *R. similis* is 650-800µm long and 20-24µm in diameter. The position of vulva is at approximately 54% of the body length from the head. The head morphology of the nematode is observed as rounded, slightly flattened and offset by a slight constriction with a support of sclerotized framework. The female nematode has a long (18µm), plainly visible stylet with prominent knobs. A dorsal overlapping of pharyngeal glands is observed in the nematodes. *R. similis* shows sexual dimorphism with the males, which 500-600µm long are being more slender than females. The head of the male nematode is well rounded and non-sclerotized and set off by a conspicuous constriction. In contrast to female nematodes, the males have a stylet, which is slender and indistinct (12µm) with small knobs.

1.3.4 Biology

With the exception of males, all motile stages of *R. similis* are infective. All developmental stages of the nematode are capable of entering the roots. They are usually located in the root cortex although they can also invade the stele in banana (Haegeman *et al*., 2010). The first stage juveniles (J1) moult within the egg into second stage juveniles (J2) which hatch from the egg. The hatched J2 locates a host root and penetrates it at the tender growing tip. Once inside the root, the J2 feeds and completes its life cycle by undergoing three further moults to become either an adult male or female (Figure 1.2) depending on the conditions (Trinh *et al*., 2004). The males, which have a rudimentary stylet, are not capable of invading roots or to cause damage to any extent.

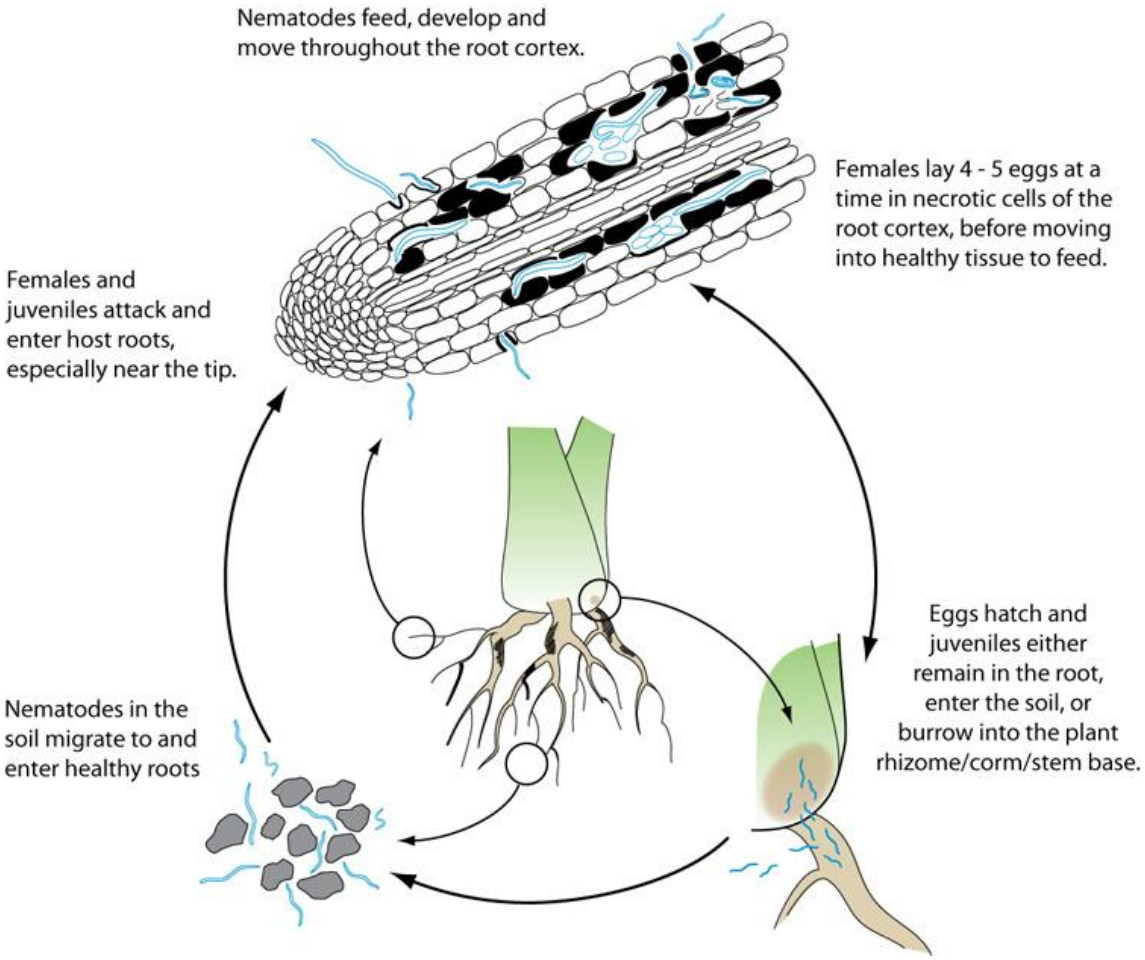

Figure 1.2. Life cycle of burrowing nematode, *Radopholus similis* (*www.apsnet.org/edcenter/intropp/lessons/Nematodes/Pages/Burrowingnematode.aspx*)

R. similis survives well in adverse conditions by adopting three strategies: 1) an extensive host range, 2) a short life cycle allowing rapid reproduction and 3) the ability of females to reproduce for one or two generations in the absence of males (MacGowan, 1977). According to Kaplan and Opperman (2000) this ability to reproduce in the absence of males is as a result of hermaphroditism rather than parthenogenesis. When the adult female does not find a mate within 60 days, self-fertilization occurs (Kaplan and Opperman, 2000) and eggs are laid predominantly within the host roots.

A female can lay an average of four eggs a day for up to two weeks (Haegeman *et al*., 2010). It takes 20 to 25 days for *R. similis* to complete a cycle from egg to egg at a temperature range of 25 to 32°C (Fallas and Sarah, 1995).

1.3.5 Symptoms and damage

Inter- and intracellular migration of the nematodes in the plant roots, feeding mainly on the cytoplasm of cortical cells, results in plant cell wall collapse forming cavities and tunnels, dark brown necrotic lesion in the cortex and nematode-filled spaces separating the stele from the cortex (Duncan and Moen, 2006; Haegeman *et al*., 2010). This is followed by secondary infections, mainly by *Fusarium oxysporum* and *Rhizoctonia solani* (Duncan and Moen, 2006; Haegeman *et al*., 2010.). Severe infection with the nematodes results in stunting and wilting of the host plant (known as the 'black head' disease in bananas). In severe cases, infection often leads to toppling of the plant due to the weakened stem base (Figure 1.3). These effects can cause massive crop losses in banana ranging from 5% to 75% (Price 2006; Haegeman *et al*., 2010). In the case of citrus trees, the infestation results in fewer and smaller leaves and more dead twigs due to reduced uptake of water and nutrients. *R. similis* is considered as one of the ten most damaging PPN world-wide (Munera *et al*., 2010). It is often called burrowing nematode.

R. similis is of great economic importance in the banana growing areas especially in Australia, Central and South America, Africa and Pacific and Carribean islands. As a result of devastating effects of *R. similis*, the European and Mediterranean Plant protection Organisation (EPPO) has declared the banana race as an A2 quarantine organism while the citrus race as A1 since the latter is not present in Europe (Haegeman *et al*., 2010).

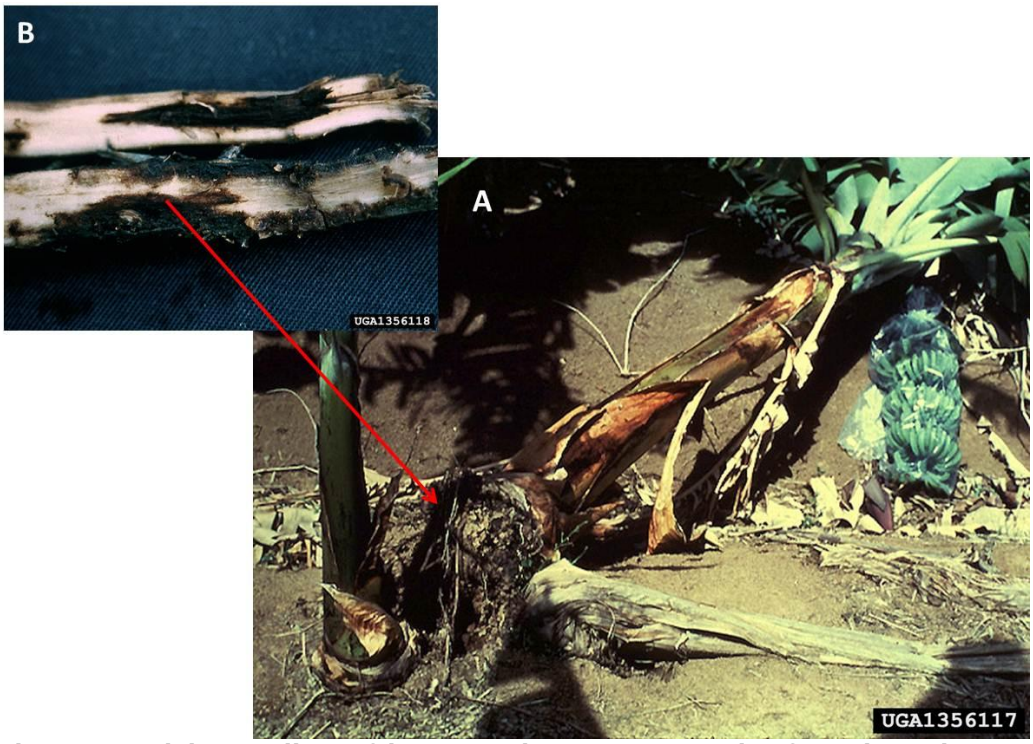

Figure 1.3. (A) Toppling of banana plants as a result of weakened root system caused by severe infection with *R. similis* and *P. coffeae*. (Nemapix Archive, Bugwood.org) (B) Dark brown necrotic lesions on banana roots caused by the migration of nematodes. (Michael McClure, University of Arizona, Bugwood.org)

1.3.6 Control measures

A major component of *R. similis* control is to reduce the initial nematode population before planting and use nematode free planting material. In this respect tissue culture-derived plants that are nematode-free offer a solution. Infected corms, suckers or seedlings can be cleaned prior to planting by superficially removing (paring) diseased tissue (Gowen and Quénéhervé, 1990; Speijer *et al*., 2001; Chabrier and Quénéhervé, 2008). Fallowing, crop rotation with non-host crops such as sweet potato or pineapple, and flooding can be other approaches to reduce the population.

The results of these practices are, however, often poor because in areas with permanent crops, cultural techniques like fallow, flooding or crop rotation are limited (Gowen and Quénéhervé, 1990).

Another drawback to the use of crop rotation is the broad host range of *R. similis* enabling it to survive in weeds (Quénéhervé *et al*., 2006) as well as its ability to survive in the absence of a host plant in the soil for more than six months without becoming anhydrobiotic (Chabrier *et al*., 2010). Application of nematicides is the primary way to control these nematodes since it can increase banana yield by approximately 50% relative to untreated controls (Fogain, 2000). The application of nematicides is, however, limited because they are expensive, detrimental to the environment and also there is a reduced availability of nematicides on the market due to increasing concerns about groundwater contamination and toxicity (Perry and Moens, 2006).

As an alternative ecologically-friendly approach, biological control agents such as arbuscular mycorrhizal fungi (AMF) have been widely used in the control of migratory nematodes (Vos *et al*., 2012; Koffi *et al*., 2012). Additionally, Atkinson *et al.,* (2004) showed that transformation of Cavendish banana with a rice cystatin, a nematode proteinase inhibitor, increased nematode resistance by 70%. Alternatively, screening for nematode resistance or tolerance in different banana cultivars is being performed (De Schutter *et al*., 2001; Dochez *et al*., 2006, 2009; Gaidashova *et al*., 2010). The introduction of resistance into commercial cultivars by conventional breeding (Dochez *et al*., 2009) has limited application in banana. Hence genetic engineering (Section 1.8) can be a promising approach for improved sustainability of banana production (Quénéhervé *et al*., 2009).

1.4 Nematode under study: *Pratylenchus coffeae*

The root lesion nematode, *P. coffeae* has an extremely wide host range and is also one of the major pests of banana.

Recent surveys in Africa have shown an increasing incidence of *P. coffeae* in West African regions and urgent need for developing resistant banana against these nematodes (Coyne, 2009).

In addition to banana, this nematode infects other crops such as abacá, yams, ginger, turmeric and coffee. *P. coffeae* is believed to have originated from the Pacific and Pacific Rim countries but it is distributed worldwide having been detected in tropical and subtropical regions (Bridge *et al*., 1997). It is considered a significant pest in Southeast Asia, Central and South America, South Africa and Ghana. However, *P. coffeae* is not found so commonly in commercial plantations of the Cavendish group, but is often reported in association with cultivars belonging to the plantain group (Luc *et al*., 1990; Bridge *et al*., 1997; Gowen, 2000).

1.4.1 Taxonomical position

P. coffeae was first identified from coffee roots in Java, Indonesia (Zimmerman, 1898; Filipjev and Schuurmans Stekhoven, 1941) and it was initially described as *Tylenchus coffeae*. The genus *Pratylenchus* was erected by Filipjev in 1936. In 1941, Filipjev and Schuurmans Stekhoven transferred *T. coffeae* to the genus *Pratylenchus.* According to Siddiqi (2000), *P. coffeae* is classified under Tylenchida (class Secernentea) with the genus *Pratylenchus* being grouped under the family Pratylenchidae within the Hoplolaimoidea superfamily of the Tylenchida order of the phylum Nematoda. However, De Ley and Blaxter, (2002) has classified *P. coffeae* as belonging to the family Pratylenchidae within the Tylenchoidea superfamily of the Rhabditid order of the phylum Nematoda. Recent phylogenetic studies (Bert *et al*., 2008; Holterman *et al*., 2009), have shown that a close phylogenetic relationship exist between *Meloidogyne* (root-knot nematodes) and *Pratylenchus* while *Radopholus* shows a sister-relationship with cyst-forming nematodes (Figure 1.1).

Thus, the migratory endoparasitic family, Pratylenchidae which includes *Radopholus* and *Pratylenchus* appears to be polyphyletic.

1.4.2 Morphological characteristics

The morphology of female nematode serves as the basis for the identification of *P. coffeae* since they possess more diagnostic features than males (Loof, 1991). The body length of the nematode ranges from 450 to 700µm. The stylet is short and stout (14µm) with well-developed basal knobs. The labial region is strongly sclerotized, low and flattened. The dorsal glands overlap the intestine ventrally with the vulva positioned posterior at 70-80% of body length. They are monoprodelphic (the genital system consists of a single, anteriorly directed tract). Female nematodes have a broadly rounded or indented tail tip while male nematodes have a short, convex-conoid tail.

1.4.3 Biology

P. coffeae is a migratory endoparasite with all stages found within the root cortex. The female nematodes and juveniles feed mainly on cortical cells leading to the formation of cavities containing nematodes of all stages and as a result black necrotic lesions are formed. The nematodes are commonly called root lesion nematodes because of the pronounced black lesions that form on the affected roots. The life cycle of *P. coffeae* is 27 days at temperatures ranging from 25 to 30°C (Loos, 1962; Agrios, 1997). From egg to adult, the nematode moults four times. The first moult takes place within the egg, the following three moults occur outside the egg (Figure 1.4). Eggs hatch in 6 to 8 days at 28-30^{o}C in water. These nematodes can survive in the absence of host plants for up to 24 months in anhydrobiotic state of suspended metabolic activity and survive in soil (Radewald *et al*., 1971; Castillo and Vovlas, 2007).

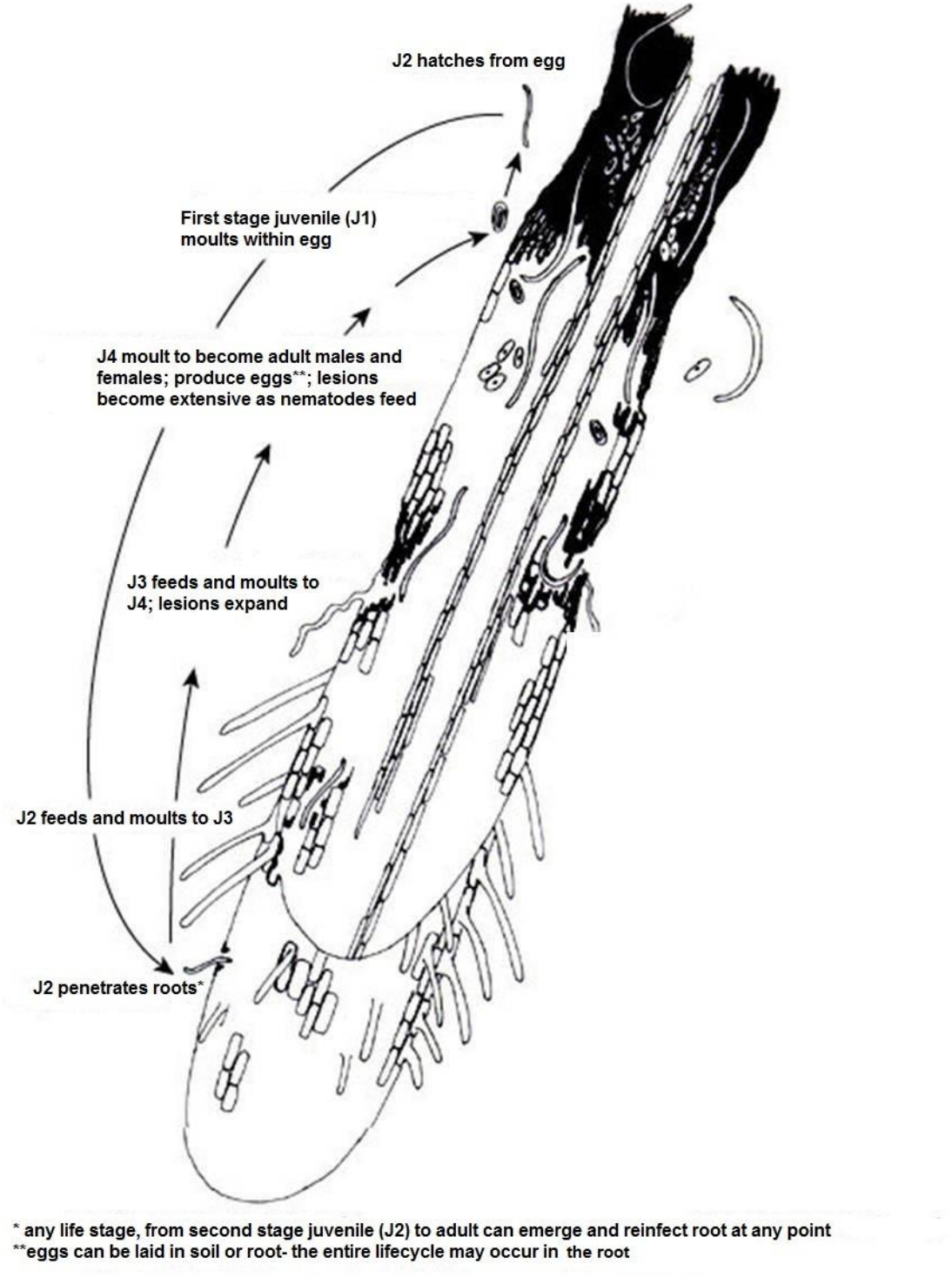

Figure 1.4. Life cycle of root-lesion nematode *Pratylenchus coffeae* (*www.apsnet.org/edcenter/intropp/lessons/Nematodes/Pages/LesionNematode.aspx*)

1.4.4 Symptoms and damage

All mobile stages of *P. coffeae* from the second-stage juvenile (J2) onwards may enter the roots and invade via all locations along the seedling tap roots, including root cap, apical meristem, region of elongation, region of maturation and mature tissues. When large numbers of the nematodes invade a root at a single location, both the epidermal and cortical tissues are destroyed, resulting in an exposed lesion extending to the stele tissues (Radewald *et al*., 1971). Males are necessary for reproduction and they continually migrate in and out of the roots destroying tissue in the same manner as migrating females and juveniles; however they do not form cavities (Radewald *et al*., 1971).

P. coffeae reduces the root's efficiency to absorb water and nutrients by damaging root cells during penetration and feeding (Vaast *et al*., 1998). Coffee roots infected with *P. coffeae* turn yellow then brown, they become stunted and plants have few and small chlorotic leaves. In the case of banana and plantain, *P. coffeae* causes purple or black necrotic lesions leading to the damage of inner cortex. These necrotic lesions enlarge and become more deeply coloured over time. Roots heavily infested with *P. coffeae* are often accompanied by secondary rotting and root breakage. The above ground symptoms of damage include stunting of plants, lengthening of the vegetative cycle, reduction in size and number of leaves and in bunch weight, reduction of the productive life of the plantation, and toppling (Figure 1.2) (Gowen *et al*., 2005). In yam *P. coffeae* causes dry rot disease (Bridge *et al*., 1996) while in the case of citrus it causes citrus slump by reducing tree vigour which in turn causes a serious decline of the trees and small fruit sizes (Tarjan and Tomerlin, 1973).

1.4.5 Control measures

The planting of nematode-free suckers is recommended to control *P. coffeae* infestation in banana. The removal of infected external tissue along with the adhering soil (paring) and treatment of suckers with nematicidal solution or hot water are recommended to obtain nematode-free suckers for planting. Using plantlets grown from meristem culture is a reliable solution to obtain nematode free banana plants as well as to avoid the toxic effects of nematicides. These nematodes are also controlled by other control strategies used for *R. similis* (Section 1.3.6). Resistance to *P. coffeae* has been assessed in different banana cultivars. Although different techniques for field screening of Musa germplasm have been evaluated against *P. coffeae*, very few sources of nematode resistance have been identified so far (Price *et al*. 1996; Tripathi *et al*., 2013).

1.5 Molecular biology of nematodes and their interaction with plants

Substantial progress has been made over the past few years in understanding nematode parasitism of plants with application of molecular tools. Genes that enable nematodes to parasitize plants have been identified and explored by several research groups using different molecular tools. Genes encoding different cell wall degrading enzymes from different species of nematodes including sedentary nematodes such as *M. incognita*, (Béré-Maileet *et al.,* 2000), *Globodera rostochiensis* (Smant *et al*., 1998), *G. tabacum* (Goellner *et al.*, 2000), *H. glycines* (Smant *et al*., 1998; Gao *et al*., 2004), *H. avenae* (Long *et al*., 2012), as well as migratory nematodes, such as *P. penetrans* and *P. coffeae* (Uehara *et al.,* 2001: Kyndt *et al*., 2008), *R. similis* (Haegeman *et al*., 2008), *B. xylophilus* (Kikuchi *et al*., 2004; Kikuchi *et al*., 2005; Kikuchi *et al.*, 2011) and *Rotylenchulus reniformis* (Wubben *et al.,* 2010a) have been identified and characterized.

The biology and genetics of the sophisticated interaction between sedentary nematodes and their host plants have been unravelled with the help of different tools in molecular biology (Gheysen and Fenoll, 2002; Abad *et al*., 2003; Davis *et al.*, 2008; Barcala *et al.*, 2010; Jaouannet *et al.,* 2012). Additionally, identification and characterization of genes involved in reproductive and developmental processes have become more feasible due to the molecular data generated for different species of nematodes including free-living and parasitic forms. Over 1,000,000 ESTs are now available from different species of nematodes (www.nematode.net). The complete genome of free-living nematodes such as *C. elegans* (*C. elegans* sequencing consortium, 1998), *C. briggsae* (Gupta and Sternberg, 2003) and *Pristionchus pacificus* (Dieterich *et al.,* 2008) as well as parasitic nematodes *Brugia malayi* (Ghedin *et al.,* 2007), *M. incognita* (Abad *et al.*, 2008) and *M. hapla* (Opperman *et al.,* 2008), *Trichinella spiralis* (Mitreva *et al*., 2011), *B. xylophilus* (Kikuchi *et al.,* 2011) have been generated.

In addition to the primary sequence information, the functional characterization of the genes involved in developmental processes has been extensively studied using, RNA interference (RNAi) (Fire *et al*., 1998; Elbashir *et al*., 2001; Silva *et al*., 2004; Perrimon *et al*., 2010; Sioud, 2011). In *C. elegans* this technique has been widely used to provide information on the possible function of more than 20,000 genes and the phenotypes resulting from the knockout of the genes have been well documented (Zhuang and Hunter, 2011a). Recently, the RNAi technique has been applied to parasitic nematodes to enrich the knowledge on developmental and parasitism genes. The application of RNAi in PPN has made significant progress in developing novel methods for PPN control. The basic mechanism of RNAi and its applications and limitations are described in this chapter.

1.6 RNA interference

RNA interference is an ancient self-defence mechanism of eukaryotic cells to combat infection by RNA viruses (Ruiz *et al*. 1998; Voinnet 2001) and transposons (Ketting *et al.* 1999; Tabara *et al*. 1999). Furthermore, it is also found to carry out numerous additional functions depending on the organism. It has been demonstrated that it eliminates defective mRNAs by degradation (Plasterk, 2002) and also tightly regulates protein levels in response to various environmental stimuli (McManus *et al.,* 2002). RNAi generally refers to a suppression of the expression of a target gene induced by double stranded RNA (dsRNA) that shares significant homology with the target gene. The RNA molecules that induce RNAi mainly belong to two small RNA classes produced by different types of genes: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Couzin, 2002). Although siRNAs are considered to be the main players in RNAi, miRNAs, which inhibit translation of RNA into protein, have been considered as one of important players in this machinery. miRNAs are considered to be vital and evolutionarily ancient components of regulators of different developemental genes in plants and animals (Lee *et al*., 2007; Moxon *et al*., 2008; Williams, 2008)

The discovery of RNAi, was nominated as the breakthrough of the year 2002 and won the Nobel Prize in 2006 and today, there is an explosion in its application that is revolutionizing different areas of research. Andy Fire, Craig Mello and their colleagues discovered the basic mechanism of RNAi in *C. elegans* and identified important characteristics of this new phenomenon (Fire *et al*., 1998): (a) it is induced by double-stranded RNA, (b) the effect of RNAi is systemic, and (c) RNAi is heritable.

The discovery of RNAi in *C. elegans* (Fire *et al.,* 1998) has been used as the basis for understanding RNAi in other organisms. RNAi is used mainly to unravel the functions of genes by switching them “off” at the post-transcriptional level. The genetic screens can only be realistically performed on organisms with certain characteristics such as the ability to breed under laboratory conditions, short life cycle and high fecundity (Montgomery, 2004). Although it often requires months or years of dedicated work to identify the mutated gene responsible for a specific phenotype, RNAi-based methods have become a ‘back-door entrance’ to reveal the biological functions of genes in the organisms that are intractable to traditional genetic manipulations due to its relative ease and remarkable potency when it is used as a reverse genetic tool (Montgomery, 2004).

The discovery of RNAi has brought new revolution in the research studies in a diverse set of organisms including trypanosomes, the fruit fly *Drosophila*, and many other animal as well as plant species and has also helped to understand the basic connections to post-transcriptional gene silencing (PTGS) in plants (Baulcombe, 2004) and fungi (Cogoni and Macino, 1997) as well as to the endogenous regulation by miRNAs (Ambros, 2001). The findings that dsRNA could trigger PTGS in *C. elegans* led to the engineering of tobacco and rice plants to synthesize dsRNA exhibiting gene silencing in the transgenic plants (Waterhouse *et al.* 1998). This proved that evolutionarily divergent organisms (plants and nematodes) respond to the presence of dsRNA in a similar manner.

With the increasing availability of the genome sequences of many species, RNAi can contribute to a more detailed understanding of complicated physiological processes, and also to the development of resistance against many pathogens including nematodes, insects as well as viruses.

1.6.1 Mechanism of RNAi

Research to identify and characterize the genes implicated in RNAi has been performed in *C. elegans* (Smardon *et al.* 2000 Calixto *et al.,* 2010; Gent *et al*., 2010; Avgousti *et al.,* 2012; Philips *et al.,* 2012), *Arabidopsis* (Mourrain *et al*. 2000; Dunoyer *et al.,* 2010; Wang *et al.,* 2010; Pontier *et al*., 2012), *N. crassa* (Li *et al*., 2010; Lee *et al.*, 2010), *Drosophila* (Lipardi and Paterson, 2009; Miyoshi *et al*., 2010), and mammals (Hannon and Rossi, 2004; González-González *et al.,* 2008; Grimm *et al.,* 2010). Generally, RNAi can be induced by a dsRNA source from outside the cell (exogenous RNAi) as well as from transcription of coding or noncoding genomic sequences within the cell (endogenous RNAi) (Grishok *et al.,* 2005; Grishok, 2012).

In the RNAi pathways, the “core” RNA-silencing response involves processing of the trigger dsRNA into smaller 21- to 25-bp (base pair) fragments with dinucleotide 3′ overhangs by an adenosine triphosphate (ATP)-dependent enzyme named Dicer. The products of Dicer activity are referred to as short interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Hammond, 2005; Ghildiyal and Samore, 2009) which serve as “guides” in bringing the nuclease machinery to the target mRNA. These siRNAs associate with a protein complex called the RNA-induced silencing complex (RISC). The siRNA is unwound by a helicase component of RISC to allow base pairing between the antisense strand and the target mRNA (Zamore *et al*., 2000; Kaya and Doudna, 2012) leading to endonucleolytic cleavage of the target mRNA. Following cleavage, the siRNA/RISC complex becomes available to target another messenger molecule. Thus, the initial trigger dsRNA generates several siRNAs, each of which recruits and activates a RISC, which together may function catalytically to target multiple mRNAs. Dicer and RISC are some of the most evolutionarily conserved components of the RNA silencing machinery. It has been evident from recent studies that, at least in some species, additional amplification may occur.

Studies in *C. elegans* have shown that robust silencing of target RNAs is supplemented through the action of RdRPs that amplify primary silencing signals with the generation of secondary siRNAs (transitive RNAi) which show homology upstream of the primary dsRNA sequence (Sijen *et al*., 2001; Lipardi *et al*., 2001; Simmer *et al*., 2002; Tijsterman *et al*., 2002; Alder *et al*., 2003; Gent *et al*., 2009; Vasale *et al*., 2010).

Further studies in plants have revealed that transitive RNAi proceeds in both 5'-3'or 3'-5' directions, pointing out that aberrant mRNAs from altered chromatin structures serve as substrates for RdRPs. However, in flies and mammals, no cellular RdRP for the generation of secondary siRNAs has been found (Stein *et al*. 2003; Duan *et al*., 2012). Additionally, it has recently been discovered that a new class of endogenous small RNAs are generated in flies and mammals by a pathway independent of RdRP amplification (Ghildiyal *et al*., 2008; Lau *et al*., 2009; Crombach and Hogeweg, 2011). Thus different mechanisms have apparently evolved in different species for amplification of the silencing effect.

The endogenous pathways share the core components of RNAi pathway such as Dicer with exogenous RNAi pathway. However, both pathways differ in the requirement of more specialized factors associated with the production of primary and secondary siRNAs such as Argonaute proteins bound with primary siRNAs and RdRps required for the amplification of secondary siRNAs (Yigit *et al*., 2006; Gent *et al*., 2010; Vasale *et al*., 2010). It has been shown that multiple and complex endogenous silencing pathways exist in *C. elegans* (Lee *et al*., 2006). In the following section of this chapter the core pathways of exogenous RNAi is described in detail.

1.6.1.1 Classical exogenous RNAi pathway

Genome wide RNAi based screens have been performed in *C. elegans* by introducing dsRNA into the nematodes through feeding with bacteria expressing dsRNA (Timmons and Fire, 1998) and examining the phenotypes in the next generations. This allowed large scale screens for RNAi deficient mutants giving more insight on basic mechanics underlying the RNAi pathway. The investigation on how siRNAs are generated from large dsRNA molecules led to the discovery of the first RNAi deficient (*rde*) pathway mutant in *C. elegans* (Tabara *et al*., 1999).

Two of these genes, *rde- 1* and *rde-4* are essential components acting in the first step of RNAi. *Rde-4* encodes a dsRNA binding protein which promotes the specific recognition of foreign dsRNA whereas *rde-1* encodes a PAZ-PIWI/Argonaute protein (Song *et al*., 2004). When a long dsRNA (>100bp) is introduced into a cell exogenously (Figure 1.5, step 1), it is bound by the protein complex that contains RDE-4 and the Dicer, DCR-1 (Park and Fire, 2007; Habig *et al*., 2008) (Figure 1.5, step 2). The RDE-4/DCR-1 complex also interacts with two Dicer-related helicases DRH-1 and -2 (Duchaine *et al*., 2006). The double stranded siRNA generated by the Dicer activity has a 5'monophosphate on each strand and a free 3' hydroxyl group and an overhang with 2 nucleotides at 3'end of the strand (Macrae *et al*., 2006). The Argonaute protein RDE-1 binds to the double-strand siRNA produced by the DCR-1 complex and cleaves the passenger strand to produce a single-stranded guide siRNA (Figure 1.5, step 3 to 5), so called primary siRNAs (Parrish and Fire, 2001; Tomari *et al*., 2004; Steiner *et al*., 2009; Czech and Hannon, 2011).

The guide siRNA bound by RDE-1 identifies cognate mRNA and cleaves the target mRNA (Figure 1.5, step 6).

In the amplification step of primary siRNA pools, the RNA-dependent RNA polymerases (RdRps) generate many copies of secondary siRNAs from the targeted mRNA-siRNA complex (Figure 1.5, step 7 to 9). These secondary siRNAs are found to be anti-sense to the cognate mRNA and distributed towards the 5′ end of the target mRNA (Alder *et al*., 2003; Pak and Fire, 2007; Sijen *et al*., 2007; Pak *et al*., 2012). The amplification of siRNA is mediated by the putative RdRps, RRF-1 and EGO-1 in soma and germline, respectively, in *C. elegans* (Sijen *et al*., 2001; Fischer, 2010; Zhuang and Hunter, 2011a). In contrast to primary siRNAs, secondary siRNAs have a triphosphate, which is a characteristic of RdRP activity (Pak and Fire, 2007; Sijen *et al*., 2007).

The secondary siRNAs are found to be more abundant than primary siRNAs and they interact with so-called secondary Argonautes (SAGOs) (Yigit *et al*., 2006). It has been observed that these secondary siRNA-SAGO complexes are directly involved in sequence-dependent mRNA degradation (Figure 1.5, step 10a and 11a). However, Dalzell *et al*. (2011) pointed out that the SAGOs are poorly conserved in other species of nematodes possibly providing a reason why *C. elegans* RNAi is so efficient compared to that of other parasitic nematodes. In *C. elegans* the silencing events are found to be heritable and it was found that the AGO, NRDE-3 is responsible for nuclear translocation of RNAi triggers in *C. elegans*, and is involved in processes which lead to heritability of gene silencing events (Gu *et al*., 2012; Zhuang *et al*., 2013) (Figure 1.5). The NRDE-3 shuttles the secondary siRNAs to nucleus (Figure 1.5, step10b) and interacts with a complex of nuclear RNAi-silencing effector, NRDE-2 inside the nucleus inducing transcriptional gene silencing (Figure 1.5, step11b) (Guang *et al*., 2010; Burton *et al*., 2011; Zhuang *et al*., 2013).

This nuclear RNAi complex is guided by the siRNA to the nascent transcripts and prevents RNA polymerase elongation and initiates histone methyltransferase activity (Guang *et al*., 2010; Burton *et al*., 2011).

This mechanism induces heterochromatin modifications and other transcriptional gene-silencing phenomena linked to RNAi (Grishok *et al*., 2005; Claycomb *et al*., 2009; Burton *et al*., 2011). Similar to the soma restricted NRDE-3, a germline specific nuclear Argonaute, HRDE-1 has recently been identified and it has been shown that it is essential for multi-generational silencing (Buckley *et al*., 2012).

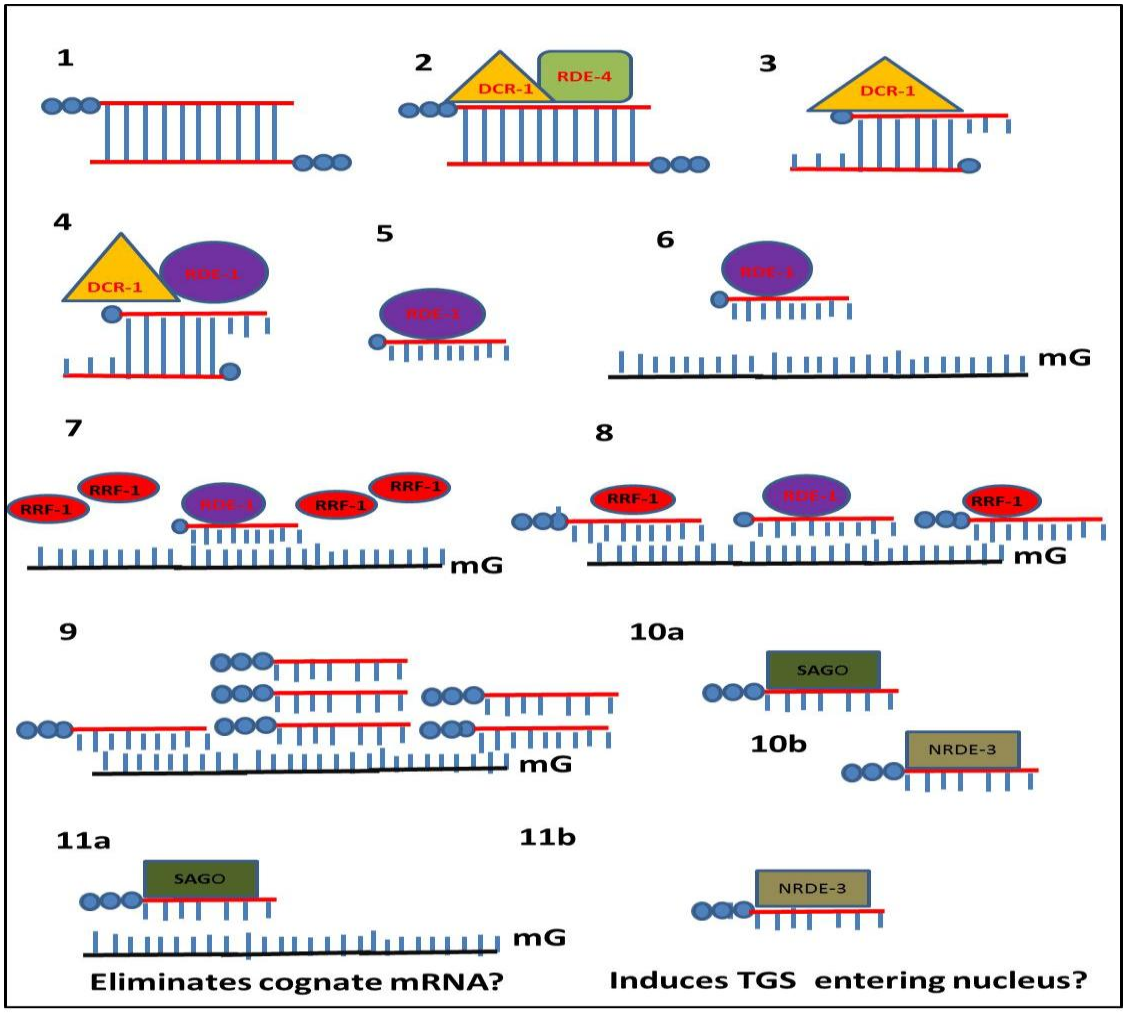

Figure 1.5. Schematic representation of classical exogenous RNAi pathway in *C. elegans* as inferred from Zhuang and Hunter (2011a). (1) *In vitro* synthesized long (>100 bp) dsRNA (red) with 5′ triphosphate (blue) ends is (2) bound by the *RDE-4* (green) and *DCR-1* (yellow) complex. (3) The endonuclease *DCR-1* dices the long dsRNA into of -20 bp ds-siRNAs. (4) Interaction with the Argonaute *RDE-1* (purple) leads to slicing of the passenger strand producing (5) a single-stranded -22 nucleotide guide siRNA bound to *RDE-1*. (6) This primary ss-siRNA guides *RDE-1* to its cognate mRNA (black). (7) In a mechanistically unclear step, the RdRP RRF-1 (red) is recruited to the *RDE-1*-siRNA-mRNA complex (8) leading to the production of many unprimed secondary siRNAs with 5′triphosphate ends. (9) Amplification of the secondary siRNAs, which are anti-sense to regions both 5′ and 3′ to the originally introduced long dsRNA. (10a), the secondary siRNAs become associated with cytoplasmic secondary Argonautes (SAGOs – olive green) via a mechanistically unclear step or (10b) the nuclear localized Argonaute NRDE-3 (tan). (11a). The secondary siRNAs then guide the cytoplasmic SAGOs to cognate mRNAs and via yet another an unkown mechanism lead to the elimination of the mRNAs. (11b) NRDE-3 shuttles the secondary siRNAs into the nucleus where they guide transcriptional gene silencing (TGS) processes. (Zhuang and Hunter, 2011a)

In addition to the key components described above, several other genes required for RNAi have been analysed by genetic screens in *C. elegans*. It has been shown that the complex containing MUT-7, a putative exoribonuclease and RDE-2/MUT-8, which acts downstream of RDE-1 and RDE-4, is required for siRNA accumulation *in vivo* (Tops *et al*., 2005; Fischer, 2010). Similarly, another essential gene, *rde-3/mut2*, encoding a polymerase-beta nucleotidyltransferase, is also required for siRNA accumulation (Chen *et al*., 2005a). In addition to this, it has also been reported by Yang *et al*., (2012) that a RDE-10/RDE-11 complex is also critical for amplifying the exogenous RNAi response in *C. elegans*. Several other genes which act downstream of secondary siRNA amplification such as *mut -7* are found to be essential for germline RNAi while others like *mut-14, -15 and -16* are required for both somatic and germline RNAi (Ketting *et al*., 1999; Tijsterman *et al*., 2002; Vastenhouw *et al*., 2003; Fischer, 2010).

RNAi screens in *C. elegans* have also identified several chromatin factors required for RNAi. These include *zfp-1, gfl-1*, encoding the proteins homologus to human GAs41 and AF10, *mes-3; mes-4 and mes-6*, encoding the chromatin binding polycomb-group proteins (Dudley *et al*., 2002; Grishok *et al*., 2005). Additionally, another nuclear RNAi component, *mrg-1*, encoding a chromodomain-containing protein, was also identified as a factor essential for persistence of RNAi over multiple generations (Vastenhouw *et al*., 2006; Fischer, 2010). Zhuang *et al* (2013) have recently identified PGL-1, another RNAi pathway component, which acts in parallel to NRDE-3 in nuclear RNAi.

1.6.1.2 Systemic RNAi

Although the basic RNAi machinery is found in a wide set of organisms, a variation in their ability to take up foreign dsRNA and use it in the RNAi pathway has been observed. The effects of RNAi can be both systemic and heritable in plants (Chuang and Meyerowitz, 2000) and *C. elegans,* but not in *Drosophila* or mammals (McEwan *et al.*, 2012). Fire *et al.* (1998) have demonstrated that RNAi is more efficient in *C. elegans* when dsRNA is injected into the intestine. The RNAi effect induced by the injection into the intestine spreads efficiently to most of the cells including the germline and is inherited by the progeny (Fire *et al*., 1998). The transmembrane proteins SID-1 and SID-2 (systemic RNAi defective) are essential components required independently for ingestion-mediated RNAi. It was shown that SID-1 is required for the uptake of silencing triggers into all cells and is expressed in the cells with direct environmental contact (Winston *et al*., 2002). It has been shown that SID-1 is required for the import but not the export of RNAi triggers (Jose *et al*., 2009). It was also found that in *C. elegans*, *sid-1* is expressed from the late embryo throughout adulthood in all non-neuronal tissues (Winston *et al*., 2002). SID-2 is less well characterized and is strongly expressed in the intestine (Britton and Murray, 2006)

In *C. elegans* neuronal cells are found to be resistant to RNAi triggered by ingested or injected dsRNA, but sensitive to neuronally expressed dsRNA, which indicates that the limiting factor for neuronal RNAi is the defect in the delivery of dsRNA to neurons. Additionally, Calixto *et al.* (2010) have shown that the transgenic expression of SID-1 in neurons enables efficient systemic RNAi. Thus the expression of SID-1 is one of the key factors for systemic RNAi in *C. elegans*. However, SID-1 alone is not sufficient to induce RNAi mediated by ingested dsRNA.

The single-pass transmembrane protein, SID-2 is also required. SID-2 is expressed exclusively in the intestinal cells and localizes strongly to the apical membrane and is required to import the ingested dsRNA from the intestinal lumen (McEwan *et al*., 2012). While SID-1 homologs were identified in mouse and human, SID-2 homologs were found only in *Caenorhabditis* nematodes. SID-2 homologs are, however, highly divergent among *Caenorhabditis* species (Winston *et al*., 2007).

Although *C. briggsae* expresses and localizes Cb-SID-2, it is unable to induce RNAi mediated by the ingested dsRNA. However, the transgenic expression of Ce-SID-2 in *C. briggsae* enables environmental RNAi suggesting either expression and/or functional differences between these two gene homologs. Additionally, McEwan *et al.* (2012) demonstrated that the expression of SID-2 in S2 cells enables the dsRNA uptake in *Drosophila*. It has also been shown that an acidic extracellular environment is required for SID-2 dependent dsRNA transport, which selectively transports dsRNA with at least 50 base pairs (McEwan *et al*., 2012). The dsRNA transporters, SID-1 and-2, act at two distinct steps; first, the ingested dsRNA is transported from the acidic intestinal lumen by SID-2 via endocytosis and in the second step, SID-1 brings these dsRNA from the internalized vesicles into the cytoplasm either by transporting directly from the vesicles or by transporting them from the body cavity space following the exocytosis of the vesicularized dsRNA (Figure 1.6). Thus both proteins, SID-1 and SID-2 function together either cooperatively or sequentially, to import ingested dsRNA (Winston *et al*., 2007; McEwan *et al*., 2012).

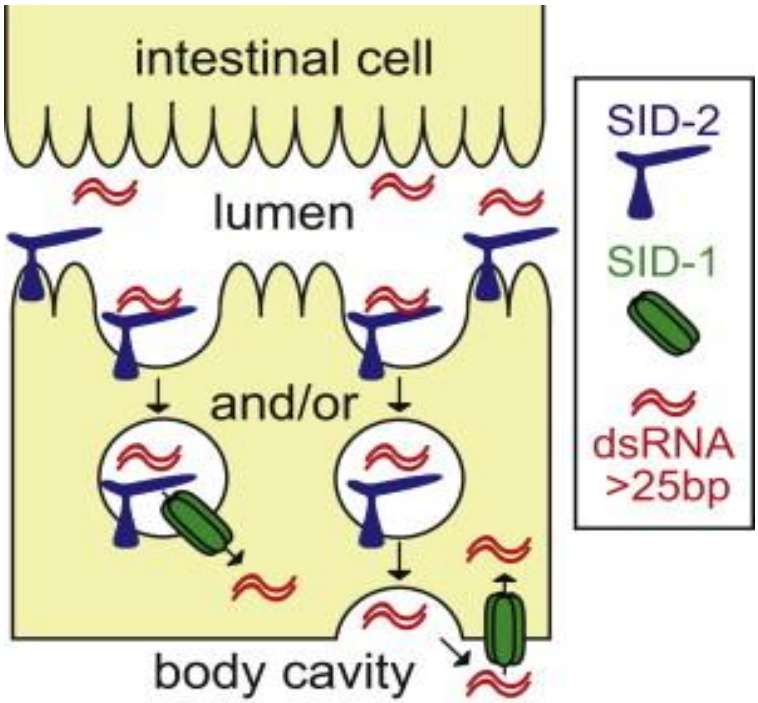

Figure 1.6. A Model of coordinated role of SID-2 and SID-1 in dsRNA uptake in *C. elegans*. Uptake of Ingested dsRNA from the intestinal lumenal space is mediated by SID-2.and the dsRNA retained in the vesicle is directly transported into the cytoplasm by SID-1 (left) or released in the pseudocoelomic fluid and imported to cells via SID-1 (right) (McEwan *et al*., 2012)

1.6.1.3 Regulators of exogenous RNAi

A number of proteins that directly or indirectly inhibit the RNAi process has been identified by RNAi screens in *C. elegans*. Mutations affecting such factors lead to enhanced RNAi in the mutant backgrounds and hence these genes are called Enhanced RNAi (*Eri*) genes.

It has been demonstrated that mutants of *rrf-3,* a gene encoding a putative RdRP, display increased sensitivity of several genes including neuronal genes to RNAi in *C. elegans* (Sijen *et al*., 2001*;* Simmer *et al*, 2002) which suggests that RRF-3 functions directly or indirectly to inhibit RNAi.

Kennedy *et al.,* (2004) identified *eri-1 C. elegans* mutants with enhanced sensitivity to RNAi in the nervous system. ERI-1 is a member of the DEMDh exonuclease subfamily, which belonging to the DEDDh family of exonucleases and it contains a SAP domain found in DNA binding proteins and also a DEDDh-like 3'- 5' exonuclease domain (Kennedy *et al*., 2004). Kennedy *et al* (2004) hypothesized that ERI-1 could normally act as an RNAi inhibitor that reduces the silencing effect. Gabel and Ruvkun (2008) demonstrated that ERI-1 is a conserved rRNA processing component that mediates 3' end maturation of the 5.8S ribosomal RNA (rRNA) in *C. elegans*. But one of the isoforms of ERI-1, which has an extended nematode-specific C-terminal sequence, mediates siRNA production and the association of the *C. elegans* Dicer ortholog, DCR-1, with a large complex that co-fractionates with the ribosome (Gabel and Ruvkun, 2008).

There are nine *Eri* loci that have been identified so far of which five are widely conserved genes (Duchaine *et al*., 2006; Fischer *et al*., 2008; Pavelec *et al*., 2009). These genes are mainly involved in the production or stability of siRNAs (Asikainen *et al*. 2007). Pavelec *et al*. (2009) grouped *Eri* genes in to; class I and class II (Table 2) on the basis of the presence or absence of germline pleiotropy. Class I proteins (ERI-1, ERI-3, RRF-3, and DCR-1(mg375Eri)) form a core ERI/Dicer complex that is required for endogenous RNAi in both the soma and germline, whereas the class II proteins (ERGO-1 and ERI-9) serve as accessory factors that modify core complex activity in tissues other than the male germline (Pavelec *et al*., 2009). Thus it is possible that the *Eri* genes may have distinct biological functions.

Additionally, it has been observed that a number of endogenous siRNAs is reduced in the *Eri* mutants of *C. elegans* while the availability of limiting components of silencing pathway such as secondary AGOs (Yigit *et al*., 2006), Dicer (Mikuma *et al*., 2004), and even the dsRNA channel SID-1 (Winston *et al*., 2002; Calixto *et al*., 2010) to exogenous siRNA is increased. Hence the current model on the mechanism of *eri* phenotypes proposes that the relatively abundant endogenous siRNAs compete with siRNAs produced from experimentally introduced dsRNA for limiting effector components of RNAi pathway (Lee *et al*., 2006; Yigit *et al*., 2006; Zhuang and Hunter, 2011a).

Although ERI-1 (Kennedy *et al*., 2004) and RRF-3 (Sijen *et al*., 2001); the DCR-1/ERI-4, (Pavelec *et al*., 2009); ERI-6/7 (Fischer *et al*. 2008); and ERGO-1/ERI-8 (Pavelec *et al*. 2009) are widely conserved among different organisms, ERI-3, ERI-9 and ERI-11 were found to be specific to *Caenorhabditis*. However, ERI-5 has shown a good conservation among nematodes (Table 1.1). Interestingly, among the Eri class of genes, only ERI-1 has been found to be well conserved among the PPN (Dalzell *et al*., 2011).

Additionally, the mutations in some members of the lin-35/Rb pathway (Table 1.1), which is involved in many cellular processes and developmental steps, have shown enhanced sensitivity to RNAi (Lu *et al*., 1998; Ceron *et al*., 2007). In *C. elegans*, mutation of this class of genes displays multiple vulva phenotypes (Ferguson and Horvitz, 1989). It has been shown that single mutants for lin-35/Rb, *lin-53* (homolog of the mammalian chromatin modifying complex subunit, RbAp48) and *dpl-1* (homolog of the mammalian transcription factor, DP) all display enhanced RNAi (Wang *et al*., 2005; Lehner *et al*., 2006; Ceron *et al*., 2007).

Grishok *et al*., (2008) demonstrated that loss of lin-35/Rb results in broad mis-regulation of endo-siRNA targets in particular, some Argonaute genes that function in exo-RNAi were upregulated. Thus, consistent with other *eri* mutants, RNAi hypersensitivity of lin-35/Rb mutants could be due to increased expression of RNAi factors and reduced competition with the endogenous pathway.

Table 1.1. Genes with enhanced RNAi phenotypes

Gene	Gene product	Other phenotypes
eri-1	Exonuclease	*T.S sterile at 25 °C X-chromosome non-disjunction
eri-2/rrf-3	RNA-directed RNA polymerase	T.S sterile at 25 °C X-chromosome non-disjunction
eri-3	Hydrolase	T.S sterile at 25 °C X-chromosome non-disjunction
eri-4/dcr-1	Helicase domain protein of *DCR-1*	T.S sterile at 25 °C Weak eri phenotype
eri-5	Tudor domain protein	Germline-specific Eri phenotype
eri-6/7	Helicase	None reported
eri-8/ergo-1	Argonaute	None reported
eri-9	RNA transferase	None reported
eri-11	Oligosaccharyl transferase	None reported
lin-35	Retinoblastoma homolog	T. S sterile and embryonic lethal, T.S arrested development, synthetic multivulva
lin-15B, dpl-1, lin-53, lin-9, lin-13, hpl-2	Syn muv B genes	Synthetic multivulva
mir-35–41	miRNA	T.S embryonic lethal

**T.S indicates temperature sensitive*

In addition to the *Eri* class of genes, it has recently been observed that some microRNAs (miRNAs), a class of small RNAs which act as regulators of endogenous genes, can also negatively regulate the exogenous RNAi pathway. The first miRNAs, *lin-4* and *let-7* were identified as endogenous regulator genes of the heterochronic pathway responsible for timing of developmental events in *C. elegans* (Ambros, 2001). At present, a large number of miRNAs have been reported in a variety of organisms, ranging from nematodes and plants to mammals (Carthew and Sontheimer, 2009).

Massirer *et al.* (2012) discovered that the mutant strain of *miR-35–41(gk262)* was hypersensitive to RNAi (Table 1.1). RNAi of *unc-22* resulted in paralysis in the *miR-35–41(gk262)* mutant background in contrast to the weaker twitching phenotype in wild-type nematodes.

Although the expression of *miR-35–41* appears to be restricted to embryos, the enhanced RNAi sensitivity has been observed in multiple tissues and different stages of development in the absence of the *miR-35–41* (Alvarez-Saavedra *et al*., 2010). It has been assumed that the cross regulation of exo-RNAi pathway and miRNA pathway is possibly due to the liberation of Dicer allowing more effective RNAi in the absence of *miR 35-41* since Dicer is the only factor described so far to be broadly required for siRNA and miRNA biogenesis (Massirer and Pasquinelli, 2013). An extensive misregulation of endo-RNAi has been observed in the mutants of *miR-35–41* (Massirer *et al*., 2012). Thus it is possible that a complex cross regulation may exist among small RNAi pathways.

MicroRNAs, experimentally introduced double stranded RNAs (dsRNA), and endogenous short interfering RNAs (endo-siRNAs) were proposed to compete for limiting shared resources, including the single *C. elegans* Dicer homolog, DCR-1, the RNA-directed RNA polymerase RRF-1, and the secondary Argonautes (SAGOs), including the *C. elegans* specific worm Argonautes (WAGOs) that mediated siRNA-dependent silencing (Figure 1.7). The competition for limiting shared resources implies that reduced flux through one pathway allows for increased access to limiting resources for the other pathways (Zhuang and Hunter, 2012).

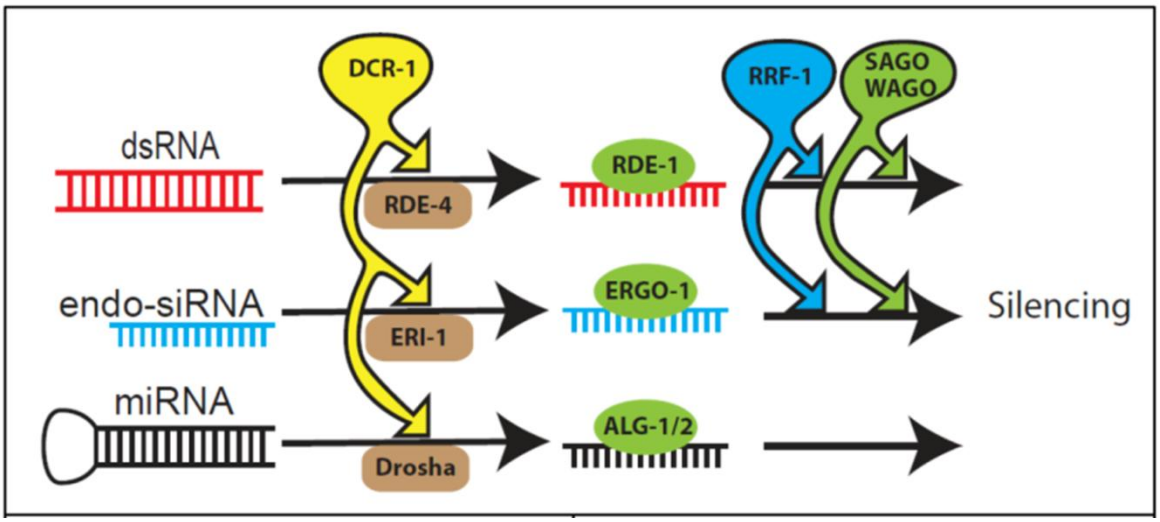

Figure 1.7. Scheme of proposed competing small RNA pathways (Zhuang and Hunter, 2012)

Although the basal RNAi machinery is conserved among different organisms, variability in RNAi efficacy has been observed (Echeverri *et al*., 2006). This can be due to biological and methodological diversity in dsRNA delivery or differences in RNAi regulatory components or other sources for such differences remain to be identified. Therefore, to maximize RNAi silencing, it is important to understand the organism-specific limitations in RNAi (Geldhof *et al*., 2007). Recent advances in deep sequencing have revealed more and more of the intricacy and potency of the endogenous small RNA network, as well as its competitive regulation of the exogenous RNAi pathway.

Mutation studies of the *eri* genes in mouse have also shown that some endogenous RNA processing can be defective in the absence of *eri* genes (Ansel *et al*., 2008). However, the impact on RNAi efficacy due to the absence of *eri* genes has not been thoroughly analysed in other organisms. Hence probably a thorough examination of the RNAi regulation perspective can give more insight into the limited utility of RNAi in other organisms.

1.7 Application of RNAi in nematodes

1.7.1 Application of RNAi as a reverse genetics tool in nematodes

RNAi based methods have been widely used in the free-living nematode *C. elegans*, where it has been deployed in genome-wide high throughput screens to identify genes involved in many cellular and developmental processes. RNAi techniques have not yet translated efficiently to animal parasitic nematodes while somewhat more progress has been made in PPN, although diversity in sensitivity to RNAi has been observed among the nematodes.

The variation in RNAi response has been observed even within the *Caenorhabditis* genus as only *C. elegans* and the uncharacterized species *C. n. sp1* are sensitive to feeding RNAi (Winston *et al*., 2007). This suggests that significant differences in the RNAi mechanism exist even amongst closely related species (Lilley *et al*., 2012; Nuez and Felix, 2012). *Caenorhabditis briggsae* was found to be insensitive to external application of dsRNAs and seems to be deficient in the uptake of dsRNAs in the intestine. It has also been shown that the expression of *C. elegans sid-2* can complement this deficiency in *C. briggsae* (Winston *et al*., 2007). Similarly most of the other close relatives of *C. elegans* were found to be insensitive to external RNAi. It has, however, been found that all species of the genus tested are sensitive to dsRNAs introduced by injection into the gonad (Winston *et al*., 2007). Insensitivity to external dsRNA has also been found in other non-parasitic nematodes such as *Oscheius tipulae* and *Pristionchus pacificus* (Louvet-Vallee *et al*., 2003; Pires da Silva, 2006; Wheeler *et al*., 2012). Other culturable free-living nematodes such as *Panagrolaimus* species, which are distantly related to *C. elegans*, have shown sensitivity to external RNAi to a lesser degree (Shannon *et al.,* 2008).

RNAi in animal- and human-parasitic nematodes has had variable levels of success. The human filarial parasite *Brugia malayi*, the rodent parasite *Nippostrongylus brasiliensis*, and the insect parasite *Heterorhabditis bacteriophora* appear to be susceptible to RNAi (Hussein *et al*., 2002; Aboobaker and Blaxter, 2003; Ciche and Sternburg, 2007; Ford *et al*., 2009). In the case of the gastrointestinal nematodes *Haemonchus contortus* and *Ostertagia ostertagi*, RNAi was effective against in 2 of 11, and 5 of 8 genes, respectively (Geldhof *et al*., 2006; Visser *et al*., 2006). Similar difficulties in eliciting an RNAi response in *Heligmosomoides polygyrus* have also been reported by Lendner *et al.* (2008).

In contrast to human and animal parasitic nematodes, RNAi has been demonstrated as a feasible technique in different species of PPN (Urwin *et al*., 2002; Kimber *et al*., 2007; Dalzell *et al*., 2010a; Arguel *et al*., 2012). The first demonstration of RNAi in PPN was performed by Urwin *et al.* (2002). They soaked J2 of the cyst nematodes, *Heterodera glycines* and *Globodera pallida*, in a solution containing dsRNA of the target gene and a neurotransmitter, octopamine to stimulate feeding in the nematodes. Since PPN lack specific mutants and transformation systems, RNAi can be a possible approach to understand the function of the genes involved in complex host-nematode interactions.

Today, RNAi as a tool for functional genomics has been demonstrated in a range of plant-parasitic nematode species including the sedentary endoparasitic nematodes *Globodera pallida, Heterodera glycines* and *Meloidogyne incognita* (Rosso *et al*., 2005; Vanholme *et al*., 2007; Dalzell *et al*., 2010a) as well as the migratory parasitic nematodes *Radopholus similis* (Haegeman *et al*., 2009) and *Bursaphelunchus xylophilus* (Park *et al*., 2008; Cheng *et al*., 2010; Kang *et al*., 2011).

In addition to dsRNA, siRNAs have also been used as triggers to induce RNAi. Recently, Dalzell *et al.* (2010a, b) used synthetic siRNAs to induce efficient gene silencing in *G. pallida* and *M. incognita*. However, these reports also showed that the efficacy of individual siRNAs targeting different regions of the same transcript was highly variable.

Additionally Arguel *et al.* (2012) reported that siRNAs can trigger knock-down of the parasitism gene *Mi-CRT*, a calreticulin gene expressed in the esophageal glands of *M. incognita*, but the silencing event was not persistent. Nevertheless, current reports have revealed the potential advantage of increasing target specificity with the use of siRNAs for functional analysis (Arguel *et al*., 2012; Atkinson *et al*., 2013).

1.7.2 Application of RNAi in PPN control

Under current production methods, the management of PPN relies mostly on the use of chemical nematicides and to a lesser extent on the use of nematode resistant plants. The effectiveness of resistant cultivars is limited by high levels of genetic diversity both within and among nematode populations. . Additionally, selection pressure often results in resistance-breaking pathotypes within the population (Mitchum *et al*., 2007; Hershman *et al*., 2008).In addition to its use as reverse genetics tool, a number of research studies have proven that RNAi can also be utilized as a strategy for nematode control by genetically engineering plants to express PPN-transcript-specific dsRNA. The PPN takes up these dsRNAs or siRNAs generated by the host plant through its stylet while it feeds on the plant cells. The imbibed dsRNA/siRNAs induce the degradation of specific nematode genes. The silencing trigger inside the nematode is amplified with the help of RNA-dependent RNA polymerase (RDRP) (Chapman and Carrington, 2007; Gent *et al*., 2010; Shi *et al*., 2013).

Host-delivered RNAi is an ideal strategy for the obligate parasitic nematodes providing a means to silence genes that are essential to the parasites and also to characterize the function of the nematode genes. The feasibility and effectiveness of host-delivered RNAi for nematode control has been confirmed by different research groups. A significant suppression of nematode reproduction has been achieved by targeting different nematode genes including genes (Huang *et al*., 2006; Sindhu *et al*., 2009; Xue *et al*., 2013), responsible for the production of nematode secretory proteins essential for parasitism, developmental genes (Klink *et al*., 2009; Li *et al*., 2010) and housekeeping genes (Yadav *et al*., 2006; Li *et al*., 2010). The first demonstration of host-delivered RNAi was performed by Yadav *et al*. (2006) in the plant-parasitic nematode *M. incognita* by expressing the dsRNAs of two genes, which encode an integrase and a splicing factor in tobacco plants. Similarly nematode resistance was also achieved against four major RKN species by expressing of dsRNA of the parasitism gene 16D10 in Arabidopsis plants (Huang *et al*., 2006). Sindhu *et al*. (2009) observed 23% to 64% reduction in *H. schachtii* females when dsRNA of four parasitism genes were expressed in transgenic Arabidopsis lines.

Recently, Hamamouch *et al.* (2012) also demonstrated that Arabidopsis plants expressing dsRNA and its processed small interfering RNA complementary to the Hg30C02 sequences exhibited a strong RNAi mediated resistance to infection by *H. schachtii*. The expression of dsRNAs of *Hg-rps-3a*, *Hg-rps-4* and *Hgspk-1*, which are required for mRNA metabolism in *H. glycines*, in soybean roots also displayed 81%–88% reductions in numbers of *H. glycines* cysts (Klink *et al*., 2009b). Similarly the importance of an mRNA splicing factor, *Prp-17* in *H. glycines* has been demonstrated by Li *et al.* (2010) generating a host-derived RNAi in soybean plants.

Although a number of studies has reported a phenotype for nematodes parasitizing plants expressing dsRNAs, very few studies have clearly demonstrated that the phenotype is due to an RNAi effect.

Some of the studies have confirmed that the nematode resistance generated was due to host-derived RNAi by showing a significant down regulation of target nematode genes from nematodes feeding on transgenic roots using real time RT-PCR analysis (Sindhu *et al*., 2009; Li *et al*., 2010a). The accumulation of target gene siRNAs in the transgenic plant roots was confirmed only in five studies (Huang *et al*. 2006; Steeves *et al*., 2006; Fairbairn *et al*., 2007; Sindhu *et al*., 2009; Li *et al*., 2010a). In some other cases, although siRNAs were not detected in the transgenic lines, low level of dsRNA was detected by northern blot analysis (Charlton *et al*., 2010). Some other studies also confirmed the presence of unprocessed transcript by amplifying intron or spacer region of the hairpin construct using RT-PCR (Patel *et al*., 2008; 2010). Charlton *et al.* (2010) further demonstrated that crossing of transgenic Arabidopsis lines expressing two different dsRNAs generates higher levels of resistance to *M. incognita* in F2 plants than either parent plant. Thus from the above reports, it is evident that it is necessary to have a large amount of dsRNAs or siRNAs at the delivery site between host and nematode to elicit effective host-derived RNAi against the nematodes.

However, the traditional transformation methodologies, which take at least several months for most of the important plant species to produce stable transgenic lines, remain a bottleneck for host –derived RNAi approach in PPN. Many research groups have come up with, high-throughput composite or chimeric hairy root systems for rapid assessment of target genes *in planta*, including soybean (Klink *et al*., 2009; Li *et al*., 2010b), sugar beet (Cai *et al*., 2003) and tomato (Remeeus *et al*., 1998).

1.8 Banana improvement for nematode resistance

Cultivated bananas are natural selections originating from South East Asia, the centre of origin of the genus Musa (Simmonds, 1962).

The edible types comprise a range of natural hybrids originating from the two species *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). Most cultivars are triploids (AAA, AAB or ABB genomes), parthenocarpic and sterile. Banana is an important food crop, which is the fourth most important food crop in the developing world after rice, wheat and maize (Frison and Sharrock). Bananas are grown in 243 countries with a total cultivated area of over 5 million hectares and total world production of 106 million metric tons in 2011 (FAOSTAT, 2011).

It is of huge economic importance for many countries particularly the poorest countries of Africa, Latin America and Asia providing the main source of income for rural communities. While 15% of the crop is exported, 85% is consumed locally in these countries. In addition to being considered a basic product for export, constituting an important source of jobs and income in most developing countries, it is an important part of the daily diet of people in the developing countries of the world. Additionally, bananas are also rich in minerals and vitamins A, C and B6 (Chandler, 1995). These benefits notwithstanding, the production of bananas is limited due to several diseases and pests including bacteria, fungi, viruses, weevils and nematodes (Dubois and Coyne, 2009).

One of the dramatic disease symptoms in banana plantations is the toppling of plants, mainly caused by *R. similis*. In addition to *R. similis*, *P. coffeae*, *P. goodeyi*, *P. speijeri*, *M. incognita*, *Helicotylenchus multicinctus* and *Rotylenchulus reniformis* have been also found to parasitize banana plants in the tropics (Gowen *et al*., 2005; Coyne, 2009; Khan and Hasan, 2010; DeLuca *et al*., 2012).

Crop losses caused by nematodes to banana are estimated at about 20% worldwide (Sasser and Freckman 1987). Considering the global importance of banana, there is a great need to develop nematode-free and high-yielding cultivars by genetic improvement of the plants.

Conventional breeding in banana remains a difficult endeavour due to long generation times, sterility, triploidy, seedlessness and limited genetic variability (Tripathi, 2003). Compared with conventional breeding methods, genetic manipulation techniques provide new opportunities for the genetic improvement of banana (Arvanitoyannis *et al*., 2008).

1.8.1 Genetic transformation of banana

Genetic transformation of banana has become an important tool for crop improvement (Sagi *et al*., 1995; Cote *et al*., 1996; Kosky *et al*., 2002; Sipen *et al*., 2011).Screening for naturally occurring nematode resistance genes and introducing them into commercially cultivated varieties is one approach to develop nematode free banana cultivars. This approach allows single or combinations of genes associated with nematode resistance to be extracted from the genome of the source organism and transferred directly into the desired variety. This enables the variety to acquire the desired trait of resistance while retaining its original characteristics.

Additionally, this approach has become very promising for the genetic improvement of banana, particularly for those cultivars that are not amenable to sexual hybridization (Jones 2000; Pillay and Tripathi 2007). Although some resistances have been identified against one of the most damaging nematode species, *R. similis*, limited sources of nematode resistance and tolerance are present in the banana gene pool (Collingborn and Gowen, 1997; Hartman *et al*., 2010). Alternatively, it is also possible to introduce nematode resistance in a cultivar by host- derived RNAi (Section 1.7.2).

Genetic transformation involves the introduction and stable integration of genes into the nuclear or chloroplast genomes with subsequent gene expression in transgenic plants. During the transformation, single or combinations of genes with desirable traits can be introduced into the plant system.

In the production of transgenic banana plants, two transformation systems have been used: particle bombardment and *Agrobacterium*-mediated transformation. In the case of particle bombardment or biolistic transformation, micro projectiles (gold or tungsten) coated with DNA are used to deliver foreign genes into plant cells, which are then selected and regenerated into transgenic plants (Becker *et al*., 2000; Cote *et al*., 1996; Sagi *et al*., 1995). However, in the case of banana transformation, this technology is limited by the availability of cell cultures with a sufficiently high capacity for plant regeneration.

In *Agrobacterium*-mediated transformation, the soil bacterium *Agrobacterium tumefaciens* is used to transform cell cultures of the plant by integrating a segment of its tumor-inducing plasmid, so-called T-DNA, which acts as a vehicle to introduce the desired gene(s) into the nuclear genome. A complex process regulated by numerous bacterial genes that are located outside the T-DNA enables the T-DNA transfer into the host plant (Gelvin, 2003). In this study *Agrobacterium*-mediated transformation of banana was used to induce resistance against *R. similis* based on host-derived RNAi approach. In the case of *Agrobacterium*-based banana transformation, wounded explants such as apical meristems, corm meristematic tissue (May *et al*., 1995) or embryogenic cell cultures (Ganapathi *et al*., 2001; Pérez -Hernandez *et al*., 2006) are co-cultivated with *A. tumefaciens* harboring the plant transformation vector in the presence of acetosyringone, an inducer of the *Agrobacterium* virulence genes. The transgenic lines that are antibiotic resistant are selected and regenerated.

The incorporation of the transgene into high molecular weight plant genomic DNA is confirmed by DNA hybridization ensuring that the transgene presence is not due to residual *Agrobacterium* persisting in the plants. The transgenic plantlets undergo multiple rounds of propagation maintaining the genotypic and phenotypic traits.

This method offers remarkable advantages such as defined integration of transgenes, reduced copy number, fewer problems with transgene co-suppression and instability and preferential integration into transcriptionally active regions of the chromosome (Gheysen *et al*., 1998; Hansen and Wright, 1999; Shibata and Liu, 2000; Hiei *et al*., 2000).

Although a number of protocols has been developed for *Agrobacterium*-mediated transformation, currently most of the banana transformation protocols are based on cell suspension cultures (Sagi *et al*. 1995; Becker *et al*. 2000; Khanna *et al*. 2004; Perez-Hernandez *et al*., 2006a). The use of cell suspension in banana transformation is, however, less attractive for routine use due to the requirement of a long time period to initiate and maintain the suspension cultures and thus it makes the transformation slow and more expensive.

Recently, Subramaniam *et al.* (2011) developed a protocol for *Agrobacterium tumefaciens*-mediated genetic transformation system using suckers as explants. In this study, the techniques such as sonication of the explants and vaccum infiltration have been adopted to increase transformation efficiency. Nevertheless, regeneration and transformation is still necessary to obtain transgenic banana plants within a short time.

1.9 Somaclonal variation: A characteristic of tissue culture regenerated plants

Variation displayed among somaclones (soma=vegetative, clone=identical copy) regenerated from *in vitro* culturing has been termed as somaclonal variations (Larkin and Scowcroft, 1981). It refers to phenotypic variation, either genetic or epigenetic in origin. The first observation of somaclonal variation was reported by Braun (1959). Variations may result from both pre-existing genetic variation within explants and variation induced during *in vitro* propagation (Evans *et al*., 1984; Vuylsteke *et al*., 1996).

Factors such as explant source, time of culture, time of subculture, number of subcultures, phytohormones, genotype, media composition, the level of ploidy and genetic mosaicism are capable of inducing *in vitro* variability (Silvarolla, 1992; Shepherd *et al*., 1996; Bairu *et al*., 2011). Although the causes of genetic instability are poorly understood, chromosome instability is believed to be one of the most common causes of tissue culture-induced variation (Roux *et al*., 2004).

A number of reports have shown that variation in chromosome numbers and structures, and chromosome irregularities during *in vitro* differentiation and among regenerated somaclones results in the loss of genes or their function, the activation of genes previously silent, and the expression of recessive genes (Larkin and Scowcroft, 1981; Hao and Deng, 2002; Mujib *et al*., 2007). Additionally, it has also been demonstrated that transpositional events, such as the activation of transposable elements, putative silencing of genes and a high frequency of methylation pattern variation among single-copy sequences, play a role in somaclonal variation (Hirochika, 1993; Barret *et al*., 2006).

There are both benefits and disadvantages to somaclonal variation. Somaclonal variation can be found in karyotype, isozyme pattern, ploidy level, growth, yield, disease resistance and resistance to adverse soil and climatic conditions (Patil and Navale, 2000). The beneficial characteristics such as resistance to disease pathotoxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites can be enriched in somaclonal mutants during *in vitro* culture. Somaclonal variation provides a valuable source of genetic variation for the improvement of crops through the selection of variants, which may show these beneficial characteristics (Mehta and Angra, 2000; Predieri, 2001; Unai *et al*., 2004).

Hwang and Ko (1988) demonstrated the use of somaclonal variation to improve resistance against *Fusarium oxysporum* in a banana cultivar. To achieve better field performance, these clones were further improved by somaclonal variation (Hwang and Tang, 1996). Thus somaclonal variation can also be useful to produce pathogen free plants and widened the genetic variability of existing cultivars. Although somaclonal variation is an important tool for the improvement of banana germplasm, it is disadvantageous when clonal uniformity is required especially in the micropropagation of considered genotypes.

The high incidence of "off-types" in the plants regenerated from *in vitro* culture is an important drawback for masspropagation of banana. Most off-types are inferior to the parental clone, because of undesirable features such as reduced growth, fertility, regeneration potential, which affects the overall performance of the plant. Somaclonal variants with different types of plant morphology such as dwarf, giant (Israeli *et al*., 1991; Vuylsteke *et al*., 1991), thin and sickly looking tall plants, twisted and crinkly leaves, narrow and drooping leaves with mosaic like symptoms, abnormal bunch

orientation (Uma *et al*., 2002), green variants lacking anthocyanidin (Vidhya and Ashalatha, 2002) have been reported.

The rate of variation can be correlated to the time the explants spent in tissue culture. Increasing the number of subcultures and their duration were found to be the factors for increasing the emergence of somaclonal variations especially in cell suspension and callus cultures (Reuveni and Israeli, 1990; Bairu *et al*., 2006; Bairu *et al*., 2010). However, there is a variation in susceptibility of *Musa* species to somaclonal variation, depending on the genotype (Israeli *et al*., 1991; Sahijram *et al*., 2003) and also the interaction between genotype and tissue culture environment (Martin *et al*., 2006).

In order to reduce the variation induced by tissue culture in banana, it is necessary to select true-to-type plant material for propagation as well as to minimize the numbers of transfers in culture. A regeneration system requiring a minimum of time in culture, but still compatible with transformation might be a possible solution to reduce the variations. A tissue culture-free transformation method may be able to eliminate somaclonal variation in banana; however, this remains a future endeavor yet to be accomplished.

1.10 Scope of study

Research on plant–nematode interactions has mainly focused on the sedentary endo-parasitic nematodes of the genus *Globodera, Heterodera and Meloidogyne*. Although the RNAi approach has been demonstrated in the migratory nematodes *R. similis* (Haegeman *et al*., 2009) and *B. xylophilus* (Park *et al*., 2008; Zhao *et al*., 2013) its feasibility in other species of migratory nematodes that are of considerable economic importance is still unknown.

Therefore the present study aims to determine the possibility of using RNAi in the control of the migratory endo-parasitic nematode *P. coffeae* and *R. similis* which are important root pathogens of banana in the tropics (Sundararaju, 2005).

As an initial step to develop resistance against *P. coffeae*, the susceptibility of this nematode is tested by *in vitro* RNAi (Chapter 2). Further screening of the genes was limited due to unavailability of the EST data of *P. coffeae*. Therefore, transcriptome analysis of *P. coffeae* was performed in order to get more insight into the genes involved in parasitic success and nematode development (Chapter 3). Variable success has been reported in RNAi mediated silencing by soaking dsRNA as well as siRNA and also there is currently insufficient knowledge on the mechanisms of RNAi in plant parasitic nematodes. A study on RNAi effectors of *P. coffeae*, *M. incognita*, *H. oryzae* in comparison with *C. elegans* was carried out in order to elucidate the components of the RNAi pathway in these nematodes (Chapter 4). Furthermore, in order to investigate the reasons for the recovery of RNAi gene silencing, the influence of RNAi regulators such as *eri-1* in the silencing of the target genes was tested in *P. coffeae* (Chapter 5).

Finally, to check the feasibility of host derived RNAi as a control strategy in *R. similis*, an attempt was made to develop transgenic banana plants expressing dsRNA of target nematode genes involved in parasitism and cell development (Chapter 6).

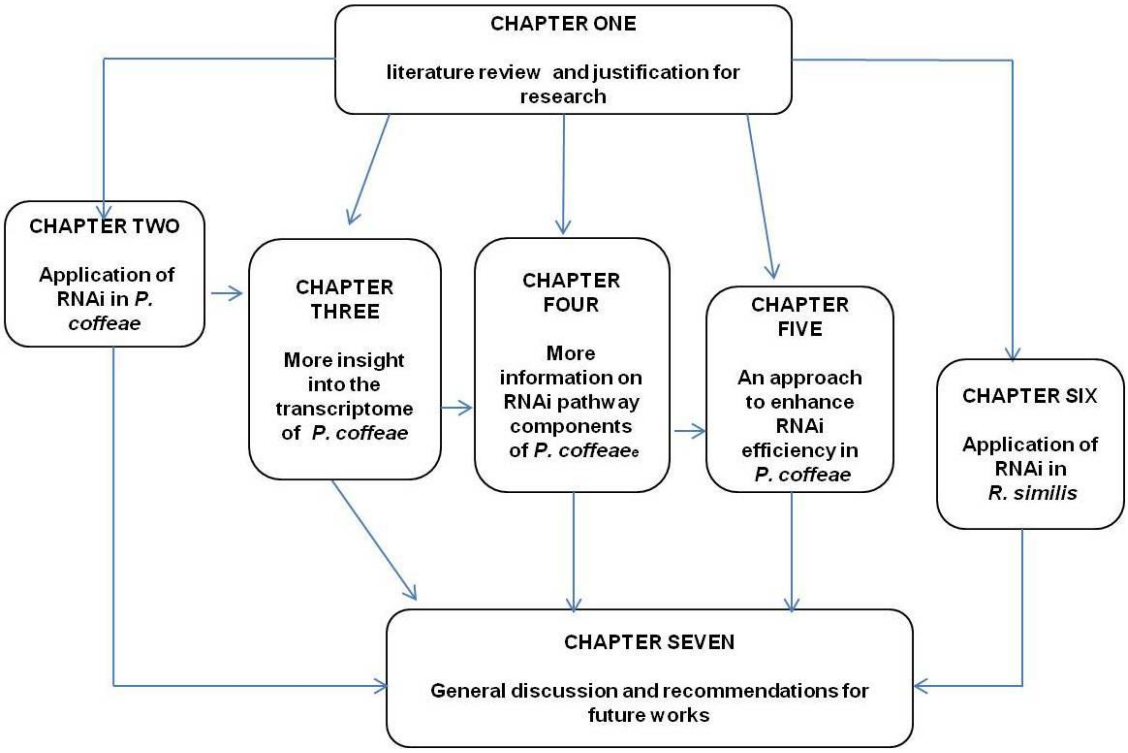

Figure 1.8. Schematic outline of thesis

CHAPTER TWO

RNA interference in *Pratylenchus coffeae*: knock-down of *Pc-pat-10* and *Pc-unc-87* impedes migration

Soumi Joseph, Godelieve Gheysen and Kuppuswamy Subramaniam

Published in: *Molecular and Biochemical parasitology* (2012), 186:51-59

2.1 Abstract

Many of the currently available nematicides used in nematode control are hazardous to the user, environment and beneficial non-target organisms. Therefore the need to develop alternative methods for nematode control such as the development of nematode-resistant crops through RNA-mediated interference (RNAi) holds great promise. The *Caenorhabditis elegans* genes, *unc-87* and *pat-10,* are essential components of the body wall muscle and are thus required for nematode movement. The *P. coffeae* orthologs of these two genes, namely *Pc-pat-10* and *Pc-unc-87* were cloned and used to test RNAi in this migratory nematode. RNAi was performed by soaking *P. coffeae* in a solution containing dsRNA of either *Pc-unc-87* or *Pc-pat-10*. The levels of both *Pc-unc-87* and *Pc-pat-10* mRNAs were significantly reduced in a sequence-specific manner in nematodes soaked for 24h. Nematodes incubated in *Pc-pat-10* dsRNA appeared straight and rigid while *Pc-unc-87* resulted in nematodes that were coiled, in contrast to the regular sinusoidal movement of the control nematodes. While 88.4±3.9% of the control nematodes successfully migrated to the bottom of the sand column in 12 hours, only 6±1.3% and 7±2.3%, respectively, of the *Pc-pat-10*(RNAi) and *Pc-unc-87*(RNAi) nematodes successfully migrated to the bottom. However a recovery in movement as well as transcript level was observed in both treatments when the nematodes were incubated in distilled water for 24h following the dsRNA soaking. The recovery rate was slower in *Pc-unc-87* when compared to *Pc-pat-10*. In summary, this study demonstrates the existence of the RNAi phenomenon in *P. coffeae* and shows that the function of *unc-87* and *pat-10* genes has been evolutionarily conserved among free-living and plant-parasitic nematodes.

2.2 Introduction

Research on plant–nematode interactions has been mainly focused on the sedentary endo-parasitic nematodes of the genus *Globodera*, *Heterodera* and *Meloidogyne*. Although the RNAi approach has been demonstrated in the migratory nematodes *R. similis* (Haegeman *et al.,* 2009) and *B. xylophilus* (Park *et al.,* 2008) its feasibility in other species of migratory nematodes that are of considerable economic importance is still unknown. Therefore the present study aims to determine the possibility of using RNAi in the control of the migratory endo-parasitic nematode *Pratylenchus coffeae* which is one of the most important root pathogens of banana in the tropics (Sundararaju, 2005). Here we report the successful elicitation of RNAi against two *P. coffeae* genes, namely *Pc-pat-10* and *Pc-unc-87*. These genes were chosen because they result in clearly different phenotypes and lethality. In *C. elegans Pat-10* encodes body wall muscle troponin C, the calcium-binding component of the troponin complex of actin thin filaments, which is essential for muscle contraction and for completion of embryonic morphogenesis and elongation (Kagawa *et al.,* 1997).

The RNAi study of this gene in *C. elegans* has shown that the knock-down of *pat-10* leads to paralysis (‘walking stick’ phenotype), larval and embryonic lethality and maternal sterility in the nematode (Gottschalk *et al.,* 2005). *C. elegans unc-87* on the other hand encodes two proteins (generated through alternative splicing) that are required to maintain the structure of myofilaments in body wall muscle cells (Kranewitter *et al.,* 2001). An RNAi study of *Unc-87* in *C. elegans* demonstrated that the knock-down effect of this gene results in uncoordinated locomotion of the nematode (“coiled” phenotype) (Simmer *et al.,* 2003).

A *Heterodera glycines* ortholog of *C. elegans unc-87* was reported to be expressed more highly in mobile second stage juveniles than in sedentary stages of the nematode which indicates the importance of this gene in movement (Mathews *et al.,* 2004).

The successful knock-down of *Pc-pat-10* and *Pc-unc-87* in our study will be a stepping stone for applications of RNAi in root-lesion nematodes.

2.3 Materials and methods

2.3.1 Nematode collection

A single population of *P. coffeae* (obtained from Prof. D. Dewaele, KuLeuven, collected from banana plantations in Ghana) was maintained and multiplied monoxenically on carrot discs at 28°C. After 8 weeks of culturing, all nematode stages (eggs, juveniles, female and male adults) were harvested from the carrot discs by maceration and sieving (Speijer and De Waele, 1997).

2.3.2 Cloning of *Pc-pat-10* and *Pc-unc-87* from *P. coffeae*

Due to the limited sequence information available at the start of the project two potentially essential *P. coffeae* genes were cloned on the basis of sequence and functional information available for *C. elegans*. The strategy was to search for genes that are sufficiently conserved for amplification using degenerate oligonucleotides as PCR primers. The second criterion was that the genes must be essential genes in *C. elegans*. Two *C. elegans* genes, namely *pat-10* and *unc-87* met both these criteria. The gene sequences from *M. incognita*, *H. glycines*, *P. penetrans*, *G. rostochiensis* and *C. elegans* were aligned and degenerate PCR primers were designed from two regions with sufficient sequence conservation.

Total RNA was extracted from the mixed life stages of *P. coffeae* using Tri-reagent (Sigma) according to the manufacturer's protocol. The first strand cDNA was synthesized using 100 ng of RNA, 10 mM dNTP, 100 µM oligodT primer, 200 units of M-MLV Reverse Transcriptase (Promega) and 20 units of Recombinant RNasin® Ribonuclease Inhibitor (Promega). A 350-bp fragment of *Pc unc-87* and a 448-bp of *Pc pat-10* was amplified from the cDNA using Taq polymerase (Bangalore Genei) and the primers KS 1875 (5'- TCTCCCGGGTTGATGACCAACTTTGGTACG) and KS 1876 (5'-TCTCCCGGGCATTYTGGTKGTCTCACGAC) for *Pc-unc-87* and KS 1869 (5'-TCTCCCGGGAYGGCTCCCAAATTGAGGA) and KS1873 (5'-TCTCCCGGGTCRCCGGCCCCATCARYTCCCA) for *Pc-Pat-10.* The PCR conditions were: 95°C for 5 min, followed by 35 cycles of 95° C for 15 sec, 55 °C for 30 sec and 72 °C for 1 min, which was followed by incubation at 72 °C for 10 min. Amplified fragments were gel-purified and inserted at the Sma I site of pBluescript SK+ plasmid vector and introduced into the DH5α strain of *Escherichia coli*. Inserts in the recombinant plasmid DNA were confirmed by DNA sequencing (Figure. 2.1). The expression of *Pc-pat-10* and *Pc-unc-87* was also checked in eggs as well as mixed stages of juveniles and adults of *P. coffeae*. A pool of approximately 50 nematodes containing mixed stages of juveniles and adult stages of males and females and another pool of approximately 50 eggs were manually picked from a batch of freshly isolated nematodes. Total RNA was isolated and cDNA was synthesized as mentioned above. A PCR was performed on these cDNAs for 35 cycles under the same PCR conditions as described previously.

2.3.3 Synthesis of double stranded RNA

The templates of sense and antisense DNA strands for generating the dsRNA of *Pc-pat-10* and *Pc-unc-87* were amplified from the vector carrying the inserts of each target gene by PCR under standard cycling condition 95°C X 5min, followed by 35 cycles of 95°C X 15s, 55°C X 30s, 72°C X 1min. The respective primers with the T7 promoter sequence incorporated at 5'end of either the sense or antisense strand were used for the PCR amplification. The PCR products were transcribed using the MEGAscript RNAi kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. The transcription products were purified by phenol: chloroform extraction and double-stranded RNA was made by incubating equimolar amounts of sense and antisense strand in boiling water for 5min, followed by 1hr at room temperature and subsequent treatment with DNase to remove the template. The dsRNA was quantified spectrophotometrically and the quality of the dsRNA was checked on a 1% agarose gel. To check dsRNA toxicity possibly affecting motility, the dsRNA of endo-1,4-beta-glucanase (*Pc-eng-1*) from *P. coffeae* was used as a control.

This gene was chosen as a dsRNA control because it does not have a direct impact on nematode motility as it encodes a cellulase enzyme involved in the breakdown of the plant cell wall during invasion. This made it possible to assess the non-specific action of dsRNA on nematode mobility. A fragment of 332bp from the 3' region of the endo glucanase cDNA from *P. coffeae* (Kyndt *et al.,* 2008) was used as the template for generating the sense and antisense strands of *Pc-eng-1.* The dsRNA was synthesized as described above.

2.3.4 *In vitro* RNAi on *P. coffeae* and analysis of mRNA levels by semi-quantitative RT-PCR followed by gel-blot analysis

Approximately 1000 juveniles and adults of *P. coffeae* freshly collected from a carrot disc culture were incubated for 24h in a 200µl solution containing 150µl of dsRNA (1µg/µl/), 50mM octopamine, 3mM spermidine and 5% gelatin. Nematodes incubated in the same solution but without the dsRNA (non-RNAi) or with dsRNA against *Pc-eng-1* served as negative controls. The nematodes were incubated at 28°C for 24h in silanized 1.5 ml microcentrifuge tubes. After incubation, the nematodes were washed thoroughly in distilled water and the total RNA extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Equal amounts of RNA from each treatment were used in cDNA synthesis, which was done as previously described. The RNA was treated with DNase (Fermentas, St-Leon-Rot, Germany) to avoid DNA contamination.

The cDNA was used as PCR template with the primers KS2702 (5'-CGAACTCGATTTTCCCACTG and KS2703 (5'-CCCCAAATTGAGGAGTACC) for the amplification of *Pc-pat-10* and KS2705 (5'-CCGGGTTGATGACCAACTT) and KS2811 (5'-GCTCTGATCAATGCTGCGC) for the amplification of *Pc unc-87*. The amplification of *Pc-eng-1* from the treated nematodes was performed using the primers KS2809 (5'-TCTTCCCAAGAATGGTGGAC) and KS2810 (5'-ACCGAGATTGAGGCAGACA). The PCR conditions were the same as above except the number of cycles, which was 22 for *Pc-pat-10*, 26 for *Pc-unc*-87 and 30 for *Pc-eng-1.* These cycle conditions were selected through a series of scoping studies to optimize the number of cycles at which the accumulation of the amplified product is in the exponential phase of the PCR cycle.

In order to obtain a better sensitivity and quantification of the transcript level, the PCR products were blotted and hybridized with radioactively labelled probe to *Pc-pat-10* and *Pc-unc-87* following standard protocols for Southern hybridization (Sambrook *et al.,* 1989). The templates for the production of DNA probes were generated by a PCR under conditions as described above on the plasmid pBluescript SK+ containing the cDNA clone of the corresponding sequences of *Pc-pat-10* and *Pc-unc-87*. The amplified PCR products were gel purified and labeled using DecaLabel™ DNA Labeling Kit (Fermentas, St-Leon-Rot, Germany) according to the manufacturer's instructions. The intensity of the hybridization signal was quantified using a phosphor imager (Bio-Rad Personal Imager Fx). The non-target genes were used as reference gene for the normalization of the cDNA, i.e. *Pc-pat- 10* and *Pc-eng1* were the references in the case of RNAi of *Pc-unc-87* and vice versa.

2.3.5 Qualitative and quantitative characterization of knock-down phenotype

For a qualitative observation of the RNAi effect on the motility phenotype, the treated nematodes were washed with sterile water and observed using a microscope equipped with time-lapse video microscopy (Axioscope 2 mot plus) and recorded using an Axiocam HRm CCD camera and Axiovision software (all from Carl Zeiss). A quantitative estimation of the motility was obtained by measuring the number of nematodes that successfully migrated through a 3-cm long column of wet sand.

Approximately 400 nematodes were added to the top of a sand column placed vertically in a collection vial containing chickpea root exudates. The numbers of nematodes that migrated through the columns into the collection vial were counted after a 12-h period. The result of the migration assay was analyzed by One-way ANOVA using GenStat Release 13 for Windows (VSN International Ltd).

Migration percentages of control nematodes (non-RNAi) were standardized to 100% and, subsequently, migration percentages of dsRNA-soaked nematodes were expressed relative to the non-RNAi controls

2.4 Results

2.4.1 High degree of sequence similarity of *pat-10* and *unc-87* among different nematodes

Blastn analysis of *Pc-pat-10* and *Pc-unc-87* sequences (http://blast.ncbi.nlm.nih.gov/blast.cgi and http://nematode.net) showed that both sequences were highly nematode specific and have no similarity with plant or human sequences. The alignment of amino acid sequences retrieved from diverse groups of nematodes including plant-parasitic, animal and human parasitic nematodes as well as free-living and entomopathogenic nematodes showed that both *pat-10* and *unc-87* share high degrees of sequence similarity among these nematodes (Figure. 2.1).The *Pc-pat-10* fragment showed significant similarity with sequences of troponin C-like proteins from *P. penetrans* (PE00200) as well as with those from other plant-parasitic genera such as *H. glycines* (HG02938) and *M. incognita* (MI02503).

Additionally the *pat-10* fragment from *P. coffeae* was also similar to the corresponding mRNA sequences of the animal parasitic and the human parasitic nematodes (*Toxocara canis* (TX00450) and *B. malayi* (BM00285) respectively) as well as the entomopathogenic and free-living nematodes (*Heterorhabditis bacteriophora* (Hbac_isotig 02222) and *C. remanei* (CR02338) respectively (Figure. 2.1A).

Similarly, Blastn analysis of the amplified fragment for *Pc-unc-87* against these group of nematodes also confirmed a very significant similarity with the mRNA sequence of the UNC-87 protein from *P. penetrans* (PE00222), *H. glycines* (HG03517) and *M. incognita* (MI02660) and with other parasitic nematodes, such as *T. canis* (TX00289) and *H. bacteriophora* (Hbac_isotig 01897) and *B. malayi* (XM_001901244.1) and also with free-living nematode, *C. remanei* (CR01939) (Figure. 2.1B). This indicates that these two genes are highly conserved among nematodes. We conclude that the cloned fragments indeed represent parts of the *P. coffeae* orthologs of the *C. elegans* genes *pat-10* and *unc-87.*

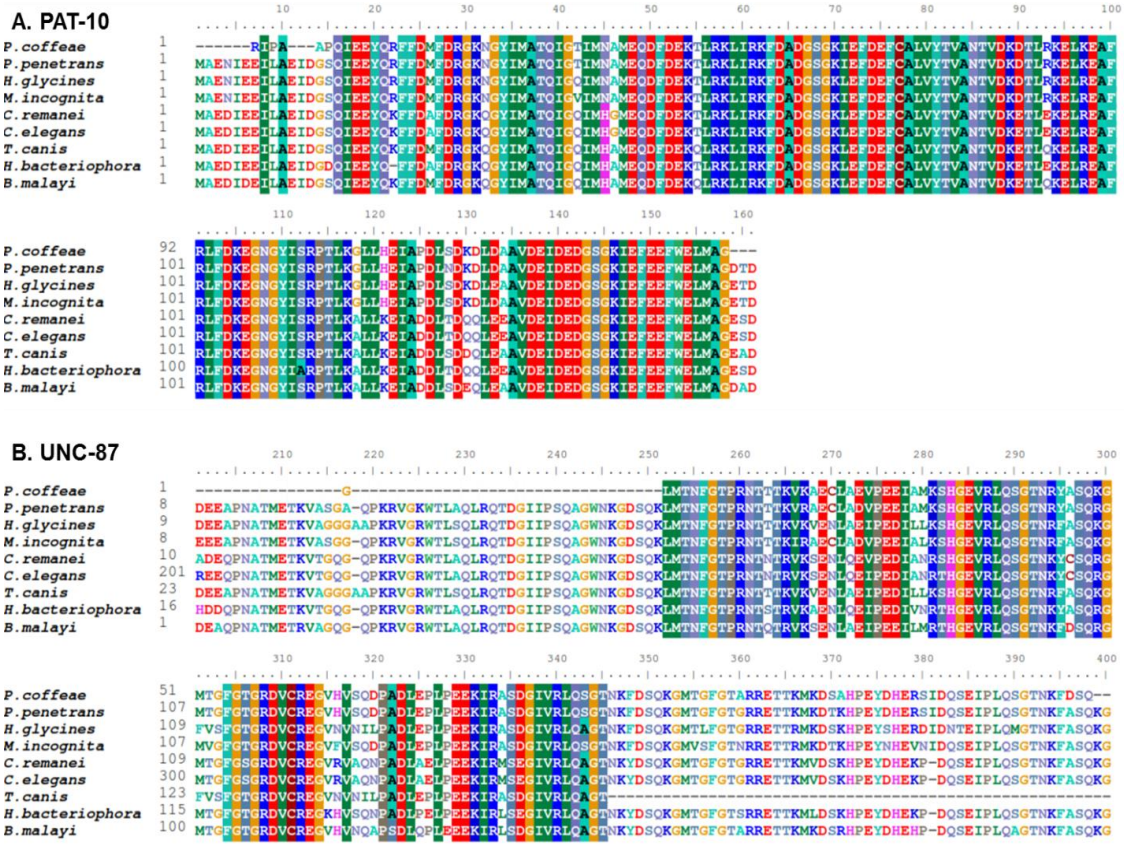

Figure 2.1. Alignment of amino acid sequences of PAT-10 (A) and UNC-87 (B) from diverse group of nematodes. The *C. elegans* nucleotide sequences available at www.wormbase.org were used to search the nematode EST database available at http://nematode.net to identify the corresponding orthologs from different groups of nematodes. These sequences were translated into amino acid sequences and aligned with the amino acid sequences of RT-PCR products obtained from *P. coffeae*. The color shaded region shows 100% amino acid conservation in all aligned sequences. The Gene/Isotig/Contig ID for the *pat-10* orthologs are: *P. penetrans* – PE00200, *H. glycines* – HG02938, *M. incognita* – MI02503, *C. remanei* - CR02338, *H. bacteriophora* - Hbac_isotig02222, *T. canis* – TX00450, and *B. malayi* –BM00285. The Gene/Isotig/Contig ID for the *unc-87* orthologs are *P. penetrans* – PE00222, *H. glycines* – HG03517, *M. incognita* – MI02660, *C. remanei* - CR01939, *H. bacteriophora* - Hbac_isotig01897, *T. canis* – TX00289, GenBank ID for *B. malayi* –XM_001901244.1. The GenBank ID for *C. elegans pat-10* and *unc-87* are FO080890.1 and FO080807.1, respectively

2.4.2 *Pc-pat-10* and *Pc-unc-87* can be efficiently silenced by RNAi

Semi quantitative RT-PCR on cDNA of *P. coffeae* showed that both genes were expressed in eggs as well as in mobile stages (Figure. 2.2).

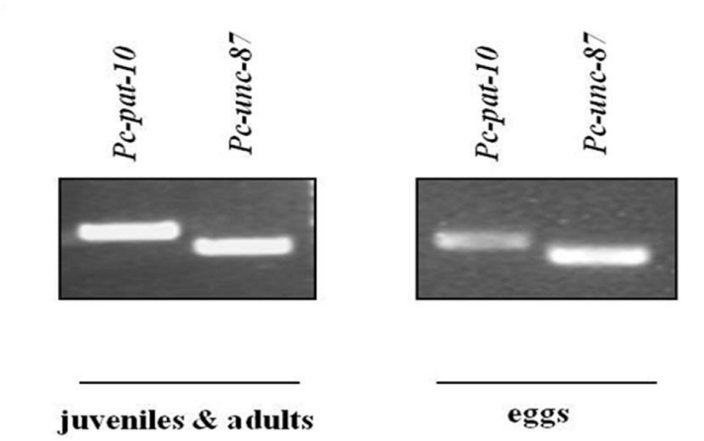

Figure 2.2. Amplification of *Pc-pat-10* and *Pc-unc-87* mRNA from mobile stages and eggs of *P. coffeae*. The mobile stages included juveniles, adult male and female nematodes that are migratory in nature. The PCR amplification was performed for 35 cycles in both cases. *Pc- pat-10* and *Pc-unc-87* were expressed in the eggs as well as in the mobile stages. gDNA contamination was checked by performing a PCR on DNase treated RNA samples (data not shown)

In non-RNAi control nematodes we could readily detect *Pc-pat-10* mRNA by 22 cycles of PCR amplification while *Pc-unc-87* mRNA required 26 cycles to yield comparable signal intensity. The transcript levels of both genes were drastically reduced in nematodes incubated in the corresponding dsRNA-containing solution compared to the control soakings. The soaking with the dsRNA of *Pc-pat-10* elicited a reduction only in the mRNA level of *Pc-pat-10,* but not in the expression of *Pc-unc-87* (Figure. 2.3A) or *Pc-eng-1*. Similarly, the nematodes incubated in *Pc-unc-87* dsRNA resulted in significant reduction only in the case of *Pc-unc-87* mRNA (Figure. 2.3B).

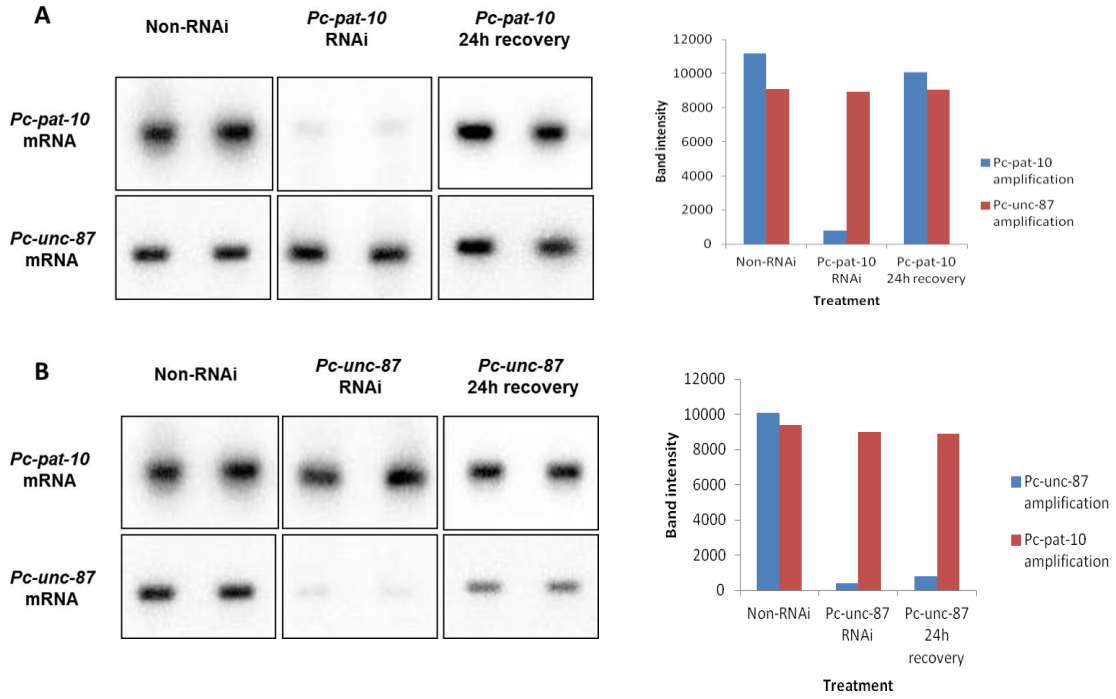

Figure 2.3. Analysis of the mRNA level by semi-quantitative RT-PCR/gel blots following 24h of dsRNA treatment and 24h of recovery in water. (A) Following 24h of incubation with dsRNA of *Pc-pat-10*; graphical representation of band intensity for each amplification (upper right) (B) Following 24h of incubation with dsRNA of *Pc-unc-87*; graphical representation of band intensity for each amplification (lower right). Non-RNAi: *P. coffeae* nematodes incubated in soaking solution without dsRNA; *Pc-pat-10* RNAi: nematodes incubated in dsRNA of *Pc-pat-10*; *Pc-pat-10* 24h recovery: the nematodes recovered in water for 24h following the dsRNA treatment with *Pc-pat-10*; *Pc-unc-87* RNAi: nematodes incubated in dsRNA of *Pc-unc-87*; *Pc-unc-87* 24h recovery: the nematodes recovered in water for 24h following the dsRNA treatment with *Pc-unc-87* . Each time the non-targeted gene was used as reference gene to show equal RNA loading. *i.e. Pc-unc-87* has been used as the reference gene for equal cDNA loading in *Pc-pat-10* RNAi and vice versa.The assay was repeated three times with consistent results.

Additionally, the RNAi treatment with the dsRNA of *Pc-eng-1* did not elicit any cross silencing of neither *Pc-pat-10* nor *Pc-unc-87* and vice versa confirming that both genes were knocked down in a gene specific manner (Figure. 2.4). Consistent results showing a significant knock-down of both genes was obtained from five soaking assays that were performed independently keeping parameters such as dsRNA concentration, soaking duration, incubation temperature and soaking components constant.

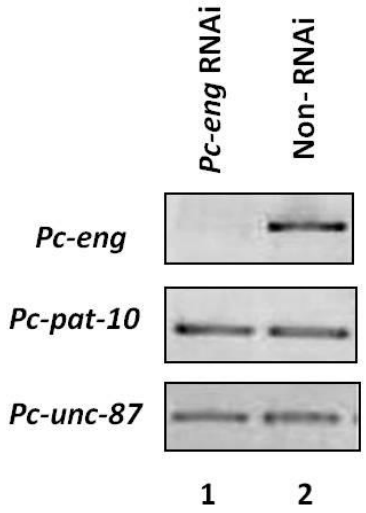

Figure 2.4. A semi-quantitative RT-PCR showing the transcript level of *Pc-eng-1*, *Pc-pat-10* and Pc-*unc-87* after 24h of treatment with the dsRNA of *Pc-eng-1* (*Pc-eng* RNAi). The optimized number of cycles at which the accumulation of the amplified product is in the exponential phase of the PCR cycle were 22 for *Pc-pat-10*, 26 for *Pc-unc*-87 and 30 for *Pc-eng-1.* The dsRNA of *Pc-eng-1* induced a significant reduction in the transcript level of *Pc-eng-1* while the transcript level of *Pc-pat-10* and *Pc-unc-87* remained unaffected. The soaking was repeated twice with consistent results.

2.4.3 Silencing of *Pc-pat-10* and *Pc-unc-87* generates an aberrant phenotype

Pratylenchus coffeae nematodes soaked in non-dsRNA solution showed normal sinusoidal movement. In contrast, the silencing of *Pc-pat-10* resulted in profound inhibition of motility in the nematodes.

While some of the nematodes incubated in dsRNA of *Pc-pat-10* generated straight and rigid posture, others were very slow in movement (Figure. 2.5). Approximately 85% of the nematodes showed the 'pat' phenotype. Although dead nematodes were also straight, the aberrant phenotypes resulting from silencing were distinguished from dead nematodes by observing pharyngeal pumping and slight mobility in the head region. The mobile head that can bend in combination with the straight body gives a "walking stick" appearance. The nematodes incubated in dsRNA of *Pc-unc-87* showed a coiled posture and were restricted in movement in contrast to the regular sinusoidal movement (Figure. 2.5). This phenotype resembled the typical 'unc' phenotype (uncoordinated) observed (approximately 82%) after knocking down *unc-87* in *C. elegans.* The same kind of phenotype after the incubation with the dsRNA of *Pc-pat-10* and *Pc-unc-87* was observed in all repetitions of the experiment.

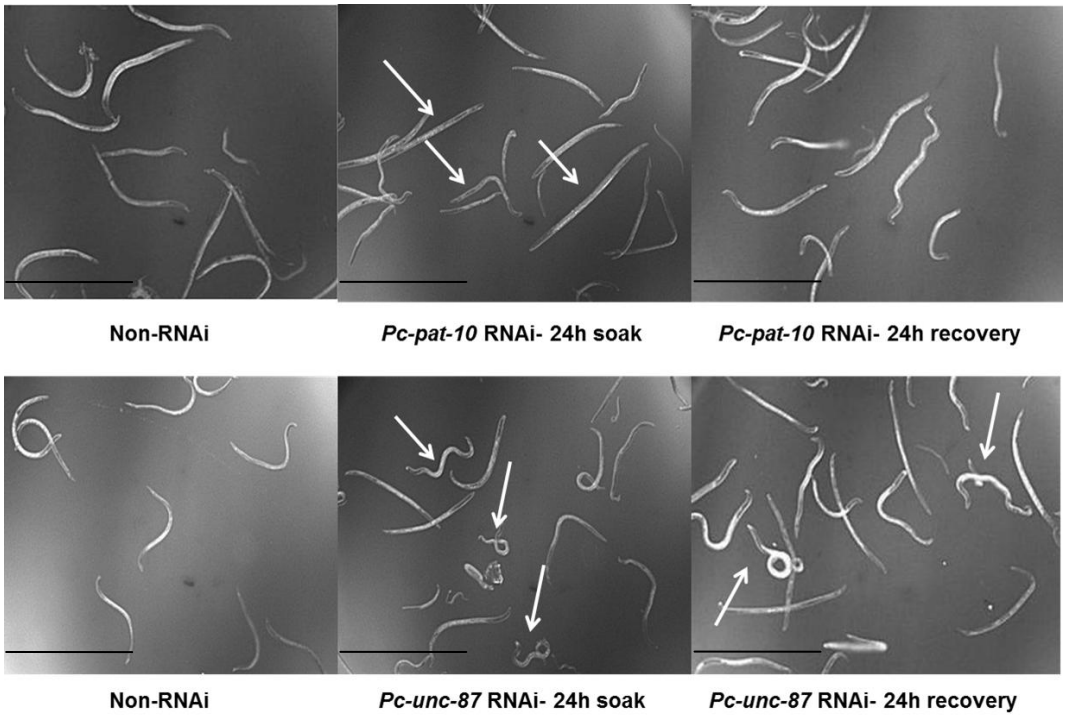

Figure 2.5. RNAi phenotype observed using time-lapse video microscopy after 24h of treatment with dsRNA of *Pc-pat-10* and *Pc-unc-87* and 24h of recovery in water after the dsRNA treatment. One snap-shot from the video are shown here. Arrows indicate the aberrant phenotype observed following the treatment. (Non-RNAi) *P. coffeae* nematodes incubated in soaking solution without dsRNA; *Pc-pat-10* RNAi: nematodes incubated in dsRNA of *Pc-pat-10*; *Pc-unc-87* RNAi: nematodes incubated in dsRNA of *Pc-unc-87.* The assay was repeated three times. Scale bar= 1mm

Earlier reports on *in vitro* RNAi in *Globodera pallida*, a cyst nematode and *Meloidogyne incognita*, a root-knot nematode have shown that high concentrations of non-specific dsRNA induce profound phenotypic changes in the infective juveniles of these nematodes (Dalzell *et al.,* 2009)]. Therefore, in order to confirm that the disruption in *P. coffeae* motility observed after RNAi is the result of specific gene silencing rather than any general toxic or inhibitory action of the dsRNA, *Pc-eng-1* encoding an *endoglucanase* that is secreted from the pharyngeal gland cells of *P. coffeae* was chosen as an additional control. This endoglucanase helps the nematode to degrade plant cell walls during the invasion and is not involved in nematode motility.

Following soaking in dsRNA of *Pc-eng-1*, no differences were observed between the locomotion of these nematodes and the control nematodes that were incubated in non-dsRNA solution while approximately 88% and 82% of the treated nematodes with dsRNA of *Pc-pat-10* and *Pc-unc-87* have shown 'pat' and 'unc' phenotypes, respectively.(Figure 2 6). This result confirms that the observed aberrant phenotypes are due to specific silencing of *Pc-pat-10* and *Pc-unc-87* rather than being caused by any non-specific action of the dsRNA.

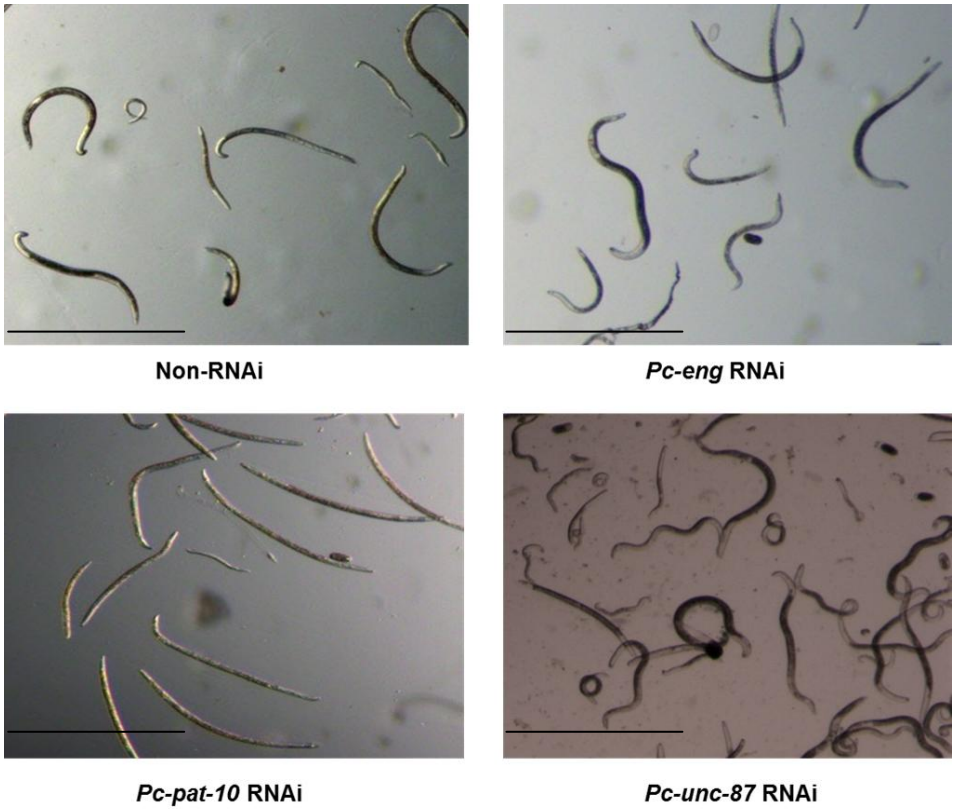

Figure 2.6. Checking the dsRNA specificity on aberrant phenotype using dsRNA of *Pc-eng-1* as a negative control. Phenotype of *P. coffeae* following 24h of incubation in soaking solution without dsRNA (Non-RNAi) and dsRNA of *Pc-eng-1* (*Pc-eng* RNAi), *Pc-pat-10* (*Pc-pat-10* RNAi) and *Pc-unc-87* (*Pc-unc-87* RNAi). While *Pc-eng* and non-RNAi show similar phenotypes, *Pc-pat-10* RNAi results in straight nematodes and *Pc-unc-87* RNAi in wavy or coiled nematodes. The assay was repeated twice. Scale bar=1mm

2.4.4 RNAi of *Pc-pat-10* and *Pc-unc-87* impedes nematode migration through a sand column

Pratylenchus coffeae incubated in 1 µg/µl *Pc-pat-10* and *Pc-unc-87* dsRNA for 24h were assessed for impaired motility by a migration assay using a wet sand column. The mean number of control nematodes that migrated was 88.4 ±3.9% after a period of 12h. However, in the case of the nematodes incubated in dsRNA constructs of *Pc-pat-10* and *Pc-unc-87* a significant reduction in the migration was noticed.

After 12h, 6.0 ±1.3 % of the nematodes incubated in dsRNA of *Pc-pat-10* and 7.4 % ± 2.3% of those incubated in dsRNA of *Pc-unc-87* successfully migrated through the sand column (Table 2.1).The assay was repeated twice and consistent results were observed in both cases. Thus the migration assay showed that the RNAi mediated knock-down of *Pc-pat-10* and *Pc-unc-87* induced 93.4% and 92.6% inhibition, respectively, in the migratory behaviour of the nematodes. Following the 24h of incubation in the dsRNA of *Pc-eng-1,* 87±2.9% of the treated nematodes were able to migrate through the sand column indicating that the dsRNA of *Pc-eng-1* does not have any inhibitory influence on the migration of the nematodes at the concentration of 1µg/µl as opposed to *Pc-pat-10* and *Pc-unc-87*.

Table 2.1. Quantitative characterization of knock-down phenotype by sand column migration assay after 24 of treatment with dsRNA

Treatment	%Migration	%Inhibition
Non-RNAi	88.4 [a]	11.6 [a]
***Pc-pat-10* RNAi**	6.0 [b]	94.0 [b]
***Pc-unc-87* RNAi**	7.4 [b]	92.5 [b]
***Pc-eng* RNAi**	87.0 [a]	13.0 [a]
significance (*P*)	< 0.001	< 0.001
cv%	4.1	6.3
s. e. d	1.771	1.771

Nematodes were assayed for impaired migration using a sand column assay after 24h of incubation in dsRNA of Pc-pat-10, Pc-unc-87 or Pc-eng-1. The number of nematodes successfully completing migration (% Migration) was compared with control nematodes incubated in soaking solution without dsRNA (non-RNAi) to determine the degree to which normal migratory behavior was disrupted (% Inhibition). Values represent means from four replicates. Any two means in a column with a letter in common are not significantly (P ≤ 0.05) different according to Tukey's multiple range test. The test was performed twice with similar results.cv and s.e.d represent the coefficient of variation and the standard error of sample mean differences respectively

2.4.5 RNAi of *Pc-pat-10* and *Pc-unc-87* does not persist in *P. coffeae*.

To investigate the durability of gene knock-down after 24h soaking with the dsRNA, the recovery of the *P. coffeae* nematodes in distilled water was observed under the microscope. While most of the nematodes (approximately 90%) that were incubated in dsRNA of *Pc-pat-10* regained the normal sinusoidal movement after 24h recovery, a few nematodes (approximately 10%) were still seen as rigid and paralyzed (Figure. 2.5). In the *Pc-unc-87* treatment, very few nematodes could regain the regular movement after 24h recovery in water (Figure. 2.5). This recovery in nematode movement and posture was confirmed by RT-PCR/gel blot analysis (Figure. 2.3A and 2.3B).

The transcript level of *Pc-pat-10* was completely recovered after 24h in water, but only~ 50% recovery was observed in the transcript level of *Pc-unc-87*. Consistent with this observation, the sand column assay also yielded similar results. While 88±8.9 % of the

control nematodes migrated through the sand column after 24h of recovery in water, 71±2 % and 56±9.8 % respectively of nematodes treated with *Pc-pat-10* dsRNA and *Pc-unc-87* dsRNA could migrate (Table 2.2).

Thus the improved migration through the sand column after 24h recovery in sterile water indicates that the knock-down effect was wearing off following the removal of the dsRNA from the soaking solution. This observation is in contrast to earlier reports of RNAi in *C. elegans* where the knock-down effect persists throughout the life of the nematode exposed to the dsRNA and can be heritable to the F1 generation (Tabara *et al.,* 1999). We conclude that the RNAi effect in *P. coffeae* is transient, and, in this respect, they are more similar to other endoparasitic nematodes than to the free-living *C. elegans.*

Table 2.2. Quantitative characterization of knock-down phenotype by sand column migration assay 24h post dsRNA treatment

Treatment	%Migration	%Inhibition
Non-RNAi	87.64 [b]	12.3 [b]
***Pc-pat-10* RNAi**	70.5 [a b]	29.4 [a b]
***Pc-unc-87* RNAi**	55.5 [a]	44.5 [a]
significance (*P*)	0.002	0.002
cv%	12.6	13.1
s. e. d	6.33	6.33

Nematodes were assayed for impaired migration using the sand column assay after 24h recovery post 24h incubation in dsRNA. The number of nematodes successfully completing the migration (% Migration) was compared with control nematodes incubated in soaking solution without dsRNA (non-RNAi) to determine the degree to which normal migratory behavior was disrupted (% Inhibition). The test was performed twice with similar results. The values represent means from four replicates. Different letters within a column represent significant (P ≤ 0.05) differences according to Tukey's multiple range tests. cv and s.e.d represent the coefficient of variation and the standard error of sample mean differences respectively.

2.5 Discussion

The results presented here clearly establish that the migratory nematode *P. coffeae* has a functional RNAi machinery, and that a loss-of-function phenotype of individual genes can readily be obtained by soaking nematodes in dsRNA solution. Expression analysis on the nematodes incubated in dsRNA of *Pc-pat-10* and *Pc-unc-87* for 24h has shown that the transcript level of both genes was significantly and specifically reduced. The silencing effect and the specificity of RNAi were clearly visible in the phenotypes of the nematodes. It was observed that the pharyngeal pumping remained active in the paralyzed nematodes of *P. coffeae* following the *Pc-pat-10* RNAi treatment. This is in agreement with reports by Terami *et al.* (1999) where pharyngeal pumping was found to be unaffected in *Pat-10* mutants of *C. elegans*.

According to Nakae and Obinata (1993) the troponin C protein encoded by *Pc-pat-10* is located in the body wall and other minor muscles, but not in the pharyngeal muscles of the nematode and thus, the pharyngeal pumping remained active even after the knock-down of *Pat-10*. Although *Pc-pat-10* and *Pc-unc-87* gene products are both associated with thin filaments of body wall muscles, the RNAi phenotype of *Pc-unc-87* is distinct from that of *Pc-pat-10.* PAT-10 is essential for the initial assembly of the sarcomere and is involved in the attachment of muscle cells to the basement membrane (Williams and Waterston, 1994). UNC-87 on the other hand serves as a structural component to maintain lattice integrity during contraction (Goetinck and Waterston, 1994)] and hence the knock-down of *Unc-87* leads to disorganized body wall muscles affecting the contraction/relaxation cycle of the muscles (Uncoordinated movement).

Recent reports have shown that it is necessary to design an optimal dose of RNAi for effectively knocking down the target genes without causing any non-specific inhibitory actions (Castanotto *et al.,* 2007; Lilley *et al.,* 2012). It has been shown that high doses of expressed short hairpin RNAs (shRNAs) cause mice mortality in a dose dependent manner (Grimm *et al.,* 2006). There are several other reports showing that exogenous siRNAs and shRNAs can compete with each other or with endogenous miRNAs for the RNAi machinery (Castanotto *et al.,* 2007; Koller *et al*., 2006). RNAi studies in *M. incognita* and *G. pallida* have also shown that even non-nematode derived double stranded RNAs induce profound phenotypic changes in these nematodes (Dalzell *et al.*, 2009). In this study no abnormal phenotype or inhibitory action on movement were observed in the nematodes incubated in the dsRNA of *Pc-eng-1* for 24h at the concentration of 1 µg/µl. The nematodes showed similar sinusoidal movement as seen in the control nematodes incubated in water.

Additionally, the RT-PCR results confirmed that the dsRNA of *Pc- eng* does not influence the transcript level of *Pc-pat-10* and *Pc-unc-87* while eliciting specific silencing of *Pc-eng-1* at the same concentration. This further confirms the earlier statement that the observed '*pat*' and '*unc*' phenotypes were induced by the specific dsRNAs. It has been shown that the maximum dsRNA concentration to avoid non-specific toxicity differs among nematodes. Toxicity of dsRNA to J2 of *M. incognita* was shown at 0.1µg/µl after 24h of soaking, while the same concentration had no effect on *G. pallida* (Dalzell *et al.,* 2009). Our results agree with the RNAi study of the migratory nematode *B. xylophilus* where it was shown that a gene specific RNAi phenotype can be induced by the soaking nematodes with dsRNA of *Bx-myo-3* and *Bx-tmy-1* at a concentration of 1 µg/µl (Park *et al.,* 2008). Only concentrations above 2µg/µl were found to be detrimental to *B. xylophilus* after 24h incubation with the control dsRNA (Cheng *et al.,* 2010).

It is possible that the discrepancy among different nematodes in their response to the dsRNA concentration may be due to the differences in their inherent ability to take up dsRNA and process these introduced dsRNAs. Genome annotation of various nematode species has revealed that diversity in the RNAi pathway components exists across various nematode clades *e.g*. *B. malayi* (Ghedin *et al.,* 2007), *M. incognita* (Abad *et al*., 2008), *Pristionchus pacificus* (Dieterich *et al.,* 2008), *M. hapla* (Opperman *et al.,* 2008).

Another observation from this study was the recovery of nematode movement and the transcript level of *Pc-pat-10* and *Pc-unc-87* following transfer of the nematodes from the dsRNA soaking solution into water. While most of the nematodes incubated in dsRNA of *Pc-pat-10* had recovered after 24h, a slower recovery was noticed in the case of nematodes incubated in *Pc-unc-87* dsRNA.

This was also reflected in the transcript level of the genes. The transcript level of *Pc-pat-10* was completely recovered after 24 h in water, but only 50% recovery was noticed in the transcript level of *Pc-unc-87*. This time–limited silencing effect has also been reported by Rosso *et al.* (2005) following the soaking of *M. incognita* juveniles in dsRNA of *Mi-crt* (calreticulin) or *Mi-pg-1* (polygalacturonase).

The silencing effect of *Mi-crt* lasted for 44h while the effect of *Mi-pg-1* remained only for 20h after soaking. In cyst nematodes a long durability of the RNAi effect (up to 14 days) was noticed after silencing of the major sperm protein gene (Urwin *et al.,* 2002) and in the case of *N. brasiliensis* 6 days for the acetylcholinesterase gene (Hussein *et al*., 2002). It appears that genes with high transcriptional activity have an RNAi effect of short duration (Rosso *et al.,* 2005; Urwin *et al.,* 2002; Hussein *et al.,* 2002). In the present study *Pc-pat-10* mRNA was readily detected by 22 cycles of PCR amplification whereas *Pc-unc-87* mRNA required 26 cycles to yield comparable signal intensity. Presuming an equally efficient RT-PCR this suggests that the *Pc-pat-10* mRNA is more abundant than *Pc-unc-87* mRNA. The difference in transcription level and turnover could be an explanation for rapid recovery of the *Pc-pat-10* transcript compared to *Pc-unc-87*.

It was noticed that the knocking down of *Pat-10* persisted long after the removal of dsRNA trigger from *C. elegans* whereas the effect of RNAi wore out after removing dsRNA of *Pc-pat-10* in the case of *P. coffeae*. There is the need to explore further the reasons for the difference in RNAi persistence between the free-living nematode *C. elegans* and the plant-parasitic nematode *P. coffeae.* It has been experimentally proven that the high efficiency of RNAi in *C. elegans* is mainly because of the amplification of primary silencing signals by an RNA dependent RNA polymerase (RdRP) encoded by *rrf-1* (Sijen *et al.,* 2001) and the systemic nature of the RNAi.

Thus, in the case of *C. elegans* a small amount of dsRNA trigger can result in profound silencing in the treated nematode as well as its progeny (Hannon, 2002; Cerutti, 2003; Zhuang and Hunter, 2012). However it has been shown that the accumulation of siRNA is negatively regulated by *eri-1,* a nuclease with siRNAse activity and also by *rrf-3,* another putative RdRP found in *C. elegans* (Simmer *et al.,* 2002; Kennedy *et al.,* 2004).

In the transcriptome analysis of *P. coffeae* (Haegeman *et al.,* 2011) *rrf-1* and *rrf-3* homologs were not found in *P. coffeae,* but a homolog for *eri-1* has been found in the nematode. Additionally the secondary Argonaute proteins (SAGOs) such as NRDE-2 and NRDE-3 which are associated with secondary siRNAs and crucial for RNAi inheritance in *C. elegans* are poorly conserved in *P. coffeae* (Haegeman *et al.,* 2011). This indicates that *P. coffeae* may be deficient in production of secondary siRNAs while the available primary siRNA to induce robust RNAi might be limited by the influence of antagonistic factors such as *eri-1*.

It has also been reported that some genes in *C. elegans* are effectively targeted by RNAi while others are resistant to RNAi. It is clear that there are multiple mechanisms behind the RNAi regulation and the most of the genes involved in the regulation have not been well explored and completely understood in PPN. It is envisaged that with a comprehensive understanding of RNAi regulatory genes in PPN, it might be possible in the future to enhance RNAi potency and persistence in these nematodes. In conclusion this study has demonstrated that *P. coffeae* is readily susceptible to RNAi and that the functions of *Pc-pat-10* and *Pc-unc-87* are evolutionarily conserved between the free-living *C. elegans* and the plant-parasitic *P. coffeae*.

However, the persistence of the RNAi effect of these genes is highly time limited in *P. coffeae* as opposed to the persistent effects in *C. elegans*.

It is therefore essential to have a greater understanding of the RNAi regulatory pathway in PPN to enhance the potency and persistence of RNAi. Alternatively, feeding the nematodes with *in planta*-produced dsRNA can provide continuous supply of siRNA in the nematodes and possibly result in a long lasting RNAi effect in the nematode. As BLASTn sequence analysis has shown that *Pc-pat-10* and *Pc-unc-87* are highly nematode specific, this minimizes the possibility of non-target effects on the plant or other organisms.

Therefore, *Pc-pat-10* and *Pc-unc-87* could be potential targets for RNAi mediated nematode control by impeding the root damage caused by nematode migration. This successful application of RNAi in *P. coffeae* opens the door to the search for novel essential target genes for controlling these migratory nematodes.

CHAPTER THREE

Analysis of the transcriptome of the root lesion nematode *Pratylenchus coffeae* generated by 454 sequencing technology

Annelies Haegeman, Soumi Joseph and Godelieve Gheysen

Molecular and Biochemical parasitology (2011), 178 (1-2):7-14.

Author's contribution: Translation and signal peptide prediction, Identifying homologs of plant cell wall modifying enzymes, PPN secretory proteins and novel parasitism genes in *P. coffeae*

3.1 Abstract

To study interactions between plants and plant-parasitic nematodes, several –omics tools have nowadays become extremely useful. To improve the knowledge on migratory nematodes, the transcriptome of *Pratylenchus coffeae* was studied through generating expressed sequence tags (ESTs) on a 454 sequencing platform. Here the generation, assembly and annotation of over 325000 reads from *P. coffeae* are presented. After assembling these reads, 56325 contigs and singletons with an average length of 353 bp were selected for further analyses. Similarity searches revealed that 26% of these sequences had significant matches to the Swiss-Prot/trEMBL database and 36% had significant matches in nematode ESTs. Over 10000 sequences were successfully annotated, corresponding to over 6000 unique Gene Ontology identifiers and 5000 KEGG orthologs. Different approaches led to the identification of different sequences putatively involved in the parasitism process. Several plant cell wall modifying enzymes were identified, including an arabinogalactan galactosidase, so far identified in cyst nematodes only. Furthermore, homologs to chorismate mutase were found, suggesting that these parasitism genes have a wider occurrence in plant-parasitic nematodes than previously assumed. In conclusion, the generated transcriptome data of *P. coffeae* will be very useful further studies including; evolutionary studies of specific gene families, such as the plant cell wall modifying enzymes, the identification and functional analysis of candidate effector genes, the development of new control strategies, *e.g*. by finding new targets for RNAi and the annotation of the upcoming genome sequence.

3.2 Introduction

Molecular analyses, especially transcriptome analyses by means of expressed sequence tags (ESTs), have recently been performed for several PPN. At the start of this study, approximately 175000 ESTs from PPN have been submitted to the NCBI database, all derived from traditional Sanger sequencing (December 2010). The sedentary root-knot nematodes (Meloidogynidae) have been extensively studied with the generation of over 70000 ESTs and the sequencing of two complete genomes of *Meloidogyne incognita* (Abad *et al*., 2008) and *Meloidogyne hapla* (Opperman *et al*., 2008).

The Pratylenchidae, a family of migratory nematodes, is the family most closely related to the Meloidogynidae (Van Megen *et al*., 2009). Approximately 15000 ESTs, of which less than 8000 are, derived from the genus *Pratylenchus*. A molecular comparison of members of the Meloidogynidae and Pratylenchidae could provide insights on the differences and similarities between sedentary and migratory nematodes. Further, in our previous study we observed that RNA interference can successfully be used as a tool to characterize the functions of genes in *P. coffeae* (Chapter 2). Functional characterization of other effectors involved in parasitism also requires additional information on EST data of the nematode. The analysis of transcriptome data might also help us to explore further on the reasons for discrepancy in RNAi response between *P. coffeae* and *C. elegans*. Keeping this in mind, we decided to characterize the transcriptome of a mixed-stage *P. coffeae* population by 454 pyrosequencing.

The latter technique has become a relatively rapid and cost-effective method for high-throughput sequencing of ESTs of non-model organisms. 454 sequencing has been used to generate ESTs mainly from animal-parasitic nematodes (Cantacessi *et al*., 2010a; Cantacessi *et al*., 2010b; Cantacessi *et al*., 2010c). At the start of this study no reports on 454 sequencing studies for PPN were available.

One of the goals of this project was to identify putative effector genes by several approaches. More specifically, we wanted to determine whether *P. coffeae* possesses a similar arsenal of plant cell wall modifying enzymes as the *Meloidogyne* species. The latter enzymes are necessary for PPN to penetrate the rigid plant cell wall. Numerous enzymes have been identified in different families of PPN, such as endo-1,4-beta-glucanase, xylanase, pectate lyase, polygalacturonase, arabinogalactan galactosidase and arabinase (Danchin *et al.,* 2010). These genes may have been acquired by horizontal gene transfer from bacteria and fungi (Jones *et al*., 2005). In *Pratylenchus* spp., so far only an endo-1,4-beta-glucanase has been identified (Kyndt *et al*., 2008), although there is also EST evidence for an expansin, a protein known to loosen the cell wall non-enzymatically (Haegeman *et al*., 2010).

3.3 Materials and methods

3.3.1 RNA extraction, cDNA synthesis and sequencing

A *P. coffeae* population from Ghana was cultured on carrot discs at a constant temperature of 25°C. RNA was isolated from mixed stages using the TRI reagent (Sigma) according to the manufacturer's instructions. First strand cDNA synthesis was done with the Super SMART PCR cDNA synthesis kit (Clontech, CA, USA) including an amplification step of 20 cycles as described in the manual. Subsequently, the amplified cDNA was purified using the Qiaquick PCR purification kit (Qiagen, Germany) and normalized using the TRIMMER kit (Evrogen). The normalized cDNA sample was sent to LGC Genomics (Berlin, Germany), where it was sequenced in two separate runs of ¼ of a picotiter plate on a 454 FLX Titanium platform (Roche, Branford, CT, USA) by a shotgun approach. The data are submitted to the NCBI Sequence Read Archive (SRA) with accession number SRA028814.

3.3.2 Cleaning and assembly

The resulting reads were processed with the CLC Genomics Workbench 4.0.2 software. SMART adapter sequences and 454 sequencing primers were trimmed from all reads. Additionally, low quality reads (<99.5% accuracy) and short reads (<50bp) were discarded. The assembly was done using standard settings.

3.3.3 Similarity searches

All contigs and singletons longer than 150 bp were blasted locally (Blastx) against Swiss-prot and trEMBL (October 2010) with an E-value cut-off of 1e-4.

Additionally, all nematode ESTs were downloaded from the EST division of Genbank, and split into three different datasets according to the nematode's lifestyle: animal-parasitic nematodes, PPN and free-living nematodes. A local tBlastx search (E<1e-4) looked for homologs for all sequences in these datasets. Since a lot of plant cell wall degrading enzymes in nematodes are thought to originate through horizontal gene transfer, we tried to identify HGT candidates. Therefore we did Blast searches against different datasets of plant, nematode and bacterial sequences. We downloaded the coding sequences of the following genomes from the RefSeq database of NCBI: *C. elegans* (NC_003279-NC_003284), *B. malayi* (NZ_AAQA00000000), *Arabidopsis thaliana* (NC_003070-NC_003075) and all completed genomes of plant pathogenic bacteria (*Pectobacterium atrosepticum*, NC_004547; *Ralstonia solanacearum*, NC_003295; *Xylella fastidosa* 9a5c, NC_002488; *Agrobacterium tumefaciens*, NC_003062; *Xanthomonas campestris* pv. campestris, NC_003902; *Xanthomonas axonopodis* pv. citri, NC_003919; *Pseudomonas syringae* pv. syringae, NC_007005; *Xylella fastidosa* Temecula 1, NC_004556; *Pseudomonas syringae* pv. tomato, NC_004578; *Leifsonia xyli* subsp. xyli, NC_006087; *Pseudomonas syringae* pv. phaseolicola, NC_005773; *Xanthomonas campestris* pv. campestris, NC_007086; *Xanthomonas campestris* pv. vesicatoria, NC_007508; *Candidatus Phytoplasma asteris,* NC_007716; *Clavibacter michiganensis* subsp. sepedonicus, NC_010407; *Candidatus Phytoplasma* mai, NC_011047; *Dickeya dadantii*, NC_012880; *Dickeya zeae*, NC_012912).

All putative proteins from the genomes of *M. incognita* and *M. hapla* were downloaded from the projects' websites (Abad *et al*., 2008; Opperman *et al*., 2008).

To look for orthologs, a reciprocal Blast strategy was used: the *Pratylenchus* sequences longer than 150 bp were blasted (Blastx, E<1e-5) against the *Meloidogyne* proteins as well as the opposite strategy (tblastn, E<1e-5). Only when both pairs of Blast hits were the same, they were considered as true orthologs.

3.3.4 Annotation

All *Pratylenchus* sequences longer than 150 bp were annotated based on the Blastx results against Swiss-prot and trEMBL. A sequence was annotated based on the top hit information, only if E<1e-5 and if the description of the top hit did not contain any terms that would suggest it is a hypothetical or unknown protein ("unknown", "putative", "uncharacterized", "hypothetical", "similar", "predicted", "probable"). Gene Ontology terms were retrieved for all unique protein identifiers from annotated sequences using QuickGO from the EBI website (http://www.ebi.ac.uk/QuickGO/GAnnotation). KEGG orthologs were identified using the KEGG Automated Annotation Server (KAAS) with default parameters (Moriya *et al*., 2007). Subsequently, KEGG BRITE mapping was applied to find the most common classifications.

3.3.5 Translation into putative proteins

To predict putative proteins, the sequences longer than 150 bp were translated using OrfPredictor (Min *et al*., 2005). To look for putative parasitism genes, the presence of signal peptides was predicted with SignalP 3.0 (Bendtsen *et al*., 2004) and transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

3.3.6 Searching for specific genes

As described above, three sequence sets putatively related to parasitism were retained: the first dataset was derived from homology to plant pathogenic bacteria and/or plants only, the second one was derived from homology to parasitic nematode ESTs exclusively, and the third one was derived from putative proteins with a signal peptide. The Blastx hits of these datasets were retrieved and manually searched for the presence of putative plant cell wall modifying enzymes. The sequences that showed similarity to these genes were locally blasted (tblastx, E<1e-5) against all *Pratylenchus* sequences longer than 150 bp to identify any additional family members.

The following putative effector genes were retrieved from Genbank and used for homology searches: 10A06 (Hewezi *et al*., 2010), 14-3-3b (Jaubert *et al*., 2002), 16D10 (Huang *et al*.,2006), 7E12 (de Lima de Souza *et al*., 2011), acid phosphatase (Huang *et al*., 2003), annexin (Patel *et al*.,2010), calreticulin (Jaubert *et al*.,2002), chitinase (Gao *et al* 2002), chorismate mutase (Lambert *et al*., 1999), CLE peptide (Gao *et al*., 2003), ERp99 (Wang *et al*., 2001), galectin (Dubreuil *et al*., 2007), glutathione peroxidase (Jones *et al*., 2004), glutathione-S-transferase (Dubreuil *et al*., 2007), map-1 (Semblat *et al*.,2001) , nodL factor (Scholl *et al*., 2003), peroxiredoxin (Robertson *et al*., 2000), SPRYSEC RBP-1 (Sacco *et al*., 2009), RING-H2 zinc finger protein (Gao *et al*., 2003), fatty acid and retinol binding protein or SEC-2 (Prior *et al*., 2001), SKP1-like protein (Gao *et al*., 2003), SXP/RAL-2 (Jones *et al*., 2000), transthyretin-like protein (Jacob *et al*., 2007), ubiquitin extension protein (Gao *et al*., 2003) and venom allergen protein (Ding *et al*., 2000). Accession numbers can be found in Table 3.4.

A tblastn search (E<1e-5) was used to identify possible homologs in the *Pratylenchus* sequences. Resulting hits were subsequently BLAST searched (Blastx, E<1e-5) against the Genbank nr database.

3.4 Results

3.4.1 Sequencing, cleaning and assembly

cDNA was isolated from a mixed population of *P. coffeae* and EST sequences were generated by 454 sequencing technology. The sequencing run resulted in a total r of 326950 reads with an average sequence length of 252 nucleotides. After adapter and quality trimming, 320703 reads remained. Assembly resulted in a total of 25987 contigs and 53179 singletons. An overview of the sequencing and assembly is presented in(Table 3.1). The contigs have an average size of 458 bp, an average coverage of 4.2 and consist on average of 10.3 reads. The number of reads included in the contigs ranges from 1 to 651 (Figure 3.1). The length of all sequences ranges from 50 to 3343 with an average of 271 bp (Figure 3.1). When sequences smaller than 150 bp are removed, the average length increases to 353 bp.

Table 3.1. Overview of sequencing run and assembly

	Sequences	Total bases	Average length (bp)
Total number of reads	326971	82445359	252
High quality trimmed reads	320703	70752552	221
Contigs	25987	11903837	458
Singletons	53179	9559767	180
Total	79166	21463604	271
Total > 150 bp	56325	19908855	353

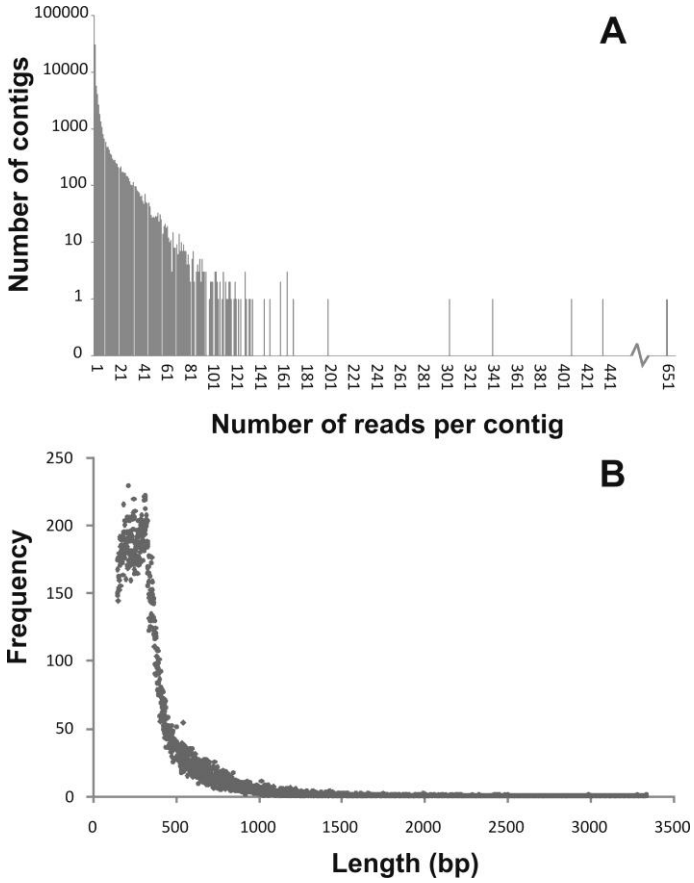

Figure 3.1. Visualization of contigs and singletons >150 bp (A) Contig size distribution showing the number of contigs with a particular number of reads (B) Frequency plot of the length distribution of the contigs and singletons

3.4.2 Similarity searches

Preliminary analyses showed that for sequences less than 150 bp it is difficult to find similarity. Therefore, we decided to continue the analysis with contigs and singletons longer than 150 bp, further referred to as the *Pratylenchus* sequences. A Blastx search (E<1e-4) was done locally against the Swiss-prot and trEMBL database.

This resulted in 14580 sequences with a significant hit (25.9%), of which 9346 were derived from contigs, while 5234 were from singletons. Of the 14580 best matches, 10087 were unique. Another tblastx search identified sequences homologous to nematode ESTs (E<1e-4). Three different databases containing nematode ESTs were constructed: one with all free-living nematodes (FLN), one with all PPN and one with all animal-parasitic nematodes (APN). Of all sequences, 20325 had significant hits in one or more of these nematode databases (36.1%) while 36000 did not have any hits at all. The sequences which showed homology were classified according to the number of hits in each nematode EST database (Figure 3.2)). Approximately half (51.6%) of the sequences with homology to nematode ESTs had hits occurring in all three nematode EST databases, while approximately one quarter (23.7%) was specific to PPN species.

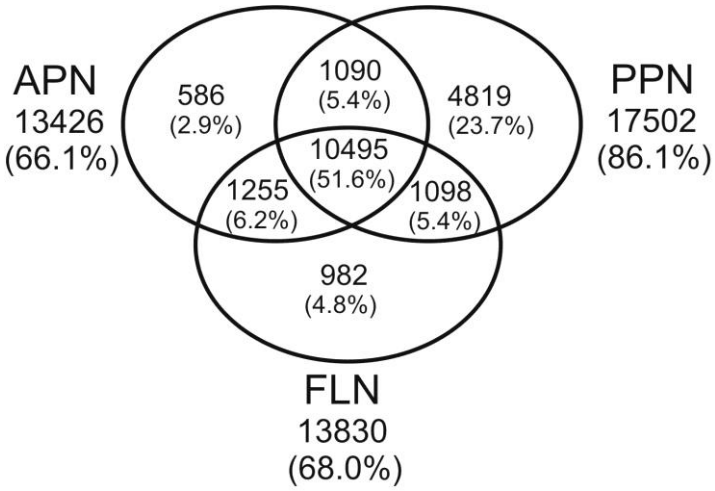

Figure 3.2. Classification of sequences with significant homology to nematode ESTs (APN: animal-parasitic nematodes, PPN: plant-parasitic nematodes, FLN: free-living nematodes)

To look for potential genes related to plant parasitism, the predicted protein sequences were downloaded from different genomes: *C. elegans*, *B. malayi*, *Arabidopsis thaliana* and 18 genomes of plant pathogenic bacteria. A Blastx search against these different datasets revealed that 13370 (23.7%) sequences have a hit against the *C. elegans* putative proteins, 12904 (22.9%) against *B. malayi*, 5503 (9.8%) against Arabidopsis and 2144 (3.8%) against the plant pathogenic bacteria. Of these hits, 348 had hits in plant pathogenic bacteria exclusively, 101 in Arabidopsis exclusively and 72 in both the bacteria and Arabidopsis. Orthologs were identified in the genomes of *M. incognita* and *M. hapla* by a reciprocal Blast strategy. The *P. coffeae* sequences had 6196 true orthologs with *M. hapla* and 5119 with *M. incognita*. To compare, *M. hapla* and *M. incognita* had 6580 orthologs according to this strategy, of which 2056 did not occur in *P. coffeae*. In total, 2746 orthologs occurred in all three datasets.

3.4.3 Annotation

Annotation was done based on the Blastx search against Swiss-Prot and trEMBL. This resulted in 10219 annotated sequences, of which 7156 were unique. Gene Ontology identifiers were searched for these unique sequences. 114100 GO terms were retrieved (of which 6146 different ones), coupled to 6810 different protein identifiers (on average 17 GO terms per protein identifier). The most abundantly present GO terms in the dataset are shown in Table 3.2. Using the KEGG Automatic Annotation Server, 5267 KEGG orthologs were identified, of which 2317 are unique. KEGG BRITE mapping revealed the most common classifications in the dataset (Figure 3.3).

Table 3.2. The ten most abundant Gene Ontology terms present in the dataset for the Cellular Component, Molecular Function and Biological Process categories

Cellular Component	**%**
Nucleus	14.2
Cytoplasm	12.8
Membrane	11.5
Integral to membrane	6.8
Plasma membrane	4.5
Mitochondrion	4.1
Intracellular	2.8
Nucleoplasm	1.9
Cytosol	1.9
Endoplasmic reticulum	1.8

Molecular Function	**%**
Protein binding	11.4
ATP binding	9.4
Nucleotide binding	4.3
DNA binding	3.3
Metal ion binding	2.7
Catalytic activity	2.7
Hydrolase activity	2.4
Oxidoreductase activity	2.1
Transferase activity	2.1
Zinc ion binding	2.1

Biological Process	**%**
Protein phosphorylation	4.0
Transport	3.6
Embryo development ending in birth or egg hatching	3.3
Oxidation-reduction process	3.2
Metabolic process	2.4
Transcription	2.2
Translation	2.0
Regulation of transcription	1.8
Reproduction	1.6
Nematode larval development	1.5

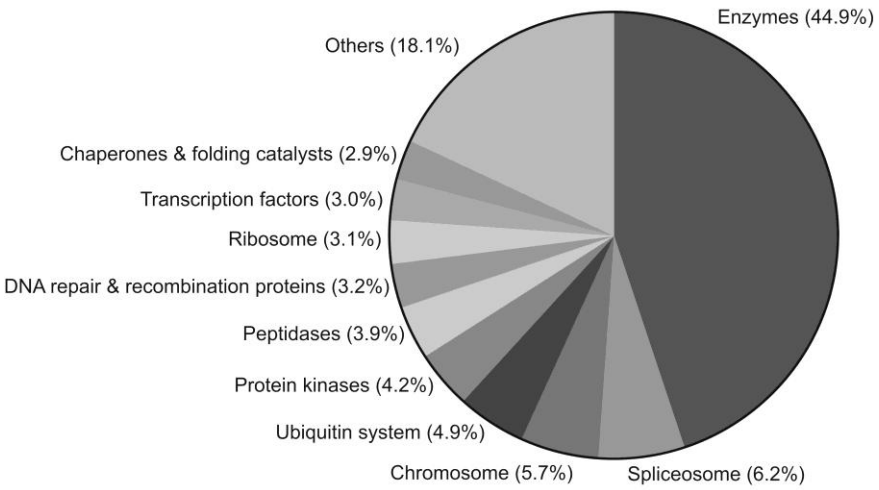

Figure 3.3. The ten most common KEGG BRITE hierarchies identified in the *Pratylenchus* sequences

3.4.4 Translation and signal peptide prediction

When translated into putative proteins, 805 of the sequences were predicted not to have an ORF. Of the 55520 sequences with ORF, 40100 coded for a putative protein longer than 50 amino acids. Only the proteins with a putative start methionine were included in signal peptide prediction. 2697 putative proteins were predicted to have a signal peptide, of which 1004 lacked a transmembrane domain.

3.4.5 Plant cell wall modifying enzymes

On a total of 56325 sequences, 667 sequences were similar to putative plant cell wall modifying enzymes (= 1.2 %) (Table 3.3). The most abundantly present enzymes are endo-1,4-glucanases or cellulases. The different contigs all show great similarity to other known GHF5 endoglucanases from nematodes, and one of the contigs appears to be the previously described gene *Pc-eng-1* (Kyndt *et al*., 2008).

Several contigs included, besides the GHF5 catalytic domain, a carbohydrate binding module (CBM). The second most abundant plant cell wall modifying protein represented in the transcripts was expansin. The ten identified contigs encoded a protein consisting of a signal peptide coupled to an expansin-like domain. Apparently none of the contigs had a CBM present. All putative proteins showed the highest similarity to an expansin-like protein from *Globodera rostochiensis*. Four contigs resembled pectate lyases. Three of them showed greatest similarity to cyst nematode pectate lyases, while one had a higher similarity to root-knot nematode pectate lyases. One contig shows high similarity to a xylanase from *R. similis.* It contains part of the catalytic domain and part of the CBM. Another contig and one singleton were similar to *Heterodera* arabinogalactan galactosidases. Finally one contig did not have similarity to any nematode genes, but shows similarity to some bacterial GHF5 proteins.

Table 3.3. Overview of plant cell wall modifying proteins identified in the *P. coffeae* EST dataset. The table contains for each protein the family it belongs to, whether it has been found before in the Pratylenchidae family, and the number of contigs, singletons and reads

Enzyme	Enzyme family	Previously found in Pratylenchidae?	Number # contigs	# singletons	# reads
Endo-1,4-beta-glucanase	GHF5	*P. coffeae*, *R. similis*, *P. vulnus* (ESTs)	15	17	242
Expansin-like protein		*P. vulnus* (ESTs)	10	2	183
Pectate lyase	PL3	-	4	0	120
Xylanase	GHF5-GHF30	*R. similis*	1	0	54
Arabinogalactan galactosidase	GHF53	-	1	1	10
Polygalacturonase	GHF28	-	2	0	7
Putative protein	GHF5	-	1	0	51

3.4.6 Plant-parasitic nematode proteins known to be secreted

Several putative secreted nematode proteins have been identified in the last decade by transcriptome and proteome analyses on different nematode species. A selection of these proteins was used for homology searches against the *Pratylenchus* sequences (Table 3.4). The resulting hits were examined carefully by blasting these against the non-redundant database. This was necessary to make a distinction between homologs to secreted proteins and homologs to endogenous nematode proteins without function in parasitism. The number of sequences identified as possible homologs is listed in Table 3.4. Of the 26 secreted proteins, 15 had putative homologs in the *P. coffeae* dataset. Two of them, chorismate mutase and SPRYSEC RBP-1 were previously only found in sedentary nematodes. The putative *P. coffeae* chorismate mutase contig showed highest similarity to chorismate mutases from bacteria from the genus Burkholderia (49% identity over 85% of the query), and the second highest similarity to nematode chorismate mutases (50% identity over 77% of the query). It had a significant match with an E-value of 7e-12 (bit score of 48) to the PFAM family chorismate mutase type 2 (PF01817). The presence of signal peptide was not confirmed in the putative chorismate mutase because the 5' part of the sequences were lacking. Four possible homologs of SPRYSEC were identified in the *P. coffeae* contigs (PF00622, E<1e-5). Two additional contigs showed highest similarity to SPRY domain containing proteins from other organisms. However, no signal peptide could be detected in the the putative SPRY domain containing proteins because the 5' parts of all sequences are lacking. However, it is also possible that these proteins are not SPRYSEC effectors in the case of *P. coffeae* and not involved in parasitism.

Table 3.4. Results from a tblastn search of selected secreted nematode proteins against the *P. coffeae* sequences.

secreted protein	accession nr	# contigs	# singletons	# reads	E-value	Presence of SP
10A06	ACU12489	No true homologs				
14-3-3b	AAL40719	2	0	80	4e-83	NA
16D10	Q06JG6	No true homologs				
7E12	AAQ10021	No true homologs				
Acid phosphatase	AAN08587	3	0	61	2e-67	NA
Annexin	AAN32888	No true homologs				
Calreticulin	AAL40720	2	0	38	3e-79	NA
Chitinase	AAN14978	No true homologs				
Chorismate mutase	AAD42163	1	0	9	2e-08	NA
CLE peptide	AAO33474	No true homologs				
ERp99	AAG21337	0	1	1	7e-18	YES
Galectin	AAB61596	1	0	6	2e-23	NA
Glutathione peroxidase	CAD38528	6	3	144	2e-77	NA
Glutathione-S-transferase	ABN64198	8	0	155	2e-51	NA
Map-1	CAC27774	No true homologs				
NodL factor	MI01045	No true homologs				
Peroxiredoxin	CAB48391	2	0	52	2e-71	NA
Sprysec RBP-1	CAM33004	4	0	38	2e-15	NA
RING-H2 zinc finger protein	AAP30834	No true homologs				
SEC-2	CAA70477	2	3	89	7e-63	YES
SKP1-like protein	AAP30763	No true homologs				
SXP/RAL-2	CAB75701	1	0	8	5e-15	NA
Transthyretin-like protein	CAM84510	9	5	235	5e-46	NA
Ubiquitin extension protein	AAO33478	0	1	1	4e-18	NA
Venom allergen protein	AAD01511	3	0	88	3e-23	YES

The accession numbers of the sequences used as query are from the Protein division of Genbank, except for nodL, which is a contig derived from EST data on www.nematode.net. The number of contigs and singletons of probable homologs are given, including the total number of reads and the best E-value. SP indicates signal peptide; NA stand for not applicable (Presence of SP in most of proteins could not be confirmed since 5' part of the sequences were missing)

3.4.7 Novel candidate parasitism/effector genes

To search for novel putative nematode parasitism genes or effectors, three different database searching strategies were applied as shown in Figure 3.4. The first approach identified 1004 putative proteins with a signal peptide and without a transmembrane domain. The second approach identified 6495 sequences that had hits to parasitic nematode ESTs exclusively. In the third approach, *Pratylenchus* sequences were compared to all proteins of *C. elegans*, *B. malayi*, *Arabidopsis thaliana* and some selected plant pathogenic bacteria. 512 sequences had hits in plants and/or pathogenic bacteria exclusively. If we consider all three approaches separately, 7680 different sequences potentially involved in parasitism can be retained.

To increase the chance of finding good candidates, two approaches can be combined, for example there are 161 candidates with a signal peptide and with hits to parasitic nematodes exclusively. If all three approaches were combined, only 4 candidates remained. When we examined the sequences retained in combined approaches, several genes known to be important in parasitism were present in the dataset, such as plant cell wall modifying enzymes, oxidoreductases and ubiquitin-like proteins. However, many other genes not known to be involved in parasitism were found (*e.g*. transport proteins, ethylene forming enzyme), as well as sequences with no significant homology and therefore unknown function. The latter sequences are interesting for further studies.

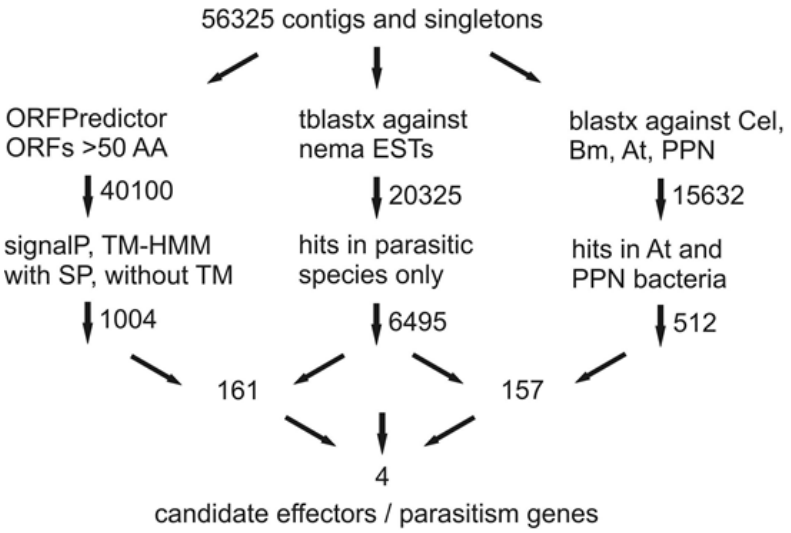

Figure 3.4. Three approaches to identify putative effectors/parasitism genes. The number of retained sequences is indicated in each step, and the final numbers are the number of sequences retained by two or three approaches combined

3.5 Discussion

Since the emergence of 454 sequencing technology, transcriptome analysis of non-model organisms by EST sequencing has become increasingly popular. To date, several EST studies of PPN have been published, most of them using the classic Sanger method (Popeijus *et al*., 2000; Dautova *et al*., 2001; Jacob *et al*., 2008; Haegeman *et al*., 2009; Kikuchi *et al*., 2007; McCarter *et al*., 2003; Huang *et al*., 2003; Mitreva *et al*., 2004: Furlanetto *et al*., 2005; Karim *et al*., 2009; Elling *et al*., 2009; Wubben *et al*., 2010b; Tyson *et al*., 2012). In animal-parasitic nematodes, three recent studies used 454 sequencing to study the transcriptomes of *Trichostrongylus colubriformis*, *Necator americanus* and *Haemonchus contortus* (Cantacessi *et al*., 2010a; Cantacessi *et al*., 2010b; Cantacessi *et al*., 2010c). Recently, Haegeman *et al*. (2012) have reported transcriptome analysis using 454 sequencing in *M. graminicola*.

Similarly, Maier *et al*. (2013) have also used 454 sequencing to elucidate transcriptome of esophageal gland cells from three different species of PPN, *Globodera rostochiensis*, *Pratylenchus penetrans,* and *Radopholus similis*.

In this study, over 325000 ESTs were derived from the migratory plant-parasitic nematode *P. coffeae*. The average read length of the reads was 252 bp, which is similar or slightly lower than reported in other studies using 454 technology (Cantacessi *et al*., 2010a; Cantacessi *et al*., 2010b; Bettencourt, 2010; Wang *et al*., 2009; Parchman *et al*., 2010). After the assembly, 56325 sequences longer than 150 bp remained with an average length of 353 bp. In a Blastx search against Swiss-prot and trEMBL, 26% of these sequences gave a significant hit and in total 18% of the sequences were successfully annotated. This percentage is relatively low, but due to the high amount of data generated, over 7000 sequences were annotated with a unique identifier. Almost half of these annotated genes were classified as “enzymes” according to KEGG. Gene Ontology mapping revealed that the most genes have basic functions such as transport, transcription; protein synthesis or modification and developmental and metabolic processes.

Potential orthologs to root-knot nematode genes were searched by comparing the *Pratylenchus* sequences to the putative protein sequences derived from the genomes of *M. incognita* and *M. hapla*. This revealed that 31% of the orthologs in common for *M. incognita* and *M. hapla* do not occur in the *P. coffeae* ESTs. Remarkably, recent upcoming data on *P. coffeae* genome has revealed that this nematode has a very small genome (19.7.Mb) and possesses only 50% of protein coding genes present in *M. hapla* (Opperman, C.H, 2013). Additionally, in comparison with *C. elegans*, *P. coffeae* has only 33% of the gene complement which indicates that *P. coffeae* seems to have a minimal

genome for a nematode. Based on this data it seems that our data has covered a substantial proportion of *P. coffeae* genome.

Several plant cell wall modifying proteins were identified in the *Pratylenchus* sequences. Previously, only an endoglucanase and some ESTs from an expansin-like protein were known in *Pratylenchus* spp (Kyndt *et al*., 2008;Haegeman *et al*., 2010). Our dataset extends the arsenal of enzymes in *P. coffeae* with xylanase, pectate lyase, polygalacturonase, arabinogalactan galactosidase and an unknown GHF5 protein. The presence of an arabinogalactan galactosidase is remarkable, since it has only been found in cyst nematodes so far, and it is not present in the available *Meloidogyne* genomes (Danchin *et al.,* 2010; Vanholme *et al*., 2009a). Our search against the contigs from the *Meloidogyne* genome has also confirmed the absence of arabinogalactan galactosidase in the root-knot genome. Because the Pratylenchidae are more closely related to the Meloidogynidae than to the Heteroderidae, the most probable evolutionary explanation is that there must have been a HGT in the common ancestor of Heteroderidae and Pratylenchidae. According to the phylogeny of van Megen *et al*. (Van Megen *et al*., 2009) this common ancestry group contains Meloidogynidae, Pratylenchidae, part of the Telotylenchidae, Heteroderidae, Rotylenchulidae, Hoplolaimidae, Dolichodoridae and Belonolamidae. Probably several lineages, including the Meloidogynidae, have subsequently lost this gene during evolution. One sequence was identified with similarity to bacterial GHF5 proteins, but without significant similarity to known nematode GHF5 endoglucanases. One nematode EST originating from *Xiphinema index* showed significant similarity to the putative new type of GHF5 proteins.

These two nematode sequences resemble putative proteins from two extremely halophilic Archaea, and two anaerobic bacteria. However, no predicted signal peptide is present in the genes of these bacteria, and none of these bacteria are involved in plant parasitism. Therefore, these new putative GHF5 proteins might not play a role in nematode parasitism.

Next to the plant cell wall modifying enzymes, other nematode secreted proteins with putative functions in the plant have been described (Table 3.4). Several genes with similarity to these putative effectors were also identified in the *P. coffeae* dataset: 14-3-3b protein, acid phosphatase, calreticulin, chorismate mutase, ERp99, galectin, glutathione peroxidase, glutathione-S-transferase, peroxiredoxin, RBP-1, SEC-2, SXP/RAL-2, transthyretin-like protein, ubiquitin extension protein and venom allergen protein. For most of these secreted proteins, no clear functional data is available yet. Interestingly, sequences similar to chorismate mutase and RBP-1 (SPRYSEC) were found, although both were thought to occur in sedentary nematodes only, and in the latter case even in cyst nematodes only. A recent report on transcriptome analysis of *Pratylenchus thornei,* (Nicol *et al*., 2012) has also identified 46.7% identity of 12 *P. thornei* contigs to chorismate mutase of *G. rostochiensis* (*Grcm-1*) and *H. schachtii* (*Hs-cm-1*). The presence of a chorismate mutase in a migratory nematode supports the hypothesis that this gene has a general role in modulating the plant's defense process rather than a role in nematode feeding site formation (Vanholme *et al*., 2009b; Jones *et al*., 2007).

The SPRYSECs are probably also involved in reducing the plant's defense responses as for one of the SPRYSECs it was shown that it can change the turnover rate of plant defense proteins (Rehman *et al*., 2009).

Potential candidate parasitism genes or effector genes were identified by three database searching strategies. These genes are interesting for future studies to elucidate more about the function of these genes, or to use as a target in nematode control. One promising control strategy is to disrupt the function of specific genes by RNAi, which has proven to be effective against nematodes (Rosso *et al*., 2009).

In conclusion, the transcriptome of *P. coffeae* is definitely useful to understand more about the biology of endoparasitic nematodes. It can be used for comparative and evolutionary studies as well as to select interesting new genes for functional studies. Moreover, the data will be valuable for the annotation of the upcoming genome (C. Opperman, personal communication).

CHAPTER FOUR

A Comparative study on RNAi effectors in the migratory endo–parasitic nematodes *Pratylenchus coffeae and Hirschmanniella oryzae*

4.1 Abstract

Despite the successful application of RNA interference in the free-living nematode, *C. elegans*, parasitic nematodes have shown variable susceptibility and efficiency towards RNAi. The aim of the study described in this chapter is to test if the differences in RNAi effector complements between *C. elegans* and plant-parasitic nematodes could have an impact on this discrepancy. In this study, a primary sequence similarity survey for orthologs of 77 *C. elegans* RNAi pathway proteins in three different species of plant-parasitic nematodes, *Pratylenchus coffeae*, *Meloidogyne incognita* and *Hirschmanniella oryzae*, was performed using the available genomic or transcriptomic data set for these nematodes. The results from this comparative study showed that all the nematodes under the study possess the basic machinery required to facilitate an RNAi response. However, some of the core proteins in the RNAi pathway were not identified in these nematodes. The core components required for the initiation of RNAi, the proteins responsible for uptake of dsRNA and spread of silencing signal, proteins associated with amplification of secondary siRNAs and heritability of RNAi events were not found in the nematodes. However, Argonautes (AGOs) responsible for endogenous RNAi seems to be well conserved among the nematodes. Based on the observations from the study, possible impacts on RNAi efficiency due to presence or absence of RNAi effector components in the nematodes have been discussed in this chapter. The insight gained from this survey can be utilized to understand the differences in RNAi pathway components between these nematodes and *C. elegans*. Additionally, this study might be useful to manipulate the RNAi response in the parasitic nematodes in order to improve the potency and efficiency of RNAi.

4.2 Introduction

RNA interference has been deployed as a successful tool to facilitate gene function studies in the free-living nematode, *Caenorhabditis elegans*. However, PPN have shown variable susceptibility and efficiency towards RNAi. While RNAi in *C. elegans* spreads throughout the organism and is transferred to its progeny, the RNAi effect in PPN is highly time limited. In our previous study (Chaper 2) the silencing effects of *Pc-pat-10* and *Pc-unc-87* were found to be transient whereas the silencing of these genes in *C. elegans* persists throughout the life of the nematode. This brings to the fore the need for further exploration on RNAi effector diversity among the PPN to give more insight into their inconsistent susceptibility and inefficiency to RNAi.

Here we investigated the differences in key RNAi pathway components among three nematode species*; Pratylenchus coffeae*, *Meloidogyne incognita* and *Hirschmanniella oryzae,* utilizing existing genomic and transcriptomic datasets. An extensive study has already been done on the diversity of RNAi effectors across different species of nematodes (Dalzell *et al*., 2011). Here the same criteria were adopted in *P. coffeae* and *H. oryzae* to investigate the occurrence of RNAi components to gain more understanding on the disparity in RNAi persistence and efficiency between *C. elegans* and these nematodes. A primary sequence similarity survey was done using 77 *C. elegans* orthologs of RNAi pathway proteins as query sequences in these three nematode species. The insight gained from this survey can be utilized to understand the differences in RNAi pathway components among these nematodes and also to manipulate the RNAi response in parasitic nematodes in order to improve the potency and efficiency of RNAi.

4.3 Materials and Methods

4.3.1 Reciprocal Blast methodology

Seventy-seven *C. elegans* proteins known to be essential components in the RNAi pathway were identified from literature (Dalzell *et al*., 2011). These proteins were separated into five core functional groups; namely, small RNA biosynthesis, dsRNA uptake and spreading, AGOs and RISC, RNAi inhibitors, and nuclear effectors. The *C. elegans* coding sequences were retrieved from WormMart (www.wormbase.org; release WS227) and used as search strings. A local tblastx (E<1e-5) was done with the *C. elegans* coding sequences as query and the assembled ESTs from *P. coffeae* and *H. Oryzae.* (unpublished data, Bauters *et al*., 2012). The genome database from *M. Incognita* (http://www.inra.fr/*Meloidogyne*_incognita/genomic_resources) was searched using Blastp to predicted protein sets, in addition to tblastn against the available contig assembly.To check if the resulting top hit was a true ortholog of the *C. elegans* gene, the resulting sequence was locally blasted (Blastx, E<1e-5) against all *C. elegans* proteins (wormpep release WS227).

4.4 Results

Each of the species considered possesses only a subset of the original search set of *C. elegans* RNAi proteins. Of the original 77 *C. elegans* search strings, *P. coffeae* and *H. oryzae* have 26 of the RNAi effector proteins while *M. incognita* possesses 30 of these proteins. This reduction could suggest that: (i) orthologs of the *C. elegans* protein may probably absent from these species; (ii) they may have diverged to such a degree that they are unrecognisable on a primary sequence level, or (iii) additional RNAi effector genes may await discovery in these species due to inadequate coverage.

The search results were placed in one of six functional groups based on the function of the genes in the RNAi pathway.

4.4.1 Small RNA biosynthesis

Proteins that are grouped under this dataset include RNase III enzymes (drosha, DRSH-1; pasha, PASH-1; Dicer, DCR-1), RNA helicases (Dicer-related helicases DRH-1 and -3), and exportins (XPO-1 and -3). They are essential components of small RNA-based genetic regulatory pathways and perform nuclear biosynthesis, nuclear export and cytoplasmic processing of small RNAs such as miRNAs and siRNAs. Of the nine proteins associated with this functional grouping in *C. elegans*, five orthologs were identified in *P. coffeae* and *H. oryzae* while seven were identified in *M. incognita*. Although most of the core proteins related to small RNA biosynthesis are well conserved in *P. coffeae*, the orthologs for Dicer related helicases, DRH-1 and 3, the dsRNA binding protein and Dicer-complex co-factor, RDE-4 and exportins, XPO-3 were not identified in *P. coffeae* (Table 4.1). However, the ortholog of DRH-3 was identified in *H. oryzae*, but the RNAse III enzyme required for nuclear biosynthesis, PASH-1 was not found in *H. oryzae* (Table 4.1). Notably, *M. incognita* was also found to be deficient in *rde-4* and *xpo-3* as in the other two nematode species.

Table 4.1. Small RNA biosynthetic proteins

		***C. elegans* orthologs**								
	Species	***drh-3***	***drsh-1***	***xpo-1***	***xpo-2***	***dcr-1***	***drh-1***	***pash-1***	***rde-4***	***xpo-3***
1	***P. coffeae***	nd	x	x	x	x	nd	x	nd	nd
2	***H. oryzae***	x	x	x	x	x	nd	nd	nd	nd
3	***M. incognita***	x	x	x	x	x	x	x	nd	nd

X' indicates presence of ortholog. nd' indicates not determined.

Table 4.2. SiRNA amplification and dsRNA uptake and spreading proteins

		***C. elegans* orthologs**										
		Amplification Proteins						**Spreading Proteins**				
	Species	***smg-2***	***smg-6***	***ego-1***	***rrf-3***	***rrf-1***	***smg-5***	***rsd-2***	***rsd-3***	***sid-1***	***rsd-6***	***sid-2***
1	***P. coffeae***	x	x	x	nd	nd	nd	nd	nd	nd	nd	nd
2	***H. oryzae***	x	nd	x	nd	nd	nd	nd	nd	nd	nd	nd
3	***M. incognita***	x	x	x	x	x	nd	nd	x	nd	nd	nd

X' indicates presence of ortholog. nd' indicates not determined.

4.4.2 Secondary siRNA amplification

Six *C. elegans* proteins involved in siRNA amplification were included under this subgrouping (Table 4.2). Interestingly, orthologs for the amplification proteins were not found in *P. coffeae* and *H. oryzae*. The RNA dependent RNA polymerase (RdRP), RRF-1 which is one of the core proteins responsible for siRNA amplification in somatic cells was not identified in *P. coffeae* and *H. oryzae* but was found in *M. incognita*. The RdRP, RRF-3 which is commonly referred to as an inhibitory RdRP of somatic RNAi was not identified in *P. coffeae* and *H. oryzae*, but was well conserved in *M. incognita*. Even though RRF-3 is considered as a potential RNAi inhibitor in exogenous RNAi, it has recently been proven to be required for biogenesis of 26G siRNAs in the endogenous RNAi pathway.

The ortholog of EGO-1, another RdRP expressed in germ cells of *C. elegans* was found in all three species studied. EGO-1 is an RdRP with core functions in transcription of ‘‘WAGO’’ (worm-specific AGO, Gu *et al*., 2009) -interacting 22G-RNAs responsible for silencing events involved in genome surveillance (Smardon *et al*., 2000; Vasale *et al*., 2010) and with additional roles in germline development (Vought *et al*., 2005), heterochromatin assembly (Maine *et al*., 2005; She *et al*., 2009), holocentric chromosome segregation (Claycomb *et al*., 2009), and P-granule function (Updike *et al*., 2009). The proteins involved with nonsense-mediated decay (NMD) such as SMG-2 (Suppressor with Morphological effects on Genitalia 2), -5, and -6 have an important role in the induction and maintenance of secondary amplification (Mango, 2001). Notably, SMG-2 and SMG-6 were well conserved in *P. coffeae* and *M. incognita* (Table 4.2). The ortholog for only one SMG protein (SMG-2) was found in the case of *H. oryzae*. However, the orthologs for SMG-5 was not identified in any of the three species.

4.4.3 dsRNA uptake and spreading

Five *C. elegans* genes putatively associated with dsRNA uptake and spread are included under this functional grouping. When *rsd-2, -3*, and *-6* are considered as the mediators of intercellular spreading of dsRNA, *sid-1* and *-2* are required for cell/environmental dsRNA uptake (Winston *et al*., 2002; Winston *et al*., 2007). Interestingly, none of the proteins were identified in *P. coffeae* and *H. oryzae*. However, the ortholog for *rsd-3* was found in *M. incognita* (Table 4.2).

4.4.4 Argonautes and RNA-induced silencing complex components

In *C. elegans,* 27 Argonautes (AGOs) that constitute the central effectors of the RNA Inducing Silencing Complex (RISC) have been identified. All of the nematode species in our dataset showed good conservation in AGOs including the miRNA interacting ALG-1 (Argonaute Plant–Like Gene), endo-siRNA (22G-RNA)-interacting WAGOs, R06C7.1 and also the worm-specific AGOs which mediate the exo-siRNA pathway, C04F12.1. However, some members of the PIWI-clade of AGOs, such as PRG-1 (Piwi-Related Gene 1), PRG-2, ERGO-1 (Endogenous RNAi deficient Argonaute 1) and the AGO/PIWI-clade secondary AGOs SAGO-1 and SAGO-2, are not well conserved in all three species of nematodes in the study.

Although the 26GRNA-interacting ALG-4 and T22H9.3 were identified in *M. incognita*, the orthologs for both AGOs were not recognized in *P. coffeae* and *H. oryzae*. , RDE-1, one of the main AGO involved in exogenous RNAi in *C. elegans*, was not identified in *P. coffeae* and *H. oryzae*. Additionally, the AGOs T23D8.7, T23B3.2, Y49F6A.1 were also not present within our parasite dataset (Table 4.3a).

Thus our study showed that the AGOs responsible for endogenous RNAi are well conserved in *P. coffeae*, *H. oryzae* and *M. incognita* while the AGOs required for exogenous RNAi are not well conserved among these nematodes. Interestingly, ERGO-1 which is involved in the function of endogenous siRNA populations in *C. elegans* (Gent *et al*., 2010) was not identified in all three nematode species.

RNAi persistence and heritability in *C. elegans* are mainly because of NRDE-3 which is responsible for nuclear translocation of RNAi triggers. The current study could not identify the ortholog for NRDE-3 in *P. coffeae* and *M. incognita* while it is well conserved in *H. oryzae* (Table 4.3b).The RISC proteins which comprise multiple dsRNA-binding proteins and exonucleases were not well conserved across the species analysed (Table 4.3b). Although an ortholog for TSN-1 (Tudor Staphylococcal Nuclease -1) (Caudy *et al*., 2003) was well conserved in all three species of nematodes, the orthologs for VIG-1 which regulates transition between larval and adult cellular fates through interaction with the *let-7* miRNA (Chan *et al*., 2008) was not identified in any of them. Among the ALG-interacting proteins which are responsible for targeting miRNA-bound ALGs to P bodies, AIN-1 (Ding *et al*., 2005; Zhang *et al*., 2007) was identified in *H. oryzae* and *M. incognita* while AIN-2, a paralogue for AIN-1, was completely absent from the three species.

Table 4.3a. Argonautes

		C. elegans orthologs Argonautes															
	species	**alg-1**	***R06C 7.1***	***C04F 12.1***	***F58G 1.1***	***alg-4***	***rde-1***	***C16 C10.3***	***ppw-1***	***sago-1***	***T22B 3.2***	***T22H 9.3***	***alg-2***	***ergo-1***	***prg-1***	***F55 A12.1***	***T23 D8.7***
1	***P. coffeae***	x	x	x	x	nd	nd	nd	nd	nd	nd	nd	x	nd	nd	nd	nd
2	***H. oryzae***	x	x	x	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	x	nd
3	***M. incognita***	x	x	x	x	x	x	nd	nd	nd	nd	x	x	nd	nd	nd	nd

X' indicates presence of ortholog. nd' indicates not identified

Table 4.3b Argonautes and RISC proteins

		***C. elegans* orthologs**											
		Argonautes								**RISC**			
	species	***nrde-3***	***sago-2***	***T23B3.2***	***Y49F6A.1***	***zk1248.7***	***prg-2***	***C06A1.4***	***C14B1.7***	***tsn-1***	***ain-1***	***vig-1***	***ain-2***
1	***P. coffeae***	nd	nd	nd	nd	x	nd	nd	nd	x	nd	nd	nd
2	***H. oryzae***	x	nd	nd	nd	x	nd	nd	nd	x	x	nd	nd
3	***M. incognita***	nd	nd	nd	nd	nd	nd	nd	nd	x	x	nd	nd

X' indicates presence of ortholog. nd' indicates not identified

4.4.5 RNAi inhibitors

Nine *C. elegans* proteins with RNAi inhibiting function are grouped under this dataset. Only two RNAi inhibitor orthologs, the DEDDh-like siRNA exonuclease ERI-1 (Kennedy *et al*., 2004) and the miRNA exonuclease XRN-2 (XRN RiboNuclease related 2), were fully conserved across all the parasitic species in question. However, an ortholog for the adenosine deaminases ADR-2 was found only in *H. oryzae*. The orthologs for other inhibitors such as ADR-1, LIN-15b, ERI-3, -5 and -6/7 were not identified in any of the three nematode species (Table 4.4).

Table 4.4. RNAi inhibitors

		***C. elegans* orthologs**								
	species	***eri-1***	***xrn-2***	***adr-2***	***xrn-1***	***adr-1***	***lin-15b***	***eri-5***	***eri-6/7***	***eri-3***
1	***P. coffeae***	x	x	nd	nd	nd	nd	nd	nd	nd
2	***H. oryzae***	x	x	x	nd	nd	nd	nd	nd	nd
3	***M. incognita***	x	x	nd	nd	nd	nd	nd	nd	nd

X' indicates presence of ortholog. nd' indicates not identified

4.4.6 Nuclear effectors

Nuclear effectors comprise those proteins that are involved with silencing events inside the nucleus. Of the 15 nuclear effectors identified in *C. elegans*, orthologs for 7 effectors were identified in the three parasitic nematode species in our study (Table 4.5).

We found that the nuclear effectors EKL-1 (Enhancer of KSR-1 Lethality-1 [KSR-1 is a Ras-ERK signaling scaffold protein] and EKL-4, CID-1 (Caffeine Induced Death homolog), GFL-1 (an ortholog of human GLIOMA-AMPLIFIED SEQUENCE-4), MES-2 (Maternal Effect Sterile-2) and RHA-1(orthologous to human RNA Helicase A) were the most highly conserved between species while EKL-5 and -6, MUT-7 (Mutator- 7) and -16, MES-3 and RDE-2 (RNAi Defective-2) were not found in the three species. It has been shown that MUT-7 and RDE-2 have a functional role in the amplification step of the RNAi pathway in *C. elegans* (Tops *et al*., 2005). An ortholog of MES-6 was identified in *P. coffeae* and *H. oryzae* but not in *M. incognita*. While a paralog of MUT-2 was found in *H. oryzae,* the ortholog of this effector was identified in *P. coffeae*. However, MUT-2 was not found in *M. incognita*. An ortholog of ZFP-1 (Zinc Finger Protein -1) which is associated with chromatin and required for RNAi (Chen *et al*., 2005a) was identified only in *P. coffeae* among the three species.

Table 4.5. Nuclear effectors

		***C. elegans* orthologs**														
	species	***mut-7***	***cid-1***	***ekl-1***	***gfl-1***	***mes-2***	***ekl-4***	***mes-6***	***rha-1***	***ekl-6***	***zfp-1***	***mut-2***	***ekl-5***	***mes-3***	***mut-16***	***rde-2***
1	***P. coffeae***	nd	x	x	x	x	x	x	x	nd	x	x	nd	nd	nd	nd
2	***H. oryzae***	nd	x	x	x	x	x	x	x	nd	nd	pg	nd	nd	nd	nd
3	***M. incognita***	nd	x	x	x	x	x	nd	x	nd	nd	nd	nd	nd	nd	nd

X' indicates presence of ortholog. nd' indicates not identified and 'pg' indicates presence of paralog

4.5 Discussion

Based on the comparative study of RNAi effectors it is evident that the migratory nematodes *P. coffeae* and *H. oryzae* possess the basic machinery required to facilitate an RNAi response. However some of the core proteins in the RNAi pathway were not identified in these nematodes. The core components required for the initiation of RNAi, RDE-1, a dsRNA binding protein and RDE-4, a PAZ-PIWI/Argonaute protein were not identified in *P. coffeae* and *H. oryzae*. RDE-4 is essential for recognition of the foreign dsRNA and the processing of dsRNAs into primary siRNAs. Together with RDE-1 it forms a complex with Dicer (DCR-1), a dsRNA specific RNaseIII ribonuclease, which is responsible for cleaving dsRNA into 21-25 nt siRNAs (Tabara *et al.*, 2002; Meister and Tuschl, 2004). The absence of RDE-4 from the three nematode species could raise a question on their ability to channel dsRNAs through a typical RNAi response. However, it is known that the *rde-4* mutant of *C. elegans* is not completely RNAi-defective. It is known that it is still possible to elicit an RNAi response in the *rde-4* mutant of *C. elegans* by introducing a higher concentration of the dsRNA trigger (Habig *et al*., 2008).

Sid-1 and *-2* have been shown to be essential for systemic RNAi by promoting dsRNA uptake and subsequent spread of dsRNA between cells. It has also been proven that *rsd-3* is involved in intercellular spread because *rsd-3* null mutants are able to take up dsRNA from gut lumen but they are not able to distribute this dsRNA into the germ line (Tijsterman *et al*., 2004). These core determinants of systemic RNAi were not identified in any of the three nematodes under the study.

Similarly, other effectors essential for RNAi spreading such as *rsd-2* and *-6* were also not identified in these nematodes. However, our previous study demonstrated high susceptibility of *P. coffeae* to RNAi by dsRNA soaking (Chapter 2). We have observed

efficient silencing of the genes expressed in different cells such as muscular and secretory gland cells. A number of studies have also demonstrated the successful application of RNAi in *M. incognita*. Similarly a recent study in *Drosophila* S2 cells, which lack *sid-1,* has shown that an efficient RNAi response can be induced in these cells by the uptake of dsRNA from the environment. In *Drosophila* this is possible because of an endocytic pathway that requires components such as clathrin heavy chain and V-ATPase (Saleh *et al*., 2006). This suggests that alternative uptake proteins or mechanisms might be involved in PPN also. Further poorly characterized morphological differences can be other factors which enable these nematodes to take up the dsRNA from the environment.

The highly efficient RNAi in *C. elegans* is possible because of the amplification of the silencing signals by two RNA directed RNA Polymerases (RdRPs). While *rrf-1* is required for production of secondary siRNA in the somatic cells (Sijen *et al.*, 2001), *ego-1* plays a similar role in the germ line (Smardon *et al.*, 2000). Interestingly, in our comparative study on the transcriptome the ortholog of *rrf-1* is not identified in *P. coffeae* and *H. oryzae* whereas an ortholog for *ego-1* is identified in these nematodes. Additionally, the *C. elegans* proteins MUT-7 and RDE-2 which are required for efficient RNAi (Tabra *et al*., 1999) are also not recognized in the transcriptome of *P. coffeae* and *H. oryzae*. The research study in *C. elegans* shows that the absence of either MUT-7 or RDE-2 leads to the reduced accumulation of siRNAs *in vivo* (Tijsterman *et al*., 2002; Sijen and Plasterk, 2003).

The other key components required for intercellular spreading of secondary siRNAs such as RSD-1, -2 and -6 and secondary siRNAs specific AGOs (SAGO-1 and-2) were also not identified in *P. coffeae*. Therefore it might be possible that the migratory nematodes *P. coffeae* and *H. oryzae* may lack an efficient siRNA amplification mechanism in somatic cells.

However, the amplification of the silencing signal may occur to some degree due to presence of *ego-1* in the germ cell of these nematodes. Additionally we observed that the RNAi of *Pc-pat-10* and *Pc-unc-87* in *P. coffeae* was time limited because both transcripts were recovered gradually 48h post dsRNA soaking (Chaper 2). Contrary to this, in *C. elegans*, the RNAi effect is even inherited to the F1 progeny (Chaper 2) and the silencing effect is usually stronger in progeny than in parents (Fire *et al*., 1998). Further the heritability of RNAi in *C. elegans* is also influenced by the occurrence of other factors such as NRDE-3. These proteins which are responsible for the heritability of silencing events are not identified in *P. coffeae*. This supports the notion that the silencing events may not be passed between the generations of *P. coffeae* unlike in *C. elegans*.

Among the proteins that constitute the RISC complex, only the gene for TSN-1 was found in the transcriptome of *P. coffeae*. PPW-1, the AGO essential for germ line RNAi which belongs to PAZ-PIWI subfamily, was also not found in *P. coffeae.* These two genes involved in exogenous RNAi were also not found in the *H. oryzae* and *M. incognita* datasets. However, dsRNA soaking in *P. coffeae* and *M. incognita* has resulted in successful RNAi response in those nematodes in spite of a few AGO components in those nematodes.

This could indicate the existence of gene redundancy in the function of individual AGOs in *C. elegans* while a reduced functionality in the parasitic nematodes. However, it is also possible that other uncharacterized AGOs with similar functional roles as those AGOs that are unidentified in the present study may exist in each nematode or it was not found just because of incomplete transcriptome. Although the AGOs responsible for endogenous regulation of gene expression such as ALG-1, R06C7.1, C04F12.1 and F58G1.1 are well conserved among the three studied nematodes, the AGO which has a key role in the function of endogenous siRNA population was not found in *P. coffeae*. This could imply that a population of small RNAs which is different from *C. elegans* might be present in the nematodes presented in the study.

Only two effectors antagonizing RNAi, *eri-1* and *xrn-2* are well conserved in the three nematode species studied here. One of the factors limiting RNAi in *C. elegans*, ADRs was not well conserved among the three analysed nematodes. However the absence of ADRs may not enhance exogenous RNAi in these nematodes because mutations in these genes did not enhance the sensitivity of RNAi triggered by exogenous dsRNA in *C. elegans*. Another antagonizing factor of RNAi*, lin-15B* which encodes Rb complex proteins was also not found in the three nematode species. Recent studies in *C. elegans* show that the absence of *lin-15B* enhances RNAi responses particularly in the nervous system (Wang *et al*., 2005).

It is known that highly differentiated neuronal cells are resistant to RNAi in *C. elegans*. The presence of RNAi inhibiting proteins in neuronal cells such as ERI-1, RRF-3 and LIN-15B is one of the reasons for this RNAi resistance in *C. elegans* (Asikainen *et al.,* 2005; Calixto *et al*., 2010). Interestingly, *rrf-3* and *lin-15B* were not found in the transcriptome of *P. coffeae* and *H. oryzae*.

In addition, unlike in *C. elegans* the *flp* genes expressed in neuromuscular cells are readily susceptible to RNAi in the plant-parasitic nematode *G. pallida* (Kimber *et al*.,

2007). However, it has been shown that RNAi sensitivity can be a property of the particular neuronal cell depending on the expression of other antagonizing factors such as *eri-1*, *lin-15B* or the factor required for systemic RNAi, *sid-1* in the cells (Asikainen *et al.,* 2005; Calixto *et al*., 2010; Zhuang and Hunter, 2011b). This implies the necessity to understand the cellular location of the proteins influencing RNAi in the parasitic nematodes.

Besides, our study displays a greatly contracted suite of RNAi effector proteins in the three nematode species presented here. For example, only one RdRP, EGO-1, which is required for germline RNAi is present in *P. coffeae* and *H. oryzae*. However, it is possible that EGO-1 can act redundantly in somatic tissues like RRF-1 in these nematodes because it has been shown that EGO-1 can function redundantly in somatic tissues of *C. elegans* when LIN-15B is absent from the nematode (Wang *et al*., 2005). Notably LIN-15B was not found in *P. coffeae* and *H. oryzae*. This indicates the need for a careful characterization of RNAi effectors in PPN to understand their expression level and distribution to see if it is different from *C. elegans*.

The study on the genes that impinge on the RNAi process shows the complexity of RNAi and its many cell biological roles, which will remain a subject for future study. With the information obtained from *C. elegans* to identify and deduce the function of genes involved in RNAi and the identification of its orthologs in PPN, it might be possible to manipulate the RNAi process in PPN. The expression of a gene could be enhanced or inhibited or restricted by cell type or time for improving the potency and persistence of the RNAi response.

CHAPTER FIVE

Silencing the RNAi effector *Pc-eri-1* can improve the efficiency and persistence of *Pc-pat-10* RNAi in *Pratylenchus coffeae*

5.1 Abstract

RNA interference is extensively used as sequence-specific tool in a wide variety of organisms to investigate the function of a gene by generating a knock-down phenotype. Although RNAi has greatly accelerated the analysis of loss-of –function phenotypes in the free-living nematode *C. elegans*, variable levels of success in RNAi have been observed across parasitic nematode species. The identification of genes that are essential for RNAi or that modulate the RNAi process has made it possible to manipulate and enhance the RNAi process. In the present study we attempted to analyze an exogenous RNAi inhibitor, *Pc-eri-1* and an RNAi enhancer, *Pc-gfl-1* that affect siRNA accumulation and RNAi effectiveness in the plant-parasitic nematode *Pratylenchus coffeae.* Therefore the method of combinatorial RNAi was used for simultaneously targeting two genes. The dsRNA of *Pc-eri-1* or *Pc-gfl-1* were co-introduced with dsRNA of the target genes, *Pc-pat-10*, *Pc-unc-87* or *Pc-eng-1* as a means of enhancing RNAi persistence in *P. coffeae*. Our results demonstrated that an enhanced RNAi persistence of *Pc-pat-10* can be achieved when *Pc-eri-1* is co-silenced. However, the silencing of *Pc-eri-1* did not show similar impact in the other genes tested and hence the down regulation of *Pc-eri-1* may only enhance the RNAi of some specific genes. Similarly to *Pc-eri-1*, the impact of *Pc-gfl-1* on RNAi was also gene-specific. Among the target genes tested here, only *Pc-pat-10* seems to be positively regulated by *Pc-gfl-1*. In summary, our findings presented here suggest that it might be possible to enhance RNAi persistence of specific genes by down regulating *eri-1* along with the target gene. Additionally the combinatorial RNAi can be a possible approach to characterize genes involved in the RNAi pathway and also a means to manipulate the RNAi response in PPN.

5.2 Introduction

Contrary to the high efficiency of RNAi in *C. elegans,* RNAi experiments in parasitic nematodes have also shown inefficient and inconsistent transcript knock-down of the target genes (Geldhof *et al.*, 2006a; Haegeman *et al.,* 2009b; Lilley *et al*., 2012). Further, differences in the susceptibilities of individual genes to RNAi have also been observed within the same nematode genus (Kimber *et al.*, 2007). It has been shown that dsRNA vary in their ability to trigger RNAi and some mRNA sequences are not effectively targeted for dsRNA-guided degradation (Cutter *et al.*, 2003; Timmons *et al.*, 2001). This shows that a better understanding of all the factors that influence successful gene silencing is still required to achieve further progress in the application of RNAi technology in nematodes.

A number of factors can be suggested to explain the intrinsic resistance and variable susceptibility to RNAi which includes variation in *in vitro* delivery methods, characteristics of inducing molecules and differences in RNAi effector protein functionality and complement of RNAi effectors between nematodes (Maule *et al,* 2011; Lendner *et al*, 2008; Viney and Thompson, 2008). The comparative genomic analysis between *C. elegans* and parasitic nematodes has identified differences in key components of the RNAi pathway between the parasitic nematodes and *C. elegans* (Rosso *et al*, 2009; Dalzell *et al*., 2011). The present study utilizes the existing information on components of RNAi effectors in *P. coffeae* to explore the influence of the RNAi regulators on effective knock-down of the target genes.

The previous study (Chapter 2) demonstrated that *Pc-pat-10* and *Pc-unc-87,* orthologs of *C. elegans* genes that are required for muscle function and locomotion, are highly susceptible to RNAi. The down regulation of these genes in *P. coffeae* resulted in loss-of-function phenotypes showing impaired locomotion.

However, the nematodes gradually recovered from the RNAi effect after removal of the dsRNA which is in contrast with dsRNA-treated *C. elegans* nematodes where RNAi effect persists throughout the life of the worm. The efficacy and persistence of RNAi in *C. elegans* is partly due to transitive RNAi in which the silencing spreads along the target mRNA from the primary RNAi trigger and the production of secondary siRNAs by RNA dependent RNA polymerase (RdRP) (Chapter 1, Section 1.6.1). Additionally, it has been shown that mutation of some genes (*Eri* genes) enhances RNAi efficiency in *C. elegans*. (Chapter 1, Section 1.6.1.3) The transcriptome data of *P. coffeae* (Chapter 4) shows that *P. coffeae* seems to have ortholog for only *eri-1* among the *Eri* class genes while orthologs for *rrf-1*, which encodes an RdRP in somatic cells was not recognized in the transcriptome analysis.

In *C. elegans* the *eri-1* mutant shows an enhanced RNAi response to different dsRNA against neuronal and non-neuronal gene targets (Kennedy *et al.*, 2004; Zhuang and Hunter, 2011b). Therefore we reasoned that if *eri-1* is down regulated, the RNAi response in *P. coffeae* can be enhanced and as a result the silencing effect may persist for a longer period of time. To verify this hypothesis we used the method of combinatorial RNAi for simultaneously targeting two genes. In our present study, the dsRNA of *Pc-eri-1* was co-introduced with dsRNA of the target genes, *Pc-pat-10*, *Pc-unc-87* or *Pc-eng-1* as a means of enhancing RNAi persistence in *P. coffeae*. Additionally, we also checked the requirement of *Pc-gfl-1,* an ortholog of the *C. elegans* nuclear RNAi effector, for effective silencing in *P. coffeae*.

The *gfl-1* of *C. elegans* encodes an ortholog of human Gloma-Amplified-Sequence-41 which is predicted to be associated with chromatin. It has been shown that the loss of *gfl-1* results in decreased RNAi in *C. elegans* (Dudley *et al.*, 2002).

The co-introduction of dsRNA of *Pc-gfl-1* with the dsRNA of the target genes may result in suppression of RNAi of the target gene. It has been proven in *C. elegans* and *Drosophila* that introduction of multiple dsRNAs can be used as an efficient method to identify genes involved in RNAi (Dudley *et al.*, 2002; Geldhof *et al.*, 2006b; Hammond *et al*., 2000; Schmid *et al.*, 2002). Although Bakhetia *et al.* (2008) tested the possibility of silencing two different genes expressed in dorsal esophageal glands of *H. glycines* simultaneously to check the additive phenotype effect using combinatorial RNAi; this is the first report of using this method in a plant-parasitic nematode to analyze the role of RNAi regulators in efficient RNAi. At present we know very little of the relative importance of the primary and secondary RNAi responses and its regulation in parasitic nematodes. Therefore our attempt to analyze RNAi regulators that affect siRNA accumulation and RNAi effectiveness may help to understand the underlying mechanism that influences the RNAi efficiency in *P. coffeae.*

5.3 Materials and methods

5.3.1 Nematode collection, RNA isolation and cDNA synthesis

A *P. coffeae* population from Ghana was maintained and multiplied as described in Chapter 2, Section 2.3.1. All the stages of nematodes including eggs, juveniles, female and male adults were collected from the discs. RNA was extracted from the mixed stages with Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was treated with DNase (Fermentas, St-Leon-Rot, Germany) to avoid DNA contamination.

First strand cDNA was synthesized using Super ScriptIII Reverse Transcriptase (Invitrogen). The reaction mixture contained 4mM dNTPs, 0.5µM oligodT primer, 10mM

DTT, 3mM $MgCl_2$ and 200U Super ScriptIII Reverse Transcriptase. The mixture was incubated for 2h at 42°C.

5.3.2 Synthesis of dsRNA and siRNA

Utilizing existing information of *P. coffeae* EST data, parts of five target genes namely *Pc-pat-10* (448bp*); Pc-eri-1* (222bp), *Pc-gfl-1* (206bp)*, Pc-unc-87* (350bp*)* and *Pc-eng-1* (332bp) were amplified from cDNA of the nematodes freshly isolated from the carrot discs with the following cycling conditions: 95°C X 5min, followed by 35 cycles of 95°C X 15s, 55°C X 30s, 72°C X 1min. The target sequences were cloned into the pGEM-T vector and confirmed by sequencing. The templates of sense and antisense DNA strands for generating dsRNA were amplified from the vector carrying the inserts of each target gene by PCR under standard conditions using the respective primers with the T7 promoter sequence incorporated at the 5'end of either the sense or antisense strand (Table 5.1).

The PCR products were transcribed *in vitro* and the dsRNAs of each target gene were synthesized using the MEGAscript RNAi kit (Ambion, Huntingdon, UK) following the manufacturer's instructions. The dsRNA was quantified spectrophotometrically and the quality of the dsRNA was checked on a 1% agarose gel. Three different siRNAs (21bp) against *Pc-pat-10* namely *Pc-patS1, Pc-patS2 a*nd *Pc-patS3* were selected from the sequence region that was used for dsRNA (Table 5.2). A 21 bp sequence starting with motif 'AA' and of G+C content between 35-60% was selected for the construction of siRNAs. The sequences were also blasted against the existing EST data to check the specificity of the sequence to avoid off target effects. The siRNAs were synthesized by *in vitro* transcription using the Silencer siRNA construction kit (Ambion, Huntingdon, UK) according to manufacturer's instructions. The quantity and quality of siRNA was measured spectrophotometrically.

Table 5.1. Primers used for amplifying dsRNA templates and semi-quantitative RT-PCR

Primers	Sequence
Pc-patT7F	TAATACGACTCACTATAGGGAGACGAACTCGATTTTCCCACTC
Pc-pat R	CCCCAAATTGAGGAGTACCA
Pc-patF	CGAACTCGATTTTCCCACTC
Pc-patT7R	TAATACGACTCACTATAGGGAGACCCCAAATTGAGGAGTACCA
Pc-patF1	GGCCATCAACTCCCAGAAT
Pc-unc T7F	TAATACGACTCACTATAGGGAGACCGGGTTGATGACCAACTT
Pc-unc R	TTTGCGAGTCGAATTTGTTG
Pc-uncF	CCGGGTTGATGACCAACTT
Pc-uncT7R	TAATACGACTCACTATAGGGAGATTTGCGAGTCGAATTTGTTG
Pc-uncF1	GCTCTGATCAATGCTGCGC
Pc-engT7F	TAATACGACTCACTATAGGGAGATCTTCCCAAGAATGGTGGAC
Pc-engR	ACCGAGATTGAGGCAGACAC
Pc-engF	TCTTCCCAAGAATGGTGGAC
Pc-engT7R	TAATACGACTCACTATAGGGAGAACCGAGATTGAGGCAGACAC
Pc-engR1	GGTGAATCCCGTGTCTTGAT
Pc-eri-1T7F	TAATACGACTCACTATAGGGAGAAACAGGAATTACACAGG
Pc-eri-1R	GCG CAA ATC GTG GGG CA
Pc-eri-1F	AACAGGAATTACACAGG
Pc-eri-1T7R	TAATACGACTCACTATAGGGAGAGCG CAA ATC GTG GGG CA
Pc-eri-1F1	GCAGTGGTATCAACGCAGAG
Pc-gfl-1T7F	TAATACGACTCACTATAGGGAGAGAGACTGGATGGGGCGAATT
Pc-gfl-1R	ATC AAA GCC CGA TAC ATC GGC
Pc-gfl-1F	GAGACTGGATGGGGCGAATT
Pc-gfl-1T7R	TAATACGACTCACTATAGGGAGAATC AAA GCC CGA TAC ATC GGC
Pc-gfl-1R1	TCACGCAATGTCCTCTTCAG
Pc-actinF	CCTCTTCCAGCCTTCCTTCT
Pc-actinR	CACCGATCCAGACGGAGTAT

5.3.3 *In vitro* RNAi Analysis

Approximately 700 nematodes (juveniles and adults) of *P. coffeae* freshly isolated from carrot discs (not older than 2 months) were soaked in 1µg/µl dsRNA diluted with sterilized water to 50µl total volume along with 50mM octopamine and 3mM spermidine. As a negative control the nematodes were incubated in the same solution but without dsRNA (non-RNAi).

For the combinatorial RNAi, an equal amount of dsRNA of the target genes was used keeping the final concentration at 1µg/µl in a total volume of 50µl. The juveniles of *P. coffeae* were soaked for 24h in a solution containing dsRNA of the gene of interest such as *Pc-pat-10*/*Pc-unc-87*/*Pc-eng-1* and also in combination with the dsRNA of *Pc-*

eri-1/Pc-gfl-1. Following 24h of exposure of the nematodes to the dsRNA, they were allowed to recover by transferring them into water on a rotator in the dark for 48h. This assay was repeated three times. In a different set up, the nematodes were soaked with dsRNA of *Pc-eri-1* for 24h prior to the incubation with *Pc-patS1* siRNA for 24h at 27°C in the dark.

The same methodology was followed for the soaking assay with other siRNAs. The soaking was done at three concentrations of siRNA, 50ng/µl, 100ng/µl and 200ng/µl in a total volume of 50µl and repeated two times. The soaking was also done in combination with the siRNA of *eri-1* for all treatments keeping the final concentration at 200ng/µl, this was repeated three times. RNA extraction and cDNA synthesis were carried out as mentioned above. The expression levels of the target genes were measured by a semi quantitative RT-PCR on the nematodes after 24h exposure to dsRNA and after 48h recovery in water and the transcript levels compared between the control (non-RNAi) and dsRNA-treated nematodes.

As a reference gene for normalizing the quantity of cDNA in each sample *Pc-actin* was amplified using the primers Pc-actinF and Pc-actinR. Primers were designed to bind outside the dsRNA region were used for amplifying each target gene. For checking the expression level of *Pc-pat-10*, the combination of the primers Pc-patF1 and Pc-patR was used while *Pc-unc-87* was amplified using Pc-uncF and Pc-uncF1. The amplification of *Pc-eng-1* and *Pc-eri-1* was done using the primer combinations Pc-engF and Pc-engR1, Pc-eri-1F and Pc-eri-1R, respectively.

The primers Pc-gfl-1F and Pc-gfl-1R1 were used for the amplification of *Pc-gfl-1* from the treated nematodes (Table5.2). The number of PCR cycles was optimized for each gene product to detect the exponential phase of the reaction. The resulting PCR products were separated on a 0.5X TAE gel containing 1.5% agarose.

Table 5.1. Short interfering sequences (siRNAs) selected against *Pc-pat-10* for RNAi in *P. coffeae*

siRNA	sequence	GC%	Homology	Sense strand	Antisense strand
Pc-patS1	AACTCCCAGAATTCCTCGAAC	47.6	*C .elegans* and *P .vulnus*	CUCCCAGAAUUCCUCGAACUU	GUUCGAGGAAUUCUGGGAGUU
Pc-patS2	AATTCGTCGAATTCGATTTTG	33.3	*M. incognita*	UUCGUCGAAUUCGAUUUUGUU	CAAAAUCGAAUUCGACGAAUU
Pc-patS3	AATTTGCGAATCAATTTTCGC	33.3	NS	UUUGCGAAUCAAUUUUCGCUU	GCGAAAAUUGAUUCGCAAAUU

5.3.4 Scoring of RNAi impact on phenotype and nematode movement

Approximately 1500 active nematodes freshly isolated from carrot discs (juveniles and adults) were incubated in dsRNA of *Pc-pat-10*, *Pc-eng-1*, and *Pc-eri-1* and also with dsRNA combinations of *Pc-pat-10* and *Pc-eri-1* as mentioned above. *Pc-eng-1* and *Pc-eri-1* dsRNAs were used as negative dsRNA controls for checking non-specific inhibitory action of dsRNA on nematode motility. Additionally the nematodes were soaked in the same solution but without dsRNA as non-RNAi control. The soaking assay was also done with siRNA of *Pc-patS1* at a concentration of 200ng/µl. Following the incubation, the nematodes were washed and diluted to 500µl with sterile water. Five aliquots (5µl) were taken for each sample and observed under an S8APO stereomicroscope (Leica). The pharyngeal pumping of the nematode was observed to distinguish dead and paralyzed (*pat*) nematodes. The percentage of the nematodes showing '*pat*' phenotype was counted in each treatment. The experiment was repeated two times.

5.4 Results

5.4.1 RNAi of *Pc-pat-10* persists after 48h of recovery following the combinatorial soaking with *Pc-eri-1* dsRNA

In previous experiments (Chapter 2) RNAi was observed to be transient in *P. coffeae*. In contrast to *C. elegans*, the RNAi effect did not persist in the nematodes after withdrawal of dsRNA of *Pc-pat-10* and *Pc-unc-87*. Both transcript levels showed a significant transcript recovery within 24 hours of removal of dsRNA. To test the influence of *Pc-eri-1* on the persistence of RNAi, *in vitro* RNAi was performed with individual dsRNA of *Pc-pat-10* in combination with the dsRNA of *Pc-eri-1.*

The semi-quantitative RT-PCR showed that when the nematodes were treated with the dsRNA of *Pc-pat-10* alone, *Pc-pat-10* mRNA level was significantly reduced after 24h in contrast to non-RNAi controls, however, recovery of the transcript was observed after 48h recovery in water (Figure. 5.1A).

On the other hand, when the nematodes were treated in a combinatorial soaking with dsRNA of *Pc-pat-10* and *Pc-eri-1*, the transcript level of *Pc-pat-10* was drastically reduced not only after 24h of dsRNA exposure but also after 48h of recovery. The mRNA level of actin (Figure. 5.1) and *Pc-unc87* (data not shown) remained unaffected in all treatments indicating a gene specific RNAi effect on *Pc-pat-10*. In the combinatorial soaking, there was no clear reduction in the transcript level of *Pc-eri-1* after 24h of dsRNA exposure but a significant down regulation of *Pc-eri-1* was observed after 48h of recovery. However, in the treatment with the dsRNA of *Pc-pat-10* alone, a slight up regulation of *Pc-eri-1* mRNA level was observed after 48h of recovery.

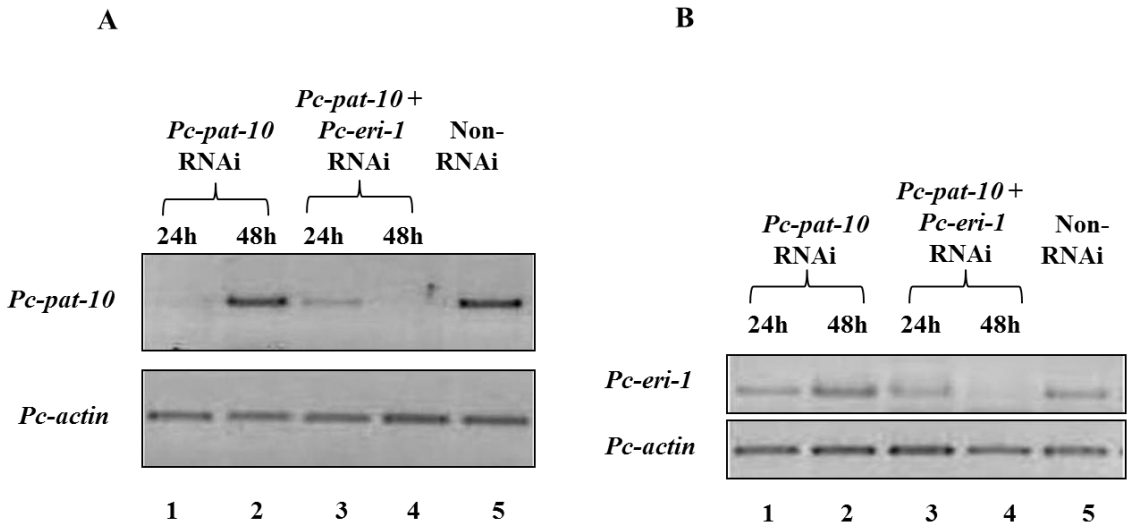

Figure 5.1. (A). RT-PCR showing the mRNA level of *Pc-pat-10* after soaking with dsRNA of *Pc-pat-10* alone and in combination with dsRNA of *Pc-eri-1*. (B) RT-PCR showing the mRNA level of *Pc-eri-1* after soaking with dsRNA of *Pc-pat-10* alone and in combination with dsRNA of *Pc-eri-1*. (24h) and (48h) indicate the soaking assays with dsRNA for 24h and recovery in water without dsRNA for 48h following the soaking assay, respectively. The soaking experiment was repeated twice 5.4.2. RNAi of *Pc-eri-1* enhanced the sensitivity of *Pc-pat-10* to siRNA.

5.4.2. RNAi of *Pc-eri-1* enhanced the sensitivity of *Pc-pat-10* to siRNA.

As a next step experiments were performed to compare dsRNA mediated silencing and siRNA mediated silencing and the influence of *eri-1*. For this purpose three concentrations of the S1 siRNA (50ng/µl, 100ng/µl, and 200ng/ml) of *Pc-pat-10* were tested following the same methodology as for the dsRNA soaking. There was no significant degree of knock-down in *Pc-pat-10* expression at any of the tested concentrations (Figure. 5.2A). In the next step experiment, three different siRNAs against the target gene were used and soaking was done with individual siRNAs as well as with all combinations of siRNAs for checking the knock-down efficacy.

The RT-PCR on cDNA of the nematodes from all treatments showed that there was no significant reduction in the mRNA level of *Pc-pat-10* (Figure. 5.2B) and very few

nematodes with abnormal phenotype were observed after 24h of soaking with the siRNA.

Since the combinatorial soaking with dsRNA of *Pc-eri-1* enhanced the RNAi persistence, the down regulation of *eri-1* prior to siRNA soaking might improve the RNAi efficacy. The soaking with dsRNA of *Pc-eri-1* alone for 24h showed significant reduction in the transcript level of the *Pc-eri-1* and the knock-down effect was still very clear after 48h of recovery in water (Figure. 5.2C). Therefore the nematodes were treated with *Pc-patS1* at the concentration of 200ng/µl following the soaking with dsRNA of *Pc-eri-1* for 24h. Notably, a significant knock-down of *Pc-pat-10* was observed after the siRNA soak (Figure. 5.2D). Thus silencing of *Pc-eri-1* has considerably enhanced the knock-down efficacy of *Pc-pat-10* siRNA. However, the silencing of *Pc- eri-1* did not influence the silencing efficacy of *Pc-patS2* and *Pc-patS3* (data not shown)

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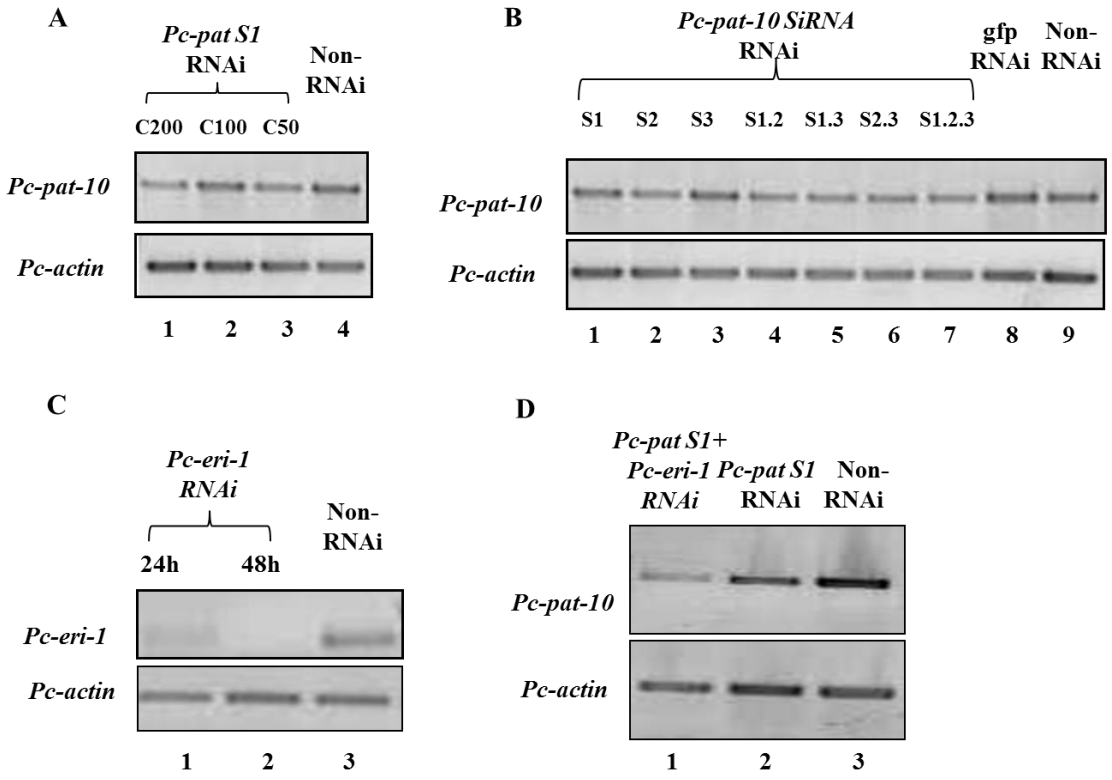

Figure 5.2. (A) RT-PCR showing amplification of *Pc-pat-10* at three concentrations of *Pc-patS1* siRNA (50ng/µl, 100ng/µl and 200ng/µl) (B) RT-PCR showing amplification of *Pc-pat-10* in all combinations of siRNAs at 200ng/µl; (S_1,S_2,S_3,$S_{1.2}$,$S_{1.3}$,$S_{2.3}$,$S_{1.2.3}$) indicate all combinations of the three siRNAs of *Pc-pat-10* (C) RT-PCR showing amplification of *Pc-eri-1* after 24h of dsRNA soaking with dsRNA of *Pc-eri-1* and a 48h recovery period. (D) RT-PCR showing amplification of *Pc-pat-10* after soaking with *Pc-patS1* alone for 24h and also in combination with dsRNA of *Pc-eri-1.* Each soaking assay was done two times

5.4.3 Recovery from the RNAi-induced pat phenotype is delayed following the combinatorial soaking of *Pc-pat-10* and *Pc-eri-1* dsRNAs.

To check if the RNAi persistence of the *Pc-pat-10* transcript level after combinatorial soaking with *Pc-eri-1* is also reflected in the prolongation of the RNAi phenotype after 48h recovery, another independent soaking assay was done.

Earlier reports on *in vitro* RNAi in *G. pallida*, a cyst nematode and *M. incognita*, a root-knot nematode have shown that high concentrations of non-specific dsRNA impair movement of the nematodes (Dalzell *et al*., 2009). Therefore, we included a control by soaking nematodes in, a gene that is expressed in the subventral glands and encodes an endoglucanase. As shown in Table 5.3, nematodes soaked in dsRNA of *Pc-eng-1* did not show any difference from the control nematodes in water.

Table 5.2. Visual scoring of 'pat' phenotype estimating the effect of *Pc-pat-10* dsRNA

Incubation period	dsRNA/siRNA	%score 'pat' phenotype
24h	Water	-
48h	Water	-
24h	*Pc-pat-10* dsRNA	65.2±8.3
48h	*Pc-pat-10* dsRNA	32.5±8.5
24h	*Pc-eng-1* dsRNA	-
48h	*Pc-eng-1* dsRNA	-
24h	*Pc-pat-10*+*Pc-eri-1*dsRNA	63.5±6.4
48h	*Pc-pat-10*+*Pc-eri-1*dsRNA	57.3±8.1
24h	*Pc-pat-10* siRNA	12.0±4.1
48h	*Pc-pat-10* siRNA	10.0±4.5
24h	*Pc-eri-1* dsRNA	-
48h	*Pc-eri-1* dsRNA	-

Nematodes were incubated in dsRNA of Pc-pat-10, Pc-eng-1, and Pc-eri-1 and also with dsRNA combinations of Pc-pat-10 and Pc-eri-1 for 24h and recovered for 48h in water without dsRNA and other soaking components. The percentage of the nematodes showing 'pat' phenotype was estimated in each treatment. The experiment was done twice keeping all parameters consistent. Pc-eng-1 and Pc-eri-1 dsRNAs were used as negative dsRNA controls for checking non-specific inhibitory action of dsRNA on nematode motility. Additionally the nematodes were soaked in the same solution but without dsRNA as non-RNAi control. Since the soaking assay with Pc-pat-10 siRNAs did not elicit a significant knock-down effect, the soaking assay was also done with the siRNA, Pc-patS1 at a concentration of 200ng/µl as an additional reference for scoring the phenotype. Approximately 700 nematodes were used for each treatment. The experiment was repeated two times.

As indicated in Table 3, 65.2±8.3% of the nematodes incubated in dsRNA of *Pc-pat-10* alone for 24h, and 63.5±6.4% of the nematodes incubated with dsRNA of *Pc-pat-10* and *Pc-eri-1* showed the 'paralyzed' phenotype.

While 32.5±8.5% of the nematodes treated with dsRNA of *Pc-pat-10* showed the RNAi phenotype after the 48h recovery period, 57.3±8.1% of the nematodes treated with dsRNAs of *Pc-pat-10* and *Pc-eri-1* showed the 'paralyzed phenotype. This indicates that about 50% of the nematodes recovered from the RNAi effect in the treatment without the silencing of *Pc-eri-1* whereas only approximately 10% of the nematodes were able to recover when *Pc-eri-1* was down regulated. The incubation of the nematodes in water or in dsRNA against *Pc-eng-1* and *Pc-eri*-1 did not elicit any inhibitory effect on nematode phenotype. The transcript level of both genes was checked by semi quantitative RT-PCR (data not shown) and the results were similar to the experiment described above.

5.4.4 A decline in *Pc-gfl-1* expression suppresses the RNAi of *Pc-pat-10*

If *Pc-gfl-1* is a requisite for RNAi in *P. coffeae*, the silencing of *Pc-gfl-1* might influence the RNAi of *Pc-pat-10*.To test this hypothesis, the nematodes were subjected to combinatorial soaking with dsRNA of *Pc-gfl-1* and *Pc-pat-10*. The nematodes were soaked for 24h in the dsRNA solution, removed and allowed to recover for 48h in water. The expression of *Pc-pat-10* and *Pc-gfl-1* was measured by semi-quantitative PCR.

The expression of *Pc-gfl-1* mRNA was significantly down regulated after 24h of soaking. The transcript was still severely silenced after 48h of recovery. However, there was no significant reduction in the transcript level of *Pc-pat-10* either after 24h or after 48h of recovery (Figure. 5.3). This suggests that dsRNA of *Pc-gfl-1* is a potent suppressor of *Pc-pat-10* RNAi and hence *Pc-gfl-1* itself could be an enhancer of RNAi.

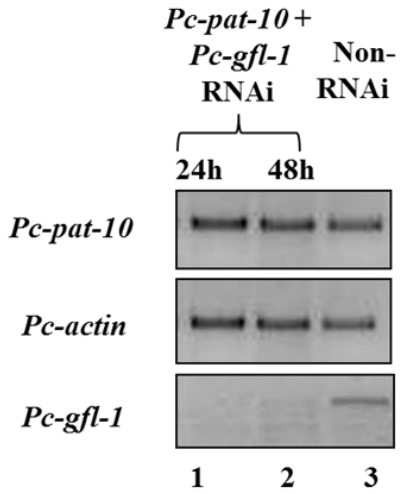

Figure 5.3. RT-PCR showing the mRNA level of *Pc-pat-10* and *Pc-gfl-1* after soaking with dsRNA of *Pc-pat-10* in combination with dsRNA of *pc-gfl-1.* (24h) and (48h) indicates the soaking assays *with* *dsRNA* for 24h and recovery in water *without* *dsRNA* for 48h following the soaking assay, respectively. As a reference gene for normalizing the quantity of cDNA in each sample *Pc-actin* was amplified. The soaking assay was done two times.

5.4.5 The silencing of *Pc-eri-1* and *Pc-gfl-1* did not influence RNAi of *Pc-eng-1*

To determine whether the silencing of *Pc-eri-1* and *Pc-gfl-1* has similar influence on RNAi of other target genes, the combinatorial soaking assay was repeated with dsRNA of *Pc-unc-87* and also with *Pc-eng-1*. The soaking with dsRNA of *Pc-eng-1* alone and also in combination with dsRNA of *Pc-eri-1* as well as *Pc-gfl-1* was done as mentioned previously. The semi quantitative RT-PCR was done on the cDNA isolated from the nematodes incubated in the dsRNA for 24h and also from those incubated in water for 48h of recovery. A very strong reduction in the mRNA level of *Pc-eng-1* was found after 24h of soaking with dsRNA of *Pc-eng-1* alone and this silencing effect strongly persisted even after 48h of recovery.

This was in contrast with the observation in the RNAi of *Pc-pat-10* where a significant recovery of the transcript was found after 48h of recovery.

The combinatorial soaking with *Pc-eri-1* also showed significant silencing of *Pc-eng-1* following the 24h of soak as well as 48h of recovery (Figure. 5.4A). However, this result does not allow coming to a conclusion on a possible influence of *Pc-eri-1* on the silencing of *Pc-eng-1*. The combinatorial soaking with dsRNA of *Pc-gfl-1* also resulted in pronounced knock-down of *Pc-eng-1* (Figure. 5.4B). Thus, dsRNA of *Pc-gfl-1* was not able to suppress the silencing of *Pc-eng-1* despite the silencing of *Pc-gfl-1*. Similarly the experiment was repeated for dsRNA of *Pc-unc-87* and the silencing of neither *Pc-eri-1* nor *Pc-gfl-1* did influence the RNAi of *Pc-unc-87* (results not shown).

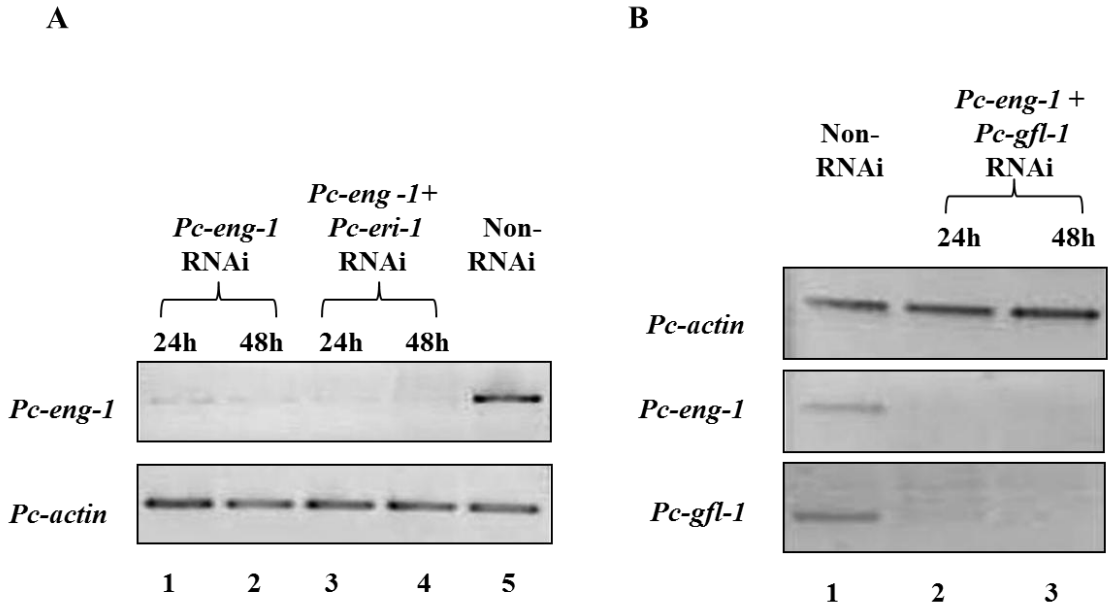

Figure 5.4. (A) RT-PCR showing the mRNA level of *Pc-eng-1* after soaking with dsRNA of *Pc-eng-1* alone and in combination with dsRNA of *Pc-eri-1.*(B) RT-PCR showing the mRNA level of *Pc-eng-1 and Pc-gfl-1* after soaking with dsRNA of *Pc-eng-1* in combination with dsRNA of *Pc-gfl-1.* (24h) and (48h) indicates the soaking assays *with* ***dsRNA* for 24h and recovery in water *without* ***dsRNA*** for 48h following the soaking assay, respectively. The assay was performed two times.**

5.5 Discussion

The susceptibility to RNAi has been tested across various species of PPN including sedentary endoparasitic and migratory nematodes (Cheng *et al.,* 2010; Dalzell *et al.*, 2010a; Haegeman *et al*., 2009b; Kang *et al*., 2011; Vanholme *et al.,* 2007; Visser *et al.*, 2006). In our previous study (Chapter 2) it was demonstrated that the migratory nematode *P. coffeae* has a functional RNAi machinery by silencing two *P. coffeae* orthologs of *C. elegans* genes, namely *pat-10* and *unc-87.* We have shown that the introduction of dsRNA, through soaking, results in significant reduction of the targeted mRNA, and this reduction is sufficient to cause loss-of-function phenotype. However, a profound recovery of the two targeted transcripts and the RNAi phenotype in both cases was observed after 24h of recovery in water.

To identify the possible involvement of RNAi effectors in this recovery we investigated the influence of two *P. coffeae* orthologs of *C. elegans* genes involved in RNAi regulation, namely *eri-1*and *gfl-1.*

Our hypothesis to explain the recovery mechanism in *P. coffeae* was that the generation of secondary siRNAs may not be very efficient in somatic cells of the nematode because most of the proteins linked to the amplification of secondary siRNAs in soma are poorly conserved in *P. coffeae*. Consequently, siRNA population available for the effective silencing is probably limited and the nematode may require a high dose of siRNAs to elicit an efficient silencing. By down regulating *Pc-eri-1* it might be possible to enhance RNAi efficiency by increasing the siRNA population available for the silencing machinery as observed in *C. elegans*. Hence the influence of *Pc-eri-1* was tested on RNAi of different genes located in different tissues of the nematode such as body wall muscles and secretory gland cells.

In the combinatorial RNAi with *Pc-pat-10* and *Pc-eri-1*, the nematode showed enhanced sensitivity to RNAi by prolonging the *Pc-pat-10* silencing effect even 48h after the withdrawal of dsRNAs from the soaking solution. Consistent with the knock-down level of the *Pc-pat-10* transcript the scoring of phenotypes showed a significant persistence of the '*pat'* phenotype in the nematodes even after 48h of recovery by co-silencing *Pc-eri-1*. To confirm further the influence of *Pc-eri-1* on siRNA accumulation, the RNAi efficiency was compared in a soak with siRNA of *Pc-pat-10* and in the combinatorial RNAi after silencing *Pc-eri-1*. The pronounced enhancement in RNAi efficiency during the soak with siRNA (*Pc-patS1)* following the silencing of *Pc-eri-1* in the nematode clearly indicates that the RNAi of *Pc-pat-10* is negatively influenced by *eri-1*.

Although *eri-1* was isolated in a screen mainly for enhanced neuronal RNAi in *C. elegans* and its expression is restricted to neurons and gonads, it has been shown that loss of *eri-1* activity causes a generalized increase in the efficacy of RNAi in most tissues. Kennedy *et al*. (2004) and Zhuang and Hunter, (2011b) demonstrated that the *eri-1* mutation in *C. elegans* enhances RNAi for mRNAs expressed in non-neuronal tissues such as body wall muscle, pharynx, intestine, muscle, and cuticle. Our results demonstrating the improved RNAi of *Pc-pat-10* expressed in body wall muscles of *P. coffeae* when *eri-1* is co-silenced are consistent with these observations in *C. elegans.* However, the silencing of *Pc-eri-1* did not show similar impact in the case of *Pc-eng-1* and *Pc-unc-87*. This implies that the silencing of *Pc-eri-1* only enhances the RNAi of specific genes. Zhuang and Hunter (2011b) also pointed out that RNAi sensitivity among *eri* mutants differs among different tissues as well as the genes expressed in the same tissue.

Based on current understanding, the mode of action of Eri class genes on regulation of exogenous RNAi is indirect by a cross regulation between exogenous and endogenous RNAi (Chapter 1, Section 1.6.1.3). The mutation of Eri genes reduces activity of other endogenous RNAi pathways, resulting in elevation of critical components of the RNAi machinery such as Dicer, RRF-1, SAGOs (Lee *et al*., 2006; Pavelec *et al*., 2009; Zhuang and Hunter, 2012). The general enhancement of RNAi irrespective of *Pc-eri-1* location and tissue/ gene specific RNAi sensitivity also supports that the influence of *Pc-eri-1* on exogenous RNAI might be indirect. And probably the competition for limiting resources may differ among the tissues due to tissue specific components of competing small RNA pathways and thus a tissue specific enhancement to RNAi has been observed when *Pc-eri-1* is co-silenced (Zhuang and Hunter 11b). However, based on the transcriptome analysis, we expect that the limiting resources for competing RNAi pathways in *P. coffeae* might be, mainly, DCR-1 because other components were not identified in the analysis.

RNAi of *Pc-eng-1* was very strong and the influence of *Pc-eri-1* RNAi on *Pc-eng-1* expression was not very clear from the results obtained in the study. Compared to RNAi of *Pc-pat-10* and *Pc-unc-87*, the individual soaking with corresponding dsRNAs produced a more efficient knock-down effect and persistence of RNAi in the case of *Pc-eng*-1, which shows that RNAi may not be always transient in *P. coffeae* and it can be gene specific. Similar to *Pc-eri-1*, we observed that the impact of *Pc-gfl-1* on RNAi was also gene-specific. Among the target genes tested here, only *Pc-pat-10* seems to be positively regulated by *Pc-gfl-1* while *Pc-eng-1* and *Pc-unc-87* were not influenced by the silencing of *Pc-gfl-1*. From our present data it is clear that only *Pc-pat-10* is more sensitive to the regulatory action of *Pc-eri-1* and *Pc-gfl-1* compared to the two other target genes.

The gene specific influence of these RNAi regulators underscores the need for their careful characterization for better utilization of these regulators as RNAi tools in *P. coffeae*.

We also observed that the RNAi induced by dsRNA is more efficient than by siRNA in *P. coffeae.* This is in contrast with the observation in other PPN where a very efficient knock-down effect by siRNA was found even at a low concentration (Dalzell *et al.,* 2010b). However, the RNAi susceptibility to siRNA varies among parasitic nematodes (Issa *et al.,* 2005). In our study a reduction in mRNA abundance of the target gene in *P. coffeae* was not observed at low concentration (50ng/µl and 100ng/µl).

Coupled with the down regulation of *Pc-eri-1*, the siRNA at the concentration of 200ng/µl induced a significant degree of knock-down effect in the nematode. In *C. elegans rde-4* (an RNAi pathway component essential for the processing and accumulation of primary siRNA) mutants have been found to accumulate higher levels of primary siRNAs after exposing the nematode to a higher concentration of dsRNA. This supports the notion that an RNAi response in the nematode varies with different conditions (Habig *et al.*, 2008). Although PPN are less efficient in taking up substantial amount of dsRNA as compared to *C. elegans*, we also expect that the optimal dose of siRNA to trigger RNAi for the target genes might differ among the parasitic nematode species depending on the RNAi regulatory mechanism of the nematode. Notably, based on transcriptome analysis of *P. coffeae, P. coffeae* is probably also *rde-4* deficient (Haegeman *et al*., 2011) and hence it is possible that the absence of RNAi effectors such as *rrf-1*,*rde-4* may also have some influence on the concentration of silencing trigger used to elicit a significant RNAi response in *P. coffeae*.

The approach of combinatorial RNAi has been successfully used in *C. elegans* to identify genes with potential roles in RNAi (Dudley *et al.*, 2002; Geldhof *et al.*, 2006b;

Min *et al.,* 2010; Timmons, 2004). The combinatorial RNAi has also been demonstrated in *Drosophila* as a tool for functional genomics by co-injecting multiple dsRNAs (Schmid *et al.,* 2002). However it has been reported that the use of combinatorial RNAi targeting two genes concurrently in *H. glycines* produced unexpected elevation in the mRNA level of one of the target genes after the RNAi (Bakhetia *et al*., 2008).

Therefore it was indeed necessary to use a threshold level of dsRNA concentration for the treatment so that it would result in a phenotype with high penetrance without generating non-specific toxicity. In our study a significant reduction in the mRNA level of both target genes simultaneously was obtained by using a total concentration of dsRNA in the soaking solution to 1µg/µl.

The earlier reports have also shown that even non-nematode derived double stranded RNAs can induce profound phenotypic changes in *M. incognita* and *G. pallida* (Dalzell *et al.,* 2009). Hence to confirm that the observed effect on mobility is the result of specific target silencing of *Pc-pat-10*, dsRNA of *Pc-eri-1* and *Pc-eng-1*, genes that do not have any influence on nematode movement in *P. coffeae* were used as negative control (see also Chapter 2). In addition, the use of relatively high dose of siRNA compared to the concentration used in *M. incognita* and *G. pallida* did not generate any inhibitory phenotype in *P. coffeae*. The juveniles incubated in all siRNAs including against *Pc-eng-1* and a non-nematode gene (*gfp)* have shown normal sinusoidal movement at the three tested concentrations of siRNA. Hence the threshold level of dsRNA concentration to generate a phenotype with high penetrance without generating nonspecific toxicity may be comparatively higher in *P. coffeae* while compared to *Globodera* and *Meloidogyne*.

In the present study, although we see that co-silencing of *Pc-eri-1* enhanced the RNAi efficiency in *Pc-pat-10* RNAi, it is necessary to test this influence on more target genes

expressed in different tissues. However, consistent with our results, Hong *et al.* (2005) proved that the rapid disappearance of siRNA was linked to up regulation of *eri-1* and *adar-1* in mice and this rebound effect of siRNA was eliminated by co-transfection of another siRNA against *eri-1*. Additionally, the notion that *eri-1* may modulate exogenous RNAi in *P. coffeae* as it does in *C. elegans* may be biologically significant, as this nematode does not encode currently recognizable RdRPs such as RRF-1, required for secondary siRNA amplification in somatic cells.

The current study raises a series of interesting questions yet to be addressed. For example, in *C. elegans*, the expression of *eri-1* is restricted to gonads and a subset of neurons and almost all genes expressed in these tissues are refractory to RNAi. Strikingly, in the plant-parasitic nematode *G. pallida* the neuronal gene *Gp-flp-12* is highly susceptible to RNAi. Furthermore, only two RNAi inhibitor orthologs of *C. elegans* genes, *eri-1* and *xrn-2*, the miRNA 5'-3' exonuclease are fully conserved in *P. coffeae* and other PPN (Dalzell *et al*., 2011). It is yet to be seen if the expression level and distribution of *eri-1* and/or other RNAi pathway components in *P. coffeae* vary from that of *C. elegans*. Further understanding of these issues may give us more insight on RNAi regulation in *P. coffeae* and other PPN. In order to improve the efficiency and potency of RNAi the influence of other RNAi pathway components on the modulation of the RNAi response in PPN should also be analyzed.

In summary, our findings presented here suggest that it might be possible to enhance RNAi potency and persistence by down regulating *eri-1* along with the target gene. Additionally the combinatorial RNAi can be a possible approach in PPN to characterize genes involved in the RNAi pathway.

CHAPTER SIX

Application of RNAi as a control strategy in *Radopholus similis*

6.1 Abstract

Application of host-derived RNAi as control strategy against different species of parasitic nematodes has been reported by different research groups. However, the feasibility of its application in other economically important migratory nematodes species has not been efficiently studied. An *in vitro* RNAi screening as a preliminary requirement for host derived RNAi has been performed to select optimal targets that are susceptible to RNAi in *Radopholus similis*, one of the major parasitic nematode of banana. The *in vitro* RNAi results from this study showed that RNA interference can be induced in *R. similis* by feeding with dsRNA as reported previously in other PPN. However, a variable level of the silencing effect was observed among the targets tested in the study. The selected target genes have also been tested for their role in inducing parasitic success of the nematode by *in vitro* infection test on a host plant, *Medicago truncatula*. Vectors for dsRNA production in banana against *R. similis* were constructed for a parasitism gene, *Rs-eng1B* and a housekeeping gene essential for nematode development, *Rs-icd-1* using the gateway recombinational cloning system. Banana plants were transformed using the dsRNA vectors and the presence and integration of the target genes were confirmed by PCR and Southern hybridization. However screening of the transgenic banana plants for nematode resistance by infection test was not performed due to poor establishment of transgenic plants. Nevertheless, the present study has demonstrated the possibility of using RNAi-mediated silencing of the genes involved in parasitism or nematode development in the control of *R. similis* parasitism.

6.2 Introduction

The application of RNAi for functional analysis of genes has been reported by several research groups in different species of PPN (Chapter 1, Section 1.7.1). As described in Chapter 2 and 5, the nematodes are exposed to dsRNA /siRNA to a limited period of time when the nematodes are soaked in the soaking solution and hence the resulting RNAi effect can be time limited. As an alternative approach to maximise the RNAi effect, dsRNA/siRNAs can be continuosly introduced into the feeding PPN by generating RNAi within the host plants. Additionally, this approach of host-generated RNAi can be an effective control strategy to suppress the parasitic success of nematodes within the host plants (Chapter 1, Section 1.7.2).

Several studies have shown extremely promising results in controlling nematode infection by host-derived RNAi approach. Almost complete resistance against root-knot nematodes was achieved by expressing hairpin RNAs targeting essential genes of the nematodes (Yadav *et al*., 2006; Li *et al*., 2010). In addition to this, studies have also shown that a high level of resistance to root-knot nematodes and cyst-forming nematodes can be obtained by targeting parasitism genes expressed in the subventral gland cells of the nematodes (Huang *et al*., 2006; Steeves *et al*., 2006; Sindhu *et al*., 2009; Klink *et al*., 2009; Xue *et al*., 2013).

However, as compared to RKN, a strong suppression of nematodes was not detected in the case of cyst nematodes. While a significant reduction in female number of between 23-64% was observed in cyst-forming nematodes, the transgenic lines expressing dsRNA of RKN genes displayed a significant reduction between 63-90% (Huang *et al*., 2006). Limited study has been done to determine the efficiency of RNAi in the migratory endoparasitic nematodes.

Haegeman *et al*. (2009b) showed that soaking of *R. similis* in dsRNA of xylanase gene resulted in 60% reduction in subsequent infection of *Medicago truncatula.* The efficiency of host-delivered RNAi has yet to be tested against migratory endo parasitic nematodes. RNA interference is used in this study in an attempt to engineer host resistance against the migratory parasitic nematode *R. similis*. In this chapter, the susceptibility of *R. similis* to RNAi is first tested by *in vitro* soaking as demonstrated in Chapters 2 and 5 for *P. coffeae*. The second aim of this study is to assess the feasibility of using RNAi to control *R. similis* in banana.

Migratory nematodes invade the roots and move intercellularly and intracellularly through the root by breaking down the cell wall. By knocking down nematode genes that encode cell wall-degrading enzymes (*e.g*. β-1, 4-endoglucanase) the invasion and subsequent establishment of the nematode in the plant could be prevented. Four different endoglucanases of the glycosyl hydrolase family 5 were identified in *R. similis* (Haegeman *et al.*, 2008). Here, two of these genes, *Rs-eng2* and *Rs-eng1B*, are used as targets for *in vitro* RNAi experiments. *Rs-eng1B* encodes for a protein with a catalytic domain and an additional Carbohydrate Binding Module (CBM), whereas *Rs-eng2* is devoid of a CBM. In addition to these *β-1, 4 endoglucanase* genes specific to parasitic nematodes, the orthologs of the *C. elegans* housekeeping genes, *Rs-icd-1* (Inhibitor of cell death-1), *Rs-rpl-1* (Ribosomal protein large subunit), *Rs-iff-2* [Initiation Factor Five (eIF-5A) homolog] and *Rs-integ* (Integrase) are also targeted for silencing. By down regulating the genes that play important roles in embryonic and larval development or nematode locomotion, the complete progression of pathogenesis could be adversely affected.

Based on this hypothesis the proposed study aims to develop an alternative control strategy against migratory nematodes by knocking down the abovementioned

parasitism or housekeeping genes. The chosen strategy is to deliver dsRNA to the nematodes through *in planta* routes by expressing the dsRNA of the target genes in banana plants. So far, this *in planta* route has not been reported for migratory nematodes. The present study, therefore, aims to test the feasibility of *in planta* RNAi for control of migratory nematodes.

6.3 Materials and methods

6.3.1 Nematode culturing, RNA isolation and cDNA synthesis

Radopholus similis was maintained on carrot discs in small petri dishes (ø 35 mm) at a constant temperature of 25°C as described by Moody *et al*. (1973). Nematodes were harvested by rinsing the petri dishes with sterile demineralised water 6-8 weeks after inoculating the discs with approximately 50 nematodes. The collected nematodes were either used immediately or stored as a pellet for RNA extraction (Chapter 5, Section.5.3.1) at 4°C following centrifugation at 12000rpm for 2min. The cDNA was synthesised as described in Chapter 5 (Section.5.4.1).

6.3.2 Selection of the target genes, synthesis of dsRNA, soaking and semi-quantitative RT-PCR

In addition to the nematode parasitism genes *Rs-eng1B* and *Rs-eng2*, the orthologs of four housekeeping gene, namely *Rs-rpl-1*, *Rs-iff-2*, *Rs-icd-1* and *Rs-integ*, which have shown a lethal RNAi phenotype in *C. elegans* (http://www.wormbase.org), have been selected. The similarity of these targets with other parasitic nematodes like *Meloidogyne* sp and *Pratylenchus* sp and plants was checked using Blast searches through the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure that the selected genes are specific to parasitic nematodes.

The target gene fragments, *Rs-eng1B* (292bp), *Rs-eng2* (221bp), *Rs-integ* (269bp), *Rs-icd-1* (267bp), *Rs-iif-1* (259bp) and *Rs-rpl-1* (233bp) were amplified from cDNA of nematodes freshly isolated from carrot discs using the primer combinations mentioned in Table 6.1a with the following cycling conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 25 s, 50°C for 25 s, 72°C for 50 s. The target sequences were cloned into the pGEM-T vector (Promega) and confirmed by sequencing. The templates of sense and antisense DNA strands for generating dsRNA were amplified from the vector carrying the inserts of each target gene by PCR under standard conditions using the respective primers with the T7 promoter sequence incorporated at the 5'end of either the sense or antisense strand (Table 6.1a). The green fluorescent protein (GFP) was used as a negative control to determine the non-specific action of dsRNA. The *in vitro* transcription and dsRNA assembly of each target gene was performed using the Megascript RNAi kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. The dsRNA was quantified spectrophotometrically and the quality of the dsRNA was checked on a 1% (w/v) agarose gel.

Approximately 1000 nematodes (juveniles and adults) of *R. similis* freshly isolated from carrot discs were soaked in a soaking solution (200µl) containing dsRNA of the target gene (1 µg/µl), 50 mM octopamine and 3 mM spermidine in separate 2 ml eppendorf tubes for each treatment. As a negative control the nematodes were incubated in the same solution but without dsRNA (non-RNAi) in addition to the *gfp*-dsRNA control.

The tubes were covered with aluminium foil and placed on a rotary incubator for 24h at room temperature. After soaking, nematodes were centrifuged for 2 min at 1000 rpm, sterilized with 0.33% (v/v) of Hospital Antiseptic Solution (HAC) for 1 h on a rotator and room temperature and subsequently, washed three times with sterile water prior to the

infection test (Section 6.3.3). Approximately 500 soaked nematodes were used for semi-quantitative RT-PCR.

The RNA extraction and cDNA synthesis were done as described in Chapter 2. The primers used for the amplification of each target gene are listed in Table 6.1b. The amount of cDNA used as template for each reaction and the number of PCR cycles were optimised for each gene product to detect the exponential phase of the reaction. The optimised number of PCR cycles for *Rs-eng 1B* and *Rs-eng2* were 32 and 28 cycles, respectively whereas the amplification of other target genes, *Rs-integ*, *Rs-icd-1*, *Rs-iff-2* and *Rs-rpl-1* was optimized at 35 cycles. The products from the PCR reactions were separated on a 0.5x TAE (20 mM Tris acetate, 1 mM EDTA) 1.5% gel.

Table 6.1a. Primers used for cloning and *in vitro* RNAi experiments

Target gene	Primer name	Sequence (5' to 3')	Tm(°C)
Primers used for cloning			
Rs-integ	Rs-integ-F Rs-integ-R	ATCAAAAGTCGTGGGAGTGC GTCCACCAGAGACTGATTGC	52 52
Rs-iif-2	Rs-iff2-F Rs-iff-2-R	TTTGCTTGCAAAACGAAGG TTCGAAGACCGGTAAACACG	50 53
Rs-rpl-1	Rs-rpl-1-F Rs-rpl-1-R	GCGCCCAATCAGTAGAGG AAAAGGACAAGCGTTTCAGC	52 52
Rs-icd-1	Rs-icd-1-F Rs-icd-1-R	GCTCTCCTATTCGTTCACAACC GATGAACTTTCTCGTCGATGG	54 53
Primers used for template amplification for *in vitro* transcription			
Gfp	Gfp-F Gfp-R Gfp-T7F Gfp-T7R	ATCCGCCACAACATCGAGG TTGTACAGCTCGTCCATGC TAATACGACTCACTATAGGGATCCGCCACAACATCG TAATACGACTCACTATAGGGTTGTACAGCTCGTCCA	53 51 65 64
Rs-eng2	EG8start EG8start-T7 EG8-R EG8-T7R	GTCAGCGCCACTTACCAGTC ACGTCCTGGGACCATGTG TAATACGACTCACTATAGGGGTCAGCGCCACTTACCAGTC TAATACGACTCACTATAGGGACGTCCTGGGACCATGTG	56 53 68 67
Rs-eng1B	EG2-CBD-F EG2Stop EG2-CBD-T7F EG2Stop-T7	CTCAGTGACCGCTTCGGTGTC TCAGCATCCACTGGTGGACACAATT TAATACGACTCACTATAGGGCTCAGTGACCGCTTCGGTGTC TAATACGACTCACTATAGGGTCAGCATCCACTGGTGGACACA	58 58 69 68
Rs-icd-1	Rs-icd-1-T7F3 Rs-icd-1-T7R3 Rs-icd-1-F3 Rs-icd-1-R3	TAATACGACTCACTATAGGGAGACCATTTCAACAATCCGAAGG TAATACGACTCACTATAGGGAGATTTGTTCCATCGTCAACAGC CCATTTCAACAATCCGAAGG TTTGTTCCATCGTCAACAGC	66 66 50 51
Rs-integ	Rs-integ-F3 Rs-integ-T7F3 Rs-integ_R3 Rs-integ-T7R3	CCTTGTTCCACTCCTTCAGC TAATACGACTCACTATAGGGAGACCTTGTTCCACTCCTTCAGC GGCAAAAATGTGTTCACAGG TAATACGACTCACTATAGGGAGAGGCAAAAATGTGTTCACAGG	53 68 49 66
Rs-iff-2	Rs-iif-2-F3 Rs-iif-2-T7F3 Rs-iif-2-R3 Rs-iif-2-T7R3	CATCCTCCTCCATTTTCACC TAATACGACTCACTATAGGGAGACATCCTCCTCCATTTTCACC ATGAGGACGGTTTTGTCAGC TAATACGACTCACTATAGGGATGAGGACGGTTTTGTCAGC	50 67 52 66
Rs-rpl-1	Rs-rpl-1-F3 Rs-rpl-1-R3 Rs-rpl-1-T7F3 Rs-rpl-1-T7R3	CGGGATCTGTTTGATCAGC AAAAGGACAAGCGTTTCAGC TAATACGACTCACTATAGGGAGACGGGATCTGTTTGATCAGC TAATACGACTCACTATAGGGAGAAAAAGGACAAGCGTTTCAGC	51 52 67 66

Rs-integ, Rs-icd-1, Rs-iff-2 andRs-rpl-1 indicate C. elegans orthologs of integrase, icd-1, iff-2 and rpl-1, respectively. A fragment from CBM domain of Rs-eng1B and a fragment from catalytic domain of Rs-eng2 were amplified.

Table 6.1b. Primers used for semi quantitative RT-PCR and Gateway cloning

Target gene	**Primer name**	**Sequence (5' to 3')**	**Tm (°C)**
Primers used for semi quantitative PCR			
Rs-actin	Rs-act-R Rs-act-F	GACCTCACTGACTACCTGATGAAGATTC ACTTCATGATCGAGTTGTAGGTGGACTCG	59 61
Rs-icd-1	Rs-icd-1-R4 Rs-icd-1-F2	CCTTCGGATTGTTGAAATGG GCTCTCCTATTCGTTCACAACC	58 56
Rs-eng1B	EG2 Sp-R EG2 Sp-F	TGATTGGGTTCGCGGATGCGACTT TCGCCTCAGCCACTGCATTGACT	59 58
Rs-eng2	EG8down EG8stop	ACTGCCGACGGAAATGGCTACGTG TCAGCAGCTCACACCGTTCTTTTGG	60 59
Rs-integ	Rs-integ-F3 Rs-integ-R2	CCTTGTTCCACTCCTTCAGC GTCCACCAGAGACTGATTGC	58 56
Rs-iff-2	Rs-iif-2-F3 Rs-iif2-R2	CATCCTCCTCCATTTTCACC TTCGAAGACCGGTAAACACG	56 58
Rs-rpl-1	Rs-rpl-1-F3 Rs-rpl-1-R1	CGGGATCTGTTTGATCAGC AGCGCAGGAAAAGAAGCG	56 60
Primers used for Gateway cloning			
Rs-eng1B	EG2CBDF-Gw EG2stop-Gw	AAAAAGCAGGCTCTCAGTGACCGCTTCGGTGTC AGAAAGCTGGGTTCAGCATCCACTGGTGGACACAATT	62 67
Rs-icd-1	Rs-icd-1-GwF Rs-icd-1-GwR	AAAAAGCAGGCTCCATTTCAACAATCCGAAGG AGAAAGCTGGGTTTTGTTCCATCGTCAACAGC	62 63
Mi-integ	Mi-Integ-GwF Mi-Integ-GwR	AAAAAGCAGGCTATGTCAAAGGCAACGTATGGA AGAAAGCTGGGTTTCAGCAATCATTTCAGGGG	62 63

Mi-integ indicates ortholog of integrase from M. incognita while Rs-integ indicates the ortholog of integrase from R. similis.

6.3.3 *In vitro* Infection Test and Analysis

Medicago truncatula L.var Jemalong was used as a model plant for *in vitro* infection studies to analyse the RNAi effect on nematode invasion. Medicago seeds were obtained from the United states Department of Agriculture (USDA).

The seeds were scarified by soaking in concentrated anhydrous H_2SO_4 for 8 min, followed by 4 times rinsing in water, and subsequent incubation in 5% (w/v) NaCl for 3 min. After rinsing the seeds again in water 6 times, they were kept at 4°C for 36 h. Following thorough rinsing with sterilized water, the treated seeds were transferred onto a Petri-dish lid using sterile micropipettes and distributed evenly on the plate (each seed in a drop of sterile water). The lid was carefully flipped over, placed on the

Petri-dish and kept in the dark for 2 days. Two day old seeds were randomly placed on Modified Strullu Romand (MSR) medium in six-well tissue culture plates, with each plate containing one seed (Elsen *et al*., 2000). Each treatment consisted of 10 biological replicates. Seedlings were grown at 25°C with a 16 h light/8 h dark cycle.

Three weeks old seedlings with a well-developed root system were inoculated with 50 dsRNA treated nematodes per plant. The soaked nematodes were inoculated on the culture medium close to the root tip of the plant. The infected plants were kept in a plant room at 25°C with light intensity, 80-100 $\mu mol.m^{-2}.s^{-1}$ and photoperiod, 16h/8h (light/dark) for 10 days when the roots were separated from the medium for selective staining of the penetrated nematodes using the method described by Byrd *et al.* (1983). Roots in six-well tissue culture plates were soaked in diluted sodium hypochlorite bleach [0.9% (v/v) NaOCl] for 4 min, thoroughly rinsed with demineralized water, covered with acid fuchsin solution (30x stock solution: 3.9 g fuchsin, 750 ml water, 250 ml acetic acid) and heated twice for 10 s in the microwave. The stained roots were transferred into the oven (56°C) for 30 min and subsequently allowed to cool for 1 h 30 min. Further, the roots were destained by replacing the stain with acidified glycerine (8 drops of HCl/100ml glycerine). This procedure stains the nematodes that have penetrated the roots and the number of nematodes invaded the roots were counted, while the nematodes in the media were counted by cutting the media into small pieces and soaking them in glycerol for 24 h.

The resultant suspension was put into a counting dish and the nematodes were counted and recorded as the number of the nematodes that were present outside the roots.

The results were analysed by One-way ANOVA using GenStat Release 13 for Windows (VSN International Ltd). The entire experiment was repeated twice for each target gene.

6.3.4 Construction of plant transformation vectors

Vectors for dsRNA production in the plant against *R. similis* were constructed for *Rs-eng1B* and *Rs-icd-1*. Additionally, a vector was constructed for dsRNA to *Mi-integ*, an *integrase* gene from *M. incognita* (Yadav *et al*., 2006) as a positive control and *gfp* (received from VIB, Gent) as a negative control. The vectors were constructed using the gateway recombinational cloning system described by Karimi *et al* (2007). The cDNA of the target gene was cloned in sense as well as antisense directions into the Gateway entry vector pK (Hells) 8-GW-I-WG-UBIL (VIB, Gent, Belgium) under the control of a maize ubiquitin (UBIL) plant promoter and separated by an intron (Figure 6.1) to generate a hairpin construct.

The fragment of each target gene was generated by PCR amplification using the primer combinations listed in Table 6.1b with the following cycling conditions: 94°C for 2 min, followed by 5 cycles of 94°C for 35 s, 45°C for 25 s, 72°C for 30 s and additional 25 cycles of 94°C for 35 s, 54°C for 25 s, 72°C for 30s. The amplified fragments were cloned up (sense) and downstream (antisense) of the *Pdk* and *cat* (reverse) introns (Table 6.2) according to the manufacturer's instructions (Invitrogen). The presence and orientation of the target gene fragments were confirmed by restriction digestion and sequencing.

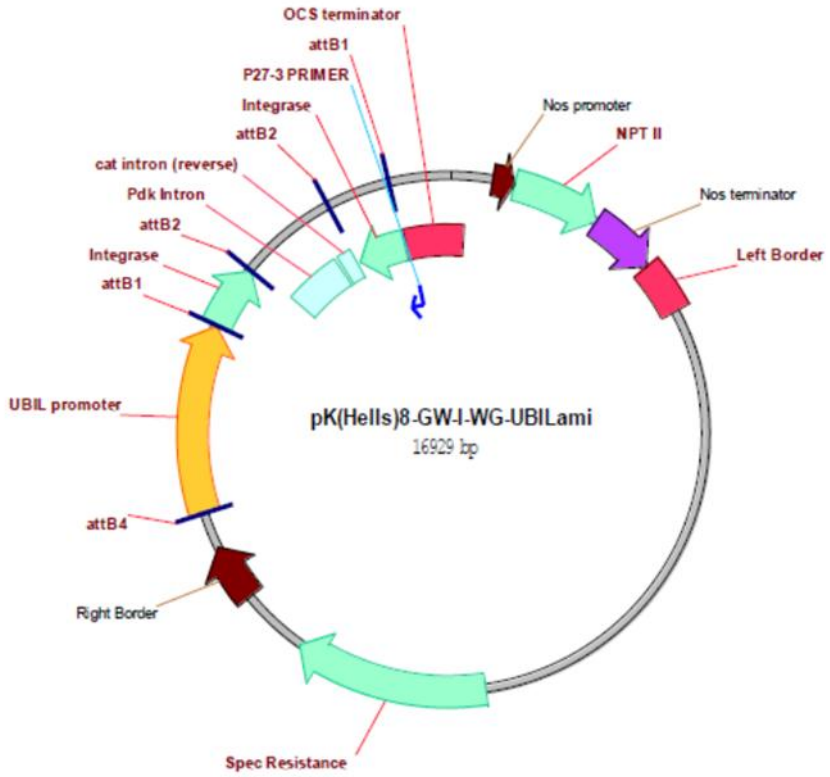

Figure 6.1. Example of a transformation vector showing the hairpin construct of the integrase gene of *Meloidogyne incognita* (*Mi-integ*). The , 285 bp integrase gene fragment (position +10 to +635 of the integrase gene); Nos, nopaline synthase; UBIL, maize ubiquitin long promoter; Spec, Spectinomycin resistance; Pdk, pyruvate dehydrogenase kinase; cat, chloramphenicol acetyltransferase intron; OCS, Octopine Synthase; NPTII, neomycin phosphotransferase II coding region; attB1, attB2 and attB3, recombination sites.

Table 6.2. Size and position of the target gene fragments in the transformation vector

Target gene	Fragment size (bp)	Fragment position in the target gene
Rs-eng1B	250	+1913 to +2451
Rs-icd-1	317	+297 to +614
Mi-integ	285	+10 to +635

Rs-eng-1B and Rs-icd-1 represent the target genes, β-1, 4-endoglucanase and icd-1, from R. similis, respectively; Mi-integ represents the target gene; integrase from M. incognita

6.3.5 *Agrobacterium* mediated transformation of banana, selection and regeneration of transgenic lines

Transgenic shoots of banana were obtained by *Agrobacterium*-mediated transformation as described by Perez Hernandez *et al.* (2006a). *Agrobacterium tumefaciens* strain EHA101 harbouring vector pFAJ3000 carrying the *gusA*^{INT} reporter gene under control of the 35S promoter (De Bondt *et al*., 1994) and those harbouring the dsRNA vectors targeting *Rs-eng1B*, *Rs-icd-1*, *Mi-integ* and *gfp* were used for transformation. The selectable marker gene, the neomycin phosphotransferase II (*nptII*) gene which confers resistance to geneticin, is driven by the Nos promoter.

Embryogenic cell suspension (ECS) of the triploid AAA dessert banana variety 'Grande Naine' (obtained from The Laboratory of Tropical Crop Improvement, KU Leuven, Belgium) was used for transformation. The transformation protocol is briefly described below. The *Agrobacterium* strains carrying the respective vectors were plated on selective semi-solid YM medium supplemented with appropriate antibiotics (100 mg/l spectinomycin and 300 mg/l streptomycin for pFAJ3000 and 50 mg/l kanamycin for the other vectors) and incubated at 28°C for 48 h.

Single bacterial colonies were cultured in selective liquid YEP medium and incubated at 210 rpm and 28°C for approximately 24 h to give an OD_{600} of 1.2. ECSs of 33% (w/v)

Settled Cell Volume (SCV) were infected with agrobacteria that had been adjusted to an OD_{600} of 0.4 in liquid ZZ medium (half strength MS medium supplemented with 5 µM 2,4-D and 1 µM zeatin; pH 5.6) supplemented with 200 µM acetosyringone (AS). A bacterial suspension of 1 ml was mixed with 200 µl of 33% (w/v) SCV ECS in each well of 24-well plates and the plates were incubated for 6 h at 25°C and 25 rpm in the dark. The cells were then evenly spread on a 50 µm sterile polyester mesh (approximately 2 x 2 cm per sample)and cocultivated for 6 days on 10 ml semi-solid ZZ medium (pH 5.8) containing 200 µM AS in a 5 cm Petri-dish at 21°C in the dark.

The transformed cells were selected by transferring them to semi-solid ZZ medium (pH 5.8) supplemented with geneticin (50 mg/ml) and timentin (200 mg/ml). The plated cultures were incubated at 25±2°C in the dark for approximately three months with a bi-weekly subculture. Independent transgenic cell colonies were picked from each plate using fine forceps and transferred onto selective embryo induction medium (RD1: half strength MS medium supplemented with 100 mg/l myo-inositol). These plates were further incubated for 2 months at 25±2°C in the dark with a monthly subculture. The differentiated cultures were transferred onto RD2 medium (half strength MS medium supplemented with 10 µM N^6-benzylaminopurine or BAP) in 5 cm Petri-dishes to induce shooting and incubated in the dark. In addition to the antibiotic selection, the transgenic cell colonies were screened by PCR to confirm the presence of the transgene. Total DNA was isolated from a group of independent colonies per vector using a modified protocol of Dellaporta *et al.* (1983) and 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 1% (w/v) DTT as extraction buffer. The DNA pellet was finally dissolved in 20 µl sterile water.

The quality and quantity of the DNA was determined spectrophotometrically using the Nanodrop® ND-1000(Isogen Life sciences). The target gene was amplified using the

primer combinations mentioned in Table 3 with the following cycling conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 60°C for 20 s, 68°C for 20 s with a final elongation step at 68°C for 2 min. The 20 µl PCR mix contained 2 µl of buffer (10x NEB), 2 µl 0f dNTPs (2 mM of each dNTP, Fermentas), 0.5 µl of forward primer and 0.5 µl of reverse primer (each at 20 µM), 0.5 µl of Taq polymerase (0.5U/µl, NEB)), 13.5 µl of water and 1 µl of (50 ng) of total DNA. The transformed vectors were used as positive controls (10 ng per PCR reaction), whereas DNA of non-transformed cell colonies and a blank sample were used as negative control for each gene tested.

6.3.6. Multiplication of transgenic lines

Individual shoots or shoot clumps originating from transgenic somatic embryos or globular cultures (via organogenesis) were transferred to sterile 50 ml growing glass test tubes containing semi-solid regeneration (REG) medium [full-strength MS basal medium including vitamins, 10 mg/l ascorbic acid, 1 µM Indole-3-acetic acid (IAA), 1 µM BAP, 30 g/l sucrose and 3 g/l phytagel, pH 5.80] and incubated at 25±2°C and 16-h photoperiod of 1000 lux to regenerate rooted *in vitro* plantlets. Each individual embryo or globular culture represents an independent transgenic line and all shoots derived from one embryo or globular cultures are genetically identical (Pérez-Hernández *et al*., 2006b). A total of 31 controls (non-transformed), 26 *gfp,* 56 *icd,* 36 *eng1B* and 40 *mi* independent lines were maintained in test tubes, and in the second subculture 6 lines with robust full-grown plantlets from each of the constructs were selected for further multiplication.

To multiply the cultures to the desired number of plants, the shoots were grown in sterile 50 ml glass test tubes containing semi-solid proliferation (PROL) medium which is identical to REG medium except for a tenfold higher BAP concentration (10 µM). The

rooted plantlets were maintained and kept healthy by regular subculture to fresh REG medium after every 4-6 weeks.

6.3.7 Histochemical GUS assay

The ECS samples that were transformed with pFAJ3000 were tested histochemically for transient GUS expression after 6 days of *Agrobacterium* co-cultivation as described by Jefferson *et al.* (1987). All samples were incubated for at least 4 h at 37°C in the assay buffer [0.1 M phosphate buffer (50 mM Na_2HPO_4 and 50 mM KH_2PO_4 at pH 7.0), 10 mM Na-EDTA, 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 0.1% (w/v) Triton X-100; Mendel *et al*., 1989] containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid cyclohexylammonium salt (X-gluc; Duchefa, Haarlem, The Netherlands). The number of blue foci was counted. Non-transformed ECS were included as negative control.

6.3.8 Molecular characterization of transgenic lines

Total DNA was isolated from 30-60 mg of leaf tissue according to a modified protocol by Dellaporta *et al*. (1983). The samples for DNA extraction were collected from *in vitro* as well as greenhouse plants. The leaf samples were macerated and cells lysed in an extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 1% (w/v) DTT]. The resulting DNA was precipitated in an equal volume of isopropanol and washed in 500 µl 70% (v:v) ethanol to remove excess salts.

Finally, the air-dried DNA pellet was re-suspended in 20 µl of sterile water. The quantity and quality of the isolated DNA were measured using the Nanodrop® ND-1000 (Isogen Life Science) spectrophotometer.

The presence of the target gene in the transformed samples was determined by PCR amplification of the target gene sequence of the hairpin constructs or part of the

ubiquitin promoter and the target gene sequence. The plasmid DNA (50 ng) harbouring the hairpin construct was used as positive control, while a blank sample and a DNA sample from an untransformed line were used as negative controls. The 20 µl PCR mix contained 2 µl buffer (10x NEB), 2 µl of dNTPs (2 mM of each dNTP), 0.5 µl each of forward and reverse primer (20 µM each), 0.5 µl of Taq polymerase (0.5 U/ µl, NEB), 13.5 µl of water and 1 µl of (50 ng) of DNA sample. The list of primers used for the analysis is shown in Table 6 3. The PCR amplification was performed under the following cycling conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s with a final elongation step at 72°C for 5min The resulting PCR products were separated on 2% (w/v) agarose gel for 25 min at 300 V in a sodium borate buffer [10 mM NaOH, pH 8.5 set using H_3BO_3 as described by Brody and Kern (2004)].

Table 6.3. Primers and the expected product sizes for PCR analysis of transgenic banana plants

Target gene/promoter -target gene[a]	Primer (5' to 3')	Expected product size(bp)	Sequence	Tm(°C)
Mi-integ	Mi-Promo-F[d] Mi-Gs-R1	285	TTTAGCCCTGCCTTCATACG AGGCCATTCTTTTCCAAAGC	52 50
PUbi-Mi-integ	UbiL Mi-Gs-R1	237	GGGCCCGGTAGTTCTACCTTC AGGCCATTCTTTTCCAAAGC	58 50
Rs-eng1B	Eng-Gs-R1 Mi-Promo-F	250	GTTCCAAATGCTCGAGATGG TTTAGCCCTGCCTTCATACG	52 52
Rs-icd-1	Rs-icd-1-R3 Rs-icd-1-F3	315	TTTGTTCCATCGTCAACAGC CCATTTCAACAATCCGAAGG	50 50
PUbi[b]-icd-1	UbiL[d] Rs-icd-1-F3	380	GGGCCCGGTAGTTCTACCTTC CCATTTCAACAATCCGAAGG	58 50
Pnos[c]-nptII	Neo-R2 pNOS-S3	149	TAGCCGAATAGCCTCTCCAC AATTCCCCTCGGTATCCAAT	54 45
gfp	GFP-F GFP-R	215	ATCCGCCACAACATCGAGG TTGTACAGCTCGTCCATGC	53 51

Mi-integ represents the target; integrase from M. incognita; Rs-eng-1B and Rs-icd-1 represent the targets, β-1, 4-endoglucanase and icd-1 from R. similis, respectively. Gfp represent the gene encoding green fluorescent protein (negative control).

[a] *Presence of the target gene (i.e. the sequence used for silencing of the corresponding nematode gene or the selectable marker gene NPTII) or promoter-target gene was investigated in the regenerated plants.*

[b] *Maize ubiquitin promoter*

[c] *Nopaline synthase promoter*

[d] *Mi-Promo F and UbiL anneals in different sites of UBIL promoter*

The stable integration of the T-DNA was confirmed by Southern hybridization analysis of four transgenic lines (mi-55, mi-90, eng1B-54 and eng1B-103) as well as one non-transgenic line (control-123) using a digoxigenin (DIG) labelled probe. Total DNA was isolated from 1 g of leaves according to Khayat *et al*. (2004) with some modifications Following the grinding of the leaf tissues in liquid nitrogen, the cells were lysed in the extraction buffer consisting of 4% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 50 mM Na-EDTA pH 8.0 and 1% (w/v) DTT. The DNA was precipitated, washed and re-suspended in 100 µl of sterile water.

The genomic DNA (11 µg) was digested to completion with *Sac*I which has a single restriction site in the T-DNA (in eng1B vector -at position 12426 and in mi vector at 11802) and separated on a 0.8% (w/v) agarose gel for 6 h at 40 V in 1x TAE (40 mM Tris acetate, 1 mM EDTA) buffer. The digested sample DNA (10 µg) was loaded per lane including the untransformed sample as the negative control and two plasmid vector samples as positive controls at the concentration of 1 and 5 copies per triploid genome, respectively. The DIG-labelled DNA marker III (Roche) was also loaded (50 ng). The DNA was blotted overnight by downward capillary transfer in 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) onto a positively charged nylon membrane (Roche) as described (Zhou *et al*., 1994) and fixed by UV crosslinking at 120,000 J/cm^2.

Hybridization and detection was done according to Remy *et al.* (2005) with some modification. Briefly, the membrane was prehybridized in 20 ml Clontech Express Hyb buffer containing 50 µg/ml yeast tRNA for 1 h. The membrane was kept overnight for hybridization in the same buffer containing the DIG-labelled probe and yeast tRNA at 20 ng/ml and 50 µg/ml, respectively.

The probes for *eng1B* (primers Eng GsR1 and Mi-Promo F), *mi* (primers Mi-Promo- F and Mi- GsR1) and *nptII* (primers Neo5 and NeoSHR,) genes were generated using

the PCR DIG-DNA Labeling Mix (Roche, Vilvoorde, Belgium). Following the hybridization, the membranes were washed twice in 2x washing buffer (2x SSC: 0.3 M NaCl, 0.03 M Na-citrate and 0.1% SDS, pH 7.0) for 5 min on the shaker (70 rpm) at 68°C. Subsequently, the membrane was washed twice with 0.1x washing buffer (0.1x SSC: 0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0) for 15 min. During detection the following solutions were used: buffer 1 [0.1 M maleic acid, 3 M NaCl pH 8 and 0.3% (v/v) Tween 20 added after autoclaving]; buffer 2 [buffer 1 containing 0.5% (w/v) blocking reagent, which is prepared at 10% (w/v) by dissolving 10 g blocking reagent in 100 ml buffer 1 by several heat pulses in the microwave oven avoiding boiling]; buffer 3 (0.1 M TrisHCl pH 9.5, 0.1 M NaCl, autoclaved].

The hybridized membrane was incubated in buffer 2 for 1 min and then for exactly 30 min in 40 ml of antibody solution [1:10,000 dilution or 4 µl Anti-DIG –AP (alkaline phosphatase; 75 U/ml) in 40 ml buffer 2]. Subsequently, the membrane was washed twice in buffer 1 each time for 30 min. Finally, the membrane was equilibrated in 20 ml buffer 3 for 10 min and then treated with 5 ml buffer 3 containing 50 µl of CSPD (Roche) substrate solution with gentle shaking for 5 min. The membrane was sealed in a plastic bag and incubated for 30 min at 37°C to bring the chemiluminescent reaction at the steady state.

The signals were detected by a liquid nitrogen-cooled slow-scan CCD camera (Versarray 512 B LN camera, Roper Scientific, Vianen, the Netherlands) at an exposure time of 1 min and 20 min and the images were processed by using image analysis software (MetaMorph® 5.0r3, Universal Imaging, USA).

The membrane was stripped by washing twice for one minute in MilliQ water, then incubated for 10 min in pre-warmed (37°C) alkaline probe stripping solution (0.2N NaOH, 0.1% SDS) before being rinsed twice for 10 min in 2x SSC solution. Re-hybridisation with the *nptII* probe was essentially done as described above.

6.3.9 Greenhouse infection and screening experiment

A total of 13 independent lines with at least 12 rooted plantlets per line were transferred to the greenhouse for the infection experiment. The plantlets were carefully removed from the test tubes and gently washed in tap water to remove the semi-solid medium. The plants were then planted in the soil mixture prepared by mixing sand and soil composite (BRIL, Type 3) at the ratio of 2:1 in 12 diameter pots containing about 1000 cm^3 soil. The plants were kept under plastic frames at 26/18°C day/night temperature and additional artificial light of 200-700 lux for 12 h until their establishment (new leaf and root formation) was noted.

The established plants were watered 2-3 times a week depending on the temperature and moisture content. Fertilization commenced when the plants were established with 50 ml of diluted liquid NPK (7:3:6) at the ratio of 1:8 per pot once every week. Three independent lines for each constructs, *Rs-icd-1* (line 30, 41 and 45), *Mi-integ* and *Gfp* (line 6, 8 and 24) along with three independent lines from untransformed plants (line 8, 28 and61) were selected for the screening experiment for nematode resistance. Due to insufficient number of established plants, only one line for *Rs-eng1B* (line 71) was selected for the screening. Six plants from each transgenic and non-transgenic line were maintained for the infection with *M. incognita* or *R. similis* according to which nematode the dsRNA present in the plants is targeted.

In addition, six plants from each line were kept uninfected to assess the susceptibility of the transgenic lines by determining the differences in root necrosis (*R. similis*) or root galls (*M. incognita*) between the infected and uninfected plants. The nematode inoculum of *M. incognita* required for the infection was maintained on transformed tomato roots *in vitro* (Verdejo *et al*., 1988) as well as on the tomato plants grown in soil in the greenhouse to rear high numbers of the nematodes. The inoculums for *R. similis* was maintained on carrot discs as described above.

6.4 Results

6.4.1 *In vitro* RNAi analysis

To assess the feasibility of RNAi for nematode control and to select suitable target genes for dsRNA constructs, mixed stages of *R. similis* were soaked for 24 h with dsRNA of each gene separately following the methodology of Urwin *et al.* (2002). Soaking the nematodes with dsRNA of *Rs-eng1B* resulted in a significant reduction of the transcript level compared to control experiments with water (Non-RNAi) or dsRNA of *gfp* (*Gfp*-RNAi) (Figure 6.2A). There was, however, no significant reduction in the transcript level of *Rs-eng2* in comparison to the controls (Figure 6.2B).

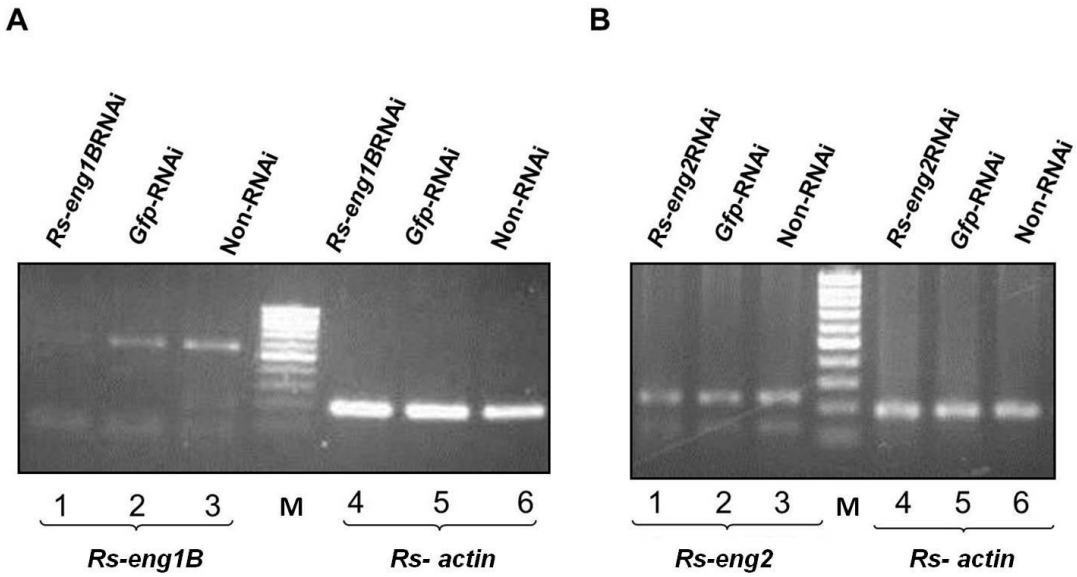

Figure 6.2. Analysis of the mRNA level by semi-quantitative RT-PCR following 24 h of treatment with (A) dsRNA of *Rs-eng1B* or controls and (B) dsRNA of *Rs-eng2* or controls, Non-RNAi: *R. similis* nematodes incubated in soaking solution without dsRNA; *Rs-eng1B*RNAi: nematodes incubated in dsRNA of *Rs-eng1B*; *Rs-eng2*RNAi: nematodes incubated in dsRNA of *Rs-eng2*; Gfp-RNAi: nematodes incubated in dsRNA of g*fp* as a dsRNA control. Transcription level of *Rs-eng1B* (567bp) (A) or *Rs-eng2* (261bp) (B) from each treatment (lane 1-3). DNA Marker (100 bp, Gene ruler, Fermentas) (M). Transcription level of *Rs-actin* (196bp) from each treatment (lane 4-6). The experiment was done two times.

Moreover, the knock-down effect was gene specific and no cross-silencing was observed. The dsRNA of *Rs-eng1B* did not elicit a knock-down effect of *Rs-eng2* and vice versa (data not shown). Similarly, a significant reduction in transcript level was seen in the case of two other target genes, *Rs-icd-1* and *Rs-integ,* while the transcription of targets *Rs-iff-2* and *Rs-rpl-1* was not affected by RNAi soaking (Figure 6.3).

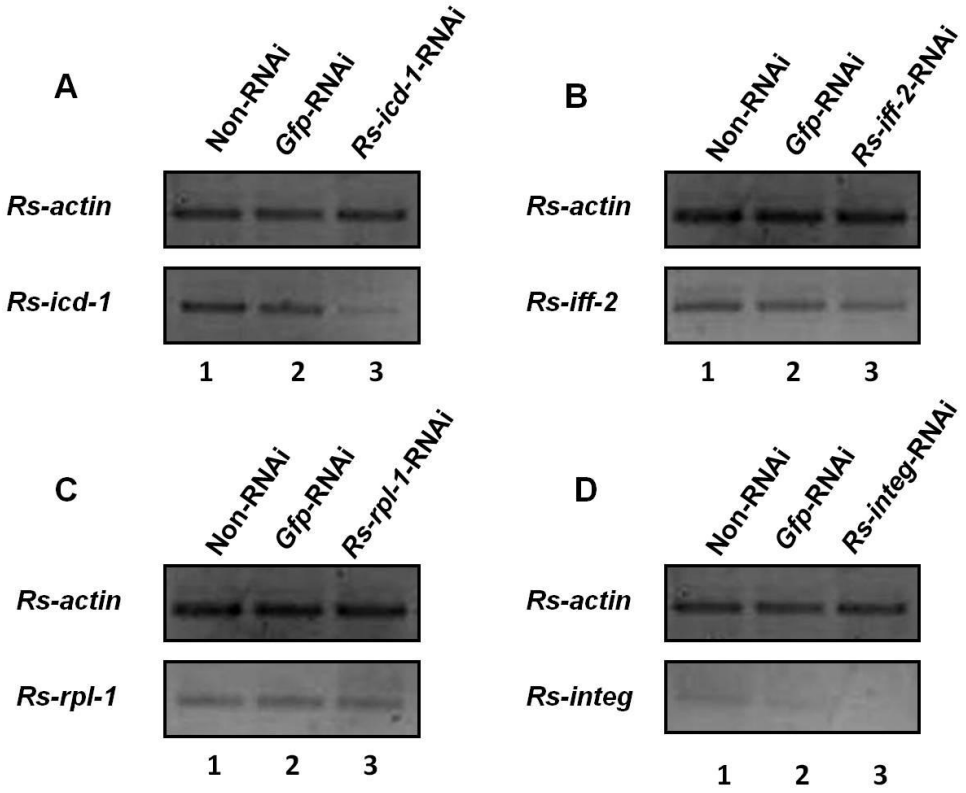

Figure 6.3. Analysis of the mRNA level of *Rs-icd-1* (A), *Rs-iff-2* (B), *Rs-rpl-1* (C) and *Rs-integ* (D) by semi-quantitative RT-PCR following 24 h of soaking with the corresponding dsRNAs. The top panel refers to the transcription level of *Rs-actin* (196 bp product) from each treatment, whereas the bottom panel refers to the transcription level of the target gene ,*Rs-icd-1* (267 bp product), *Rs-iff-2* (408 bp product), *Rs-rpl-1*(306 bp product) or *Rs-integ* (399 bp product). Non-RNAi (lane 1): *R. similis* nematodes incubated in soaking solution without dsRNA. *Gfp*-RNAi (lane 2): nematodes incubated in dsRNA of *gfp* as a dsRNA control. *Rs-icd-1*RNAi (lane 3): nematodes incubated in dsRNA of *Rs-icd-1. Rs-iif-2*RNAi (lane 3): nematodes incubated in dsRNA of *Rs-iff-2. Rs-rpl-1*RNAi (lane 3): nematodes incubated in dsRNA of *Rs-rpl-1*. *Rs-integ* RNAi (lane 3): nematodes incubated in dsRNA of *Rs-integ.* The experiment was done two times

6.4.2 Infection test

The *in vitro R. similis* infection of *Medicago truncatula* with the dsRNA treated nematodes confirmed the importance of the target gene for nematode infectivity. In agreement with the above semi-quantitative RT-PCR results (section 6.4.1), no significant difference ($P>0.05$) in the number of penetrated nematodes and egg production was observed in the case of the *Rs-eng2* dsRNA treatments compared to the control treatments (Figure 6.4A). However, a significantly lower number of nematodes was seen inside the roots in *Rs-eng1B* dsRNA treatments ($P<0.05$) as compared to the control treatments dsRNA of *gfp* (*Gfp*-RNAi) and no dsRNA (Non-RNAi) (Figure 6.4A). By contrast, the silencing of *Rs-eng1B* also resulted in 80% reduction in egg production compared with the control treatments (Figure 6.4A). Similarly, the down-regulation of *Rs-icd-1* significantly ($P<0.05$) reduced nematode invasion and egg production compared to the control treatment with dsRNA of *gfp* (Figure 6.4B).

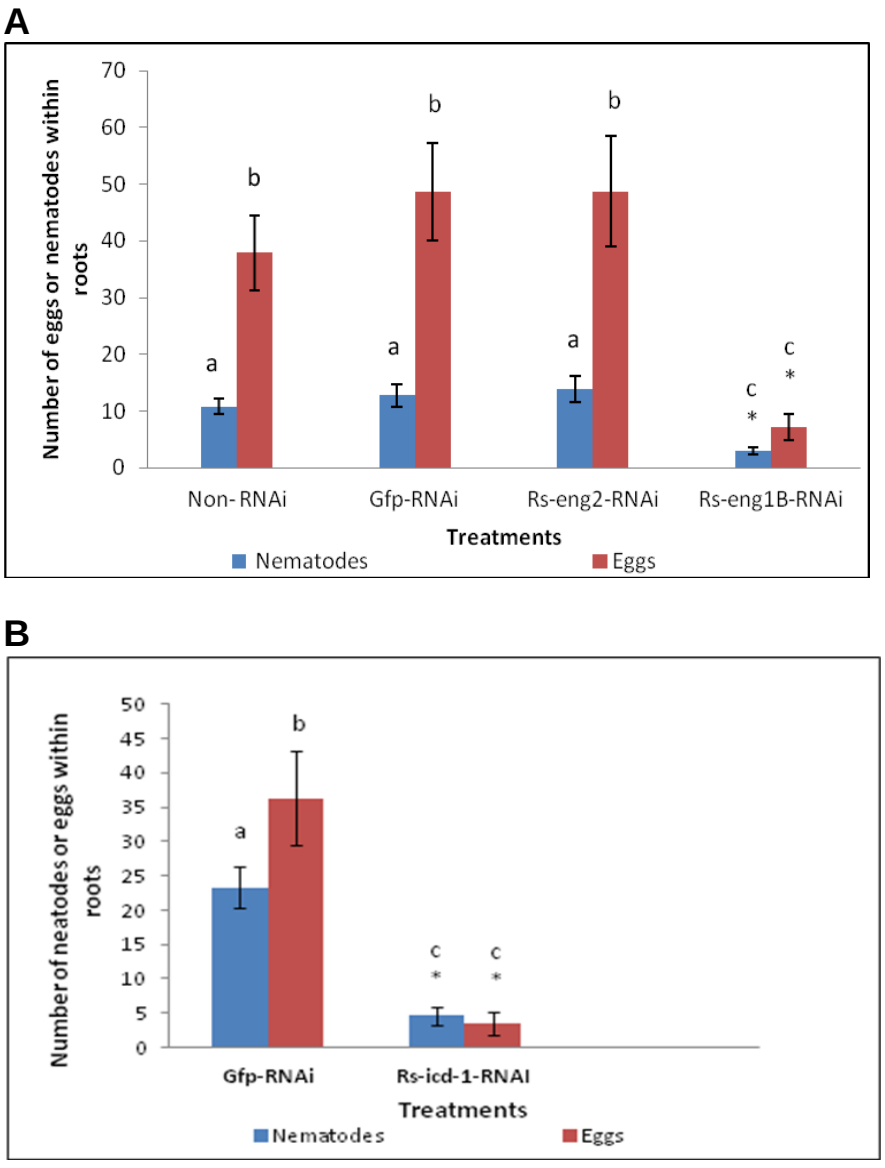

Figure 6.4. Down-regulation of *Rs-eng1B* and *Rs-icd-1* in *Radopholus similis* reduces root invasion of the nematodes in *Medicago truncatula*. (A) The number of nematodes and eggs observed inside the plant roots at 10 days post infection following 24 h soaking of the nematodes with dsRNA of *Rs-eng1B* and *Rs-eng2*.(B) The number of nematodes and eggs observed inside the plant roots at 10 days post infection following 24 h soaking with dsRNA of *Rs-icd-1*. Each plant was infected with 50 nematodes of mixed stage. Each treatment consisted of 10 biological replicates. The experiment was repeated twice. Any two means in a column with a letter in common are not significantly (P ≤ 0.05) different according to Tukey's multiple range test. 6.4.3 Transient GUS assay.

6.4.3 Transient GUS assay

ECS samples of dessert banana 'Grande Naine' were transformed with the four different vectors containing the hairpin constructs and the control vector pFAJ3000 to assess transformation frequency. After six days co-cultivation, histochemical GUS analysis showed that transient GUS expression was absent in the control, non-transformed cell clusters but was present in pFAJ3000 transformed banana cell clusters (Figure 6.5A and 6.5B, respectively). Quantification by counting the number of blue foci revealed an average ±SDEV (n=6) of 1545±830 blue foci per sample (approximately 50 mg fresh weight cells).

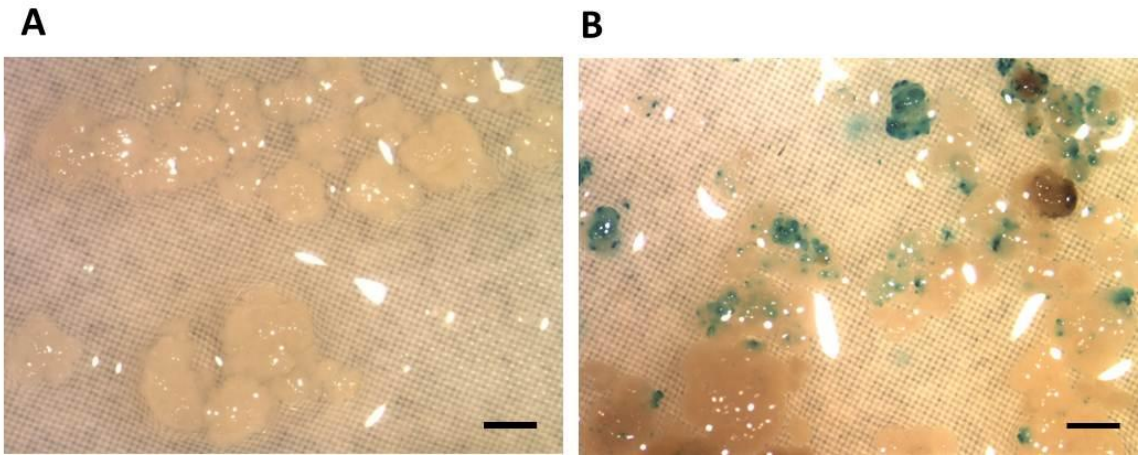

Figure 6.5. Histochemical GUS staining of 'Grande Naine' control, non-transformed (A) and EHA101 (pFAJ3000) transformed (B) cell clusters after 6 days co-cultivation. Meshes with cells were saturated with X-Gluc (1 mg/ml) solution and incubated for 4 h at 37°C. Size bar represents 1 mm.

6.4.4 Selection, regeneration and multiplication of transgenic banana

The transgenic cell colonies from ZZ selective medium (50 mg/l geneticin and 200 mg/l timentin were transferred individually to selective RD1 medium after three months selection (Figure 6.6), to obtain 120 independent lines per construct.

Embryo formation was successfully induced after two months and subsequently, the differentiated cultures were transferred to non-selective RD2 medium for shoot induction. Transgenic roots were obtained 8-10 months after transformation following transfer of shoots to REG medium.

Although a minimum of 6 independent regenerated lines were obtained per construct, plants of some lines showed poor establishment in the media (Figure 6.7). Approximately 20 plants per line were produced for the infection experiment after 2-3 months of multiplications on PROL and REG media.

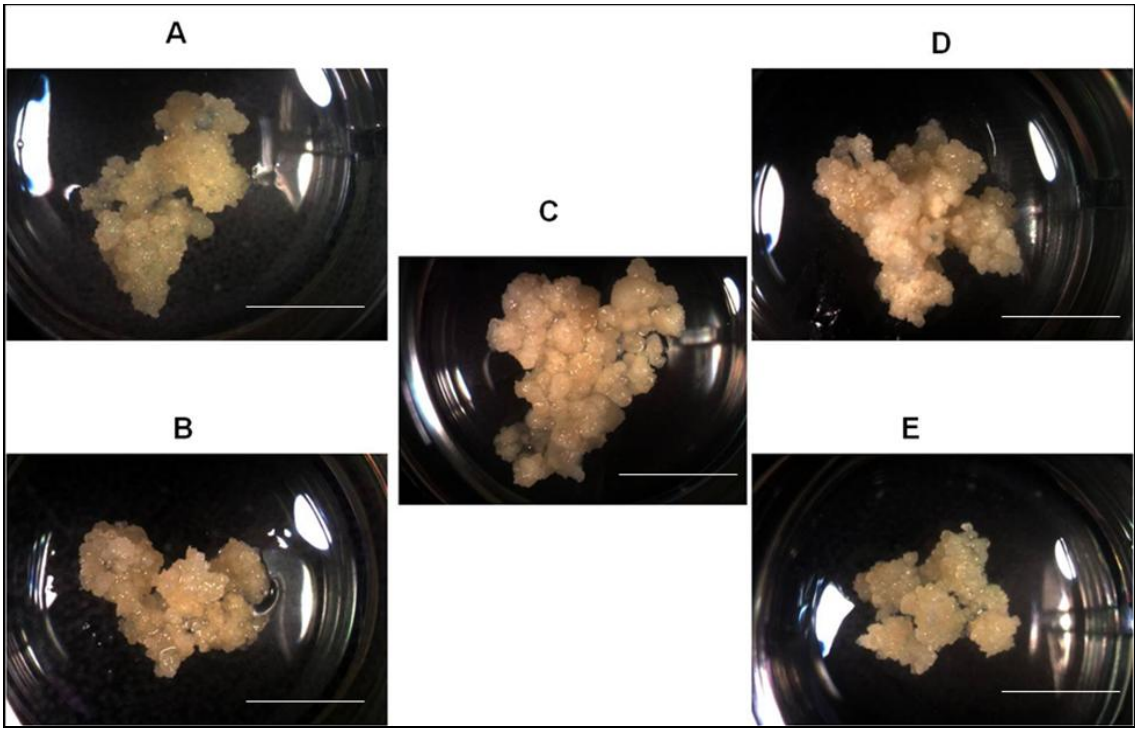

Figure 6.6. Differentiating cell cultures of transformed banana cells on selective (50 mg/l geneticin and 200 mg/l timentin) RD1 medium. Each image shows an independent line: *Rs-icd-1* (A), *Mi-integ* (B), untransformed control (C), *gfp* (D) and *Rs-eng1B* (E). Pictures were taken using a Spot RT Microscope Digital camera. Size bar represents 2 mm.

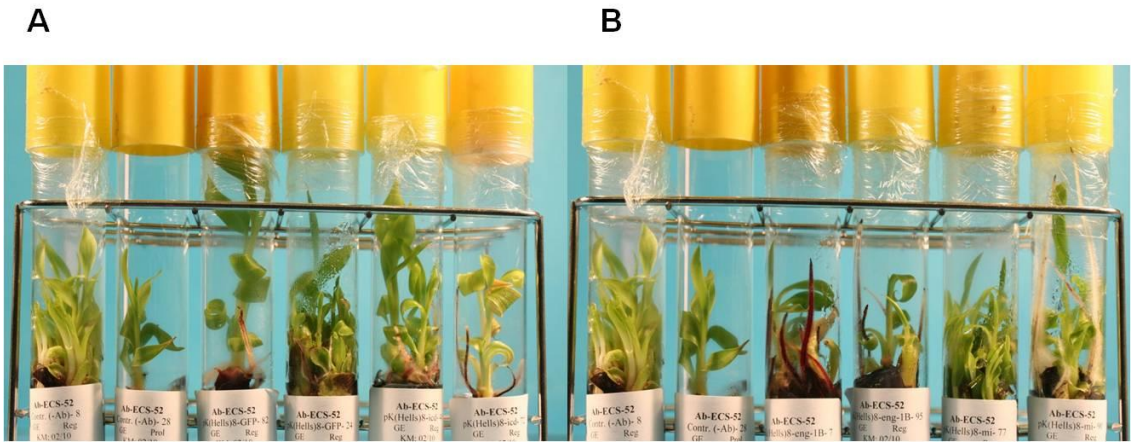

Figure 6.7. Regeneration and multiplication of transgenic banana plantlets *in vitro*. A sufficient number plantlets for each line was obtained by multiplication on REG/PROL media for 2-3 months. (A). Plants from two independent lines of control and two independent lines carrying dsRNA construct for *gfp* and *Rs-icd-1*. (B). Plants from two independent lines of control and two independent lines carrying dsRNA construct for *Rs- eng1B* and *Mi-integ,* Differential growth patterns were observed among the transgenic lines.

6.4.5 Presence and integration of the gene silencing cassettes

The presence of the target gene sequence for silencing was checked in the *in vitro* and greenhouse plants by PCR. The diagnostic products of *gfp* (215 bp), *Mi-integ (*285 bp), *Rs-icd (*315 bp) and *Rs-eng1B* (215 bp) were amplified using their specific primers from all transformed *in vitro* and greenhouse banana plants as well as in their corresponding plasmids, while in the non-transformed control lines they were absent (Figure 6.8.A, B and C). However, the PCR for the lines transformed with the *Mi-integ* silencing constructs showed a non-specific signal (Figure 6.8C) and sometimes results were inconsistent (data not shown). An additional product of approximately 420 bp in addition to the expected product size of 285 bp was seen.Further PCR screening to demonstrate the efficiency and reliability of the transformation and the resulting transgenic plants was conducted by testing for the presence of the *nptII* and promoter–target gene sequences in both *in vitro* and greenhouse (Table 6.4) lines.

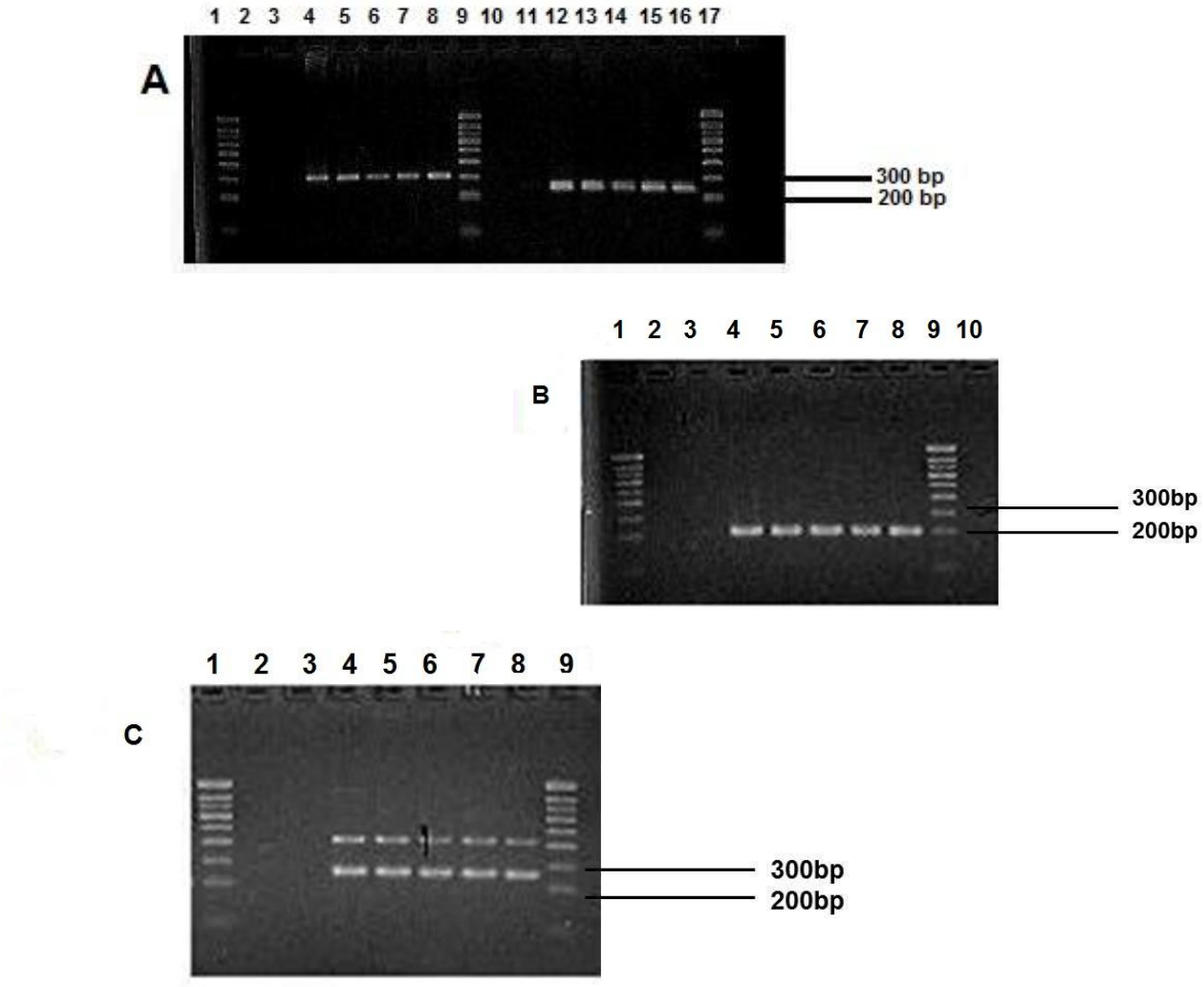

Figure 6.8. PCR screening of the *in vitro* transgenic banana plants for the presence of the target and control gene sequences used for silencing nematode genes in leaf samples using gene specific sequence primers. Panel A: target genes *Rs-icd-1* (left) and *Rs-eng1B* (right) of *R. similis:* lane 1, 100 bp Smart ladder (Eurogentec); lane, 2 blank (water) PCR control; lane 3, non-transformed control line 8; lane 4, positive *Rs-icd-1* RNAi vector control; 5 to 8: four independent *Rs-icd-1* lines (icd-9, -31, -41 and -45); lane 9, 100 bp Smart ladder; lane 10, blank (water) PCR control; lane 11, non-transformed control line 28; lane 12, positive *Rs-engIB* RNAi vector control; lanes 13 to 16: four independent *Rs-eng1B* lines (eng1B-54, -95, -103 and -104); lane 17, 100 bp Smart ladder. Panel B: *gfp* RNAi lines: lane 1, 100 bp Smart ladder; lane 2 blank (water) PCR control; lane 3, non-transformed control line 48; lane 4, *gfp* RNAi vector control; lanes 5 to 8: four independent gfp lines (gfp-6, -8, -24, -111); lane 9, 100 bp Smart ladder. Panel C: target gene *Mi-integ* of *M. incognita*: lane 1, 100 bp Smart ladder; lane 2, blank (water) PCR control; lane 3, non-transformed control line 123; lane 4, positive *Mi-integ* RNAi vector control; lanes 5 to 8: four independent Mi-integ lines (mi-21, -55, -77 and -90); lane 9, 100 bp Smart ladder.

No amplification product was obtained in non-transformed lines. We can therefore conclude that all target and control genes, *Rs- eng1B, Rs-icd-1*, *Mi-integ* and *gfp* were present in the genomic DNA of the transgenic banana plants.

Table 6.4. *In vitro* lines and green house grown lines analysed by PCR for the presence of the silencing cassette

Line tested	Target gene sequence or promoter-target sequence		
	Silencing construct[a]	***Pnos-nptII***	**promoter-target sequence[b]**
Control-8	-	-	-
Control-28	-	-	-
Control-48	-	-	-
Control-61	-	-	-
Control-123	-	-	-
Gfp-6	+	+	NA
Gfp-8	+	+	NA
Gfp-24	+	+	NA
Gfp-90	+	+	NA
Gfp-111	+	+	NA
mi-21	+	+	+
mi-55	+	+	+
mi-77	+	+	+
mi-90	+	+	+
Icd-9	+	+	+
Icd-30	+	+	+
Icd-31	+	+	+
Icd-41	+	+	+
Icd-45	+	+	+
Eng1B-54	+	+	NA
Eng1B-71	+	+	NA
Eng1B-95	+	+	NA
Eng-1B-103	+	+	NA
Eng-1B-104	+	+	NA

[a] *Primers specific for the silencing hairpin sequences; Mi; Mi (Promo-F and Mi-GsR1); eng1B; (EngGsR1 and Mi- Promo- F); icd-1; (Rs-icd-1-R3 and Rs-icd-1-F3); gfp; (GFP-F and GFP-R)*
[b] *Promoter-target sequence P_{Ubi}-Mi (UbiL and Mi-Gs-R1) or P_{Ubi} icd-1 (UbiLd and Rs-icd-1-F3). NA indicates 'not applicable'.*

To analyse the integration pattern of the transformed cassettes, Southern hybridization was performed on some selected lines. Two inserts of the *Rs-eng1B* target gene sequence are present in the line eng1B-54, while in the line eng1B-103 no signal was detected (Figure 6.9A). The double insertion in line eng1B-54 was also revealed when the hybridization was repeated with the *nptII* probe, while in the line eng1B-103 a single insertion was observed confirming its transgenic nature (Figure 6.9B). The hybridization analyses also confirmed transgene absence in the untransformed line. Integration of the *Mi* target sequence in line mi-55 and mi-90 could not be confirmed due to the high background (data not shown), while with the nptII probe the signal was too weak to decisively conclude integration of the selectable marker gene (Figure 6.9C).

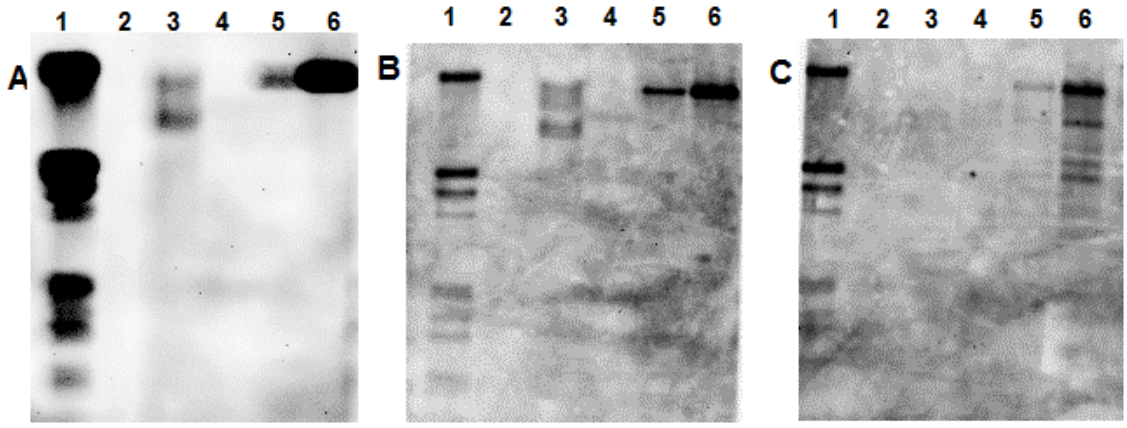

Figure 6.9. Southern hybridization analyses to verify integration of the *Rs-eng1B* target gene sequence (A), the *nptII* gene in *Rs-eng1B* transformants (B) and the *nptII* gene in the *Mi-integ* transformants (C). Genomic DNA digested with *Sac*I was hybridised with a DIG labelled probe. Panel A: Lane 1, DIG DNA marker II; lane 2, untransformed control line 123; lane 3-4, *Rs-engIB* transformed banana plant lines 54 and 103; lane 5-6, Rs-eng1B plasmid vector 1 and 5 copies, respectively. Panel B: Lane 1, DIG DNA marker II; lane 2, untransformed control line 123; lane 3-4, Rs-eng1B transformed banana plant lines 54 and 103; lane 5-6, Rs-eng1B plasmid vector 1 and 5 copies, respectively. Panel C: Lane 1, DIG DNA marker II; lane 2, untransformed control line 123; lane 3-4, *Mi-integ* transformed banana plant lines 90 and 55; lane 5-6, transformation Mi-integ plasmid vector 1 and 5 copies, respectively.

6.4.6 Plant phenotypes and infection

A very poor growth and root development of the *in vitro* plantlets was observed after a few multiplication rounds and maintenance. Only 4 independent lines with a well-established root system and 2-3 leaves were finally available for *Rs-icd-1*, *Mi-integ*, *gfp* and control plants, whereas only one line of *Rs-eng1B* was sufficiently developed for the greenhouse infection experiment. A total of 13 independent lines with at least 12 plantlets per line were obtained. Growth and development were evaluated weekly by measuring plant height and number of leaves from seven weeks onwards.

The initial assessment of the plantlets at the time of planting indicated that only two lines, namely control-61 and mi-90, were robust with well-established root systems compared to the other lines of which the plants were rather small with few roots. The majority of the plants evaluated at the standard time of inoculation (eight weeks after planting) were less than 10 cm in height (Figure 6.10A, B and C). Most of them showed a stunted growth, tip dieback and had very poor root growth. A first attempt was made to get a better growth by testing different soil conditions. The plants were also grown in compost media alone without mixing with sand. Although the plants grown in compost media showed a better survival as compared to sand: compost mix (2:1), the plants didn't show any significant improvement in plant growth and rooting (Figure 6.10D). This status of growth was sustained in the majority of the lines even after four months of maintenance in the greenhouse. A second attempt was made to check the possibility for obtaining better root growth under *in vitro* conditions. The regenerated shoots (3 independent lines from each construct) were transferred to rooting media with half strength MS media without plant growth regulators.

However, the rooting remained very weak (data not shown) and therefore, the infection of the plants with the nematode inoculum could not be performed.

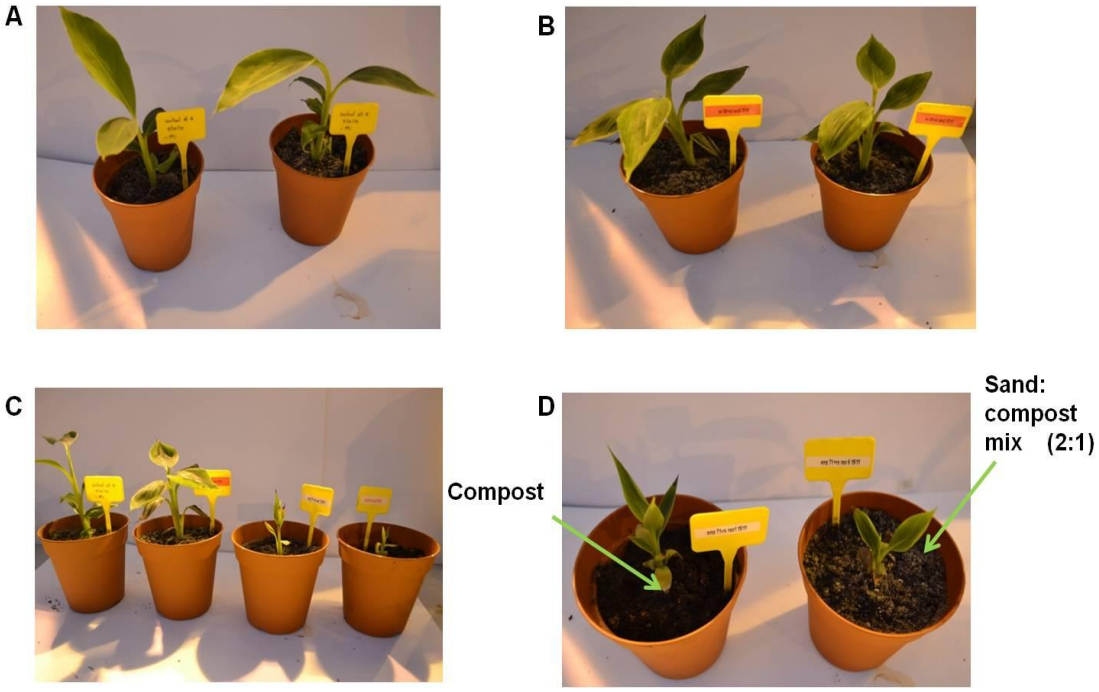

Figure 6.10. Green house plants 6 weeks after planting. (A). Plants from control line 61. (B). Plants from Mi-integrase line 90. (C). A comparison on plant growth among four lines of control, Mi-integ, Rs-eng1B and gfp. (D). A comparison on plant growth between two plants from Rs-eng71 lines in two different soil media. Plants showed slow development with poor growth of pseudostem/leaves and roots. Most of the lines showed very poor growth. The different growth media did not influence the plant growth significantly.

6.5 Discussion

The *in vitro* RNAi results show that RNA interference can be induced in the migratory endoparasitic nematode *R. similis* by feeding with dsRNA as reported previously in root- knot (Rosso *et al*., 2005) and cyst nematodes (Vanholme *et al*., 2007).

However, the silencing effect was not at the same level in all the targets: *Rs-eng 1B*, *Rs-icd-1* and *Rs-integ* showed a significant reduction in the transcript expression while *Rs-eng2*, *Rs-rpl-1* and *Rs-iff-2* did not show a significant knock-down effect after dsRNA soaking.

This shows that not all genes were affected by RNAi or that the specific target sequences were not suitable for efficient RNAi. A previous study by Chen *et al*. (2005b) demonstrated that a 244-bp dsRNA targeting an amphid secreted protein of *Globodera rostochiensis* induced greater silencing than a 309-bp dsRNA targeting the *β-1, 4-endoglucanase* as assessed by semi quantitative RT-PCR. This can be due to a number of factors including differences in spatial expression patterns, level and turnover rate of the endogenous transcript, or length or sequence of the dsRNA. Moreover, some genes might be less susceptible due to the influence of antagonising factors of RNAi or other RNAi effectors as demonstrated in the *in vitro* RNAi study of *Pratylenchus coffeae* (Chapter 5).

The infection tests have shown a high nematode mortality and reduced egg production after *Rs-eng1B* RNAi and *Rs-icd-1* RNAi soaking treatments. The results from this study indicate that the cell wall-degrading enzyme *Rs-eng1B* may play an important role in the parasitic behaviour of *R. similis* within the roots. Our results also show that the down-regulation of *Rs-icd-1* significantly reduces nematode penetration. A number of reports also have shown that the knock-down of nematode essential genes results in significant reduction in number of nematodes within the host plants (Yadav *et al*., 2006; Klink *et al.,* 2009). A study in *C. elegans* showed that RNAi of *icd-1* generates a locomotion variant exhibiting deviations in self-propelled movement on a solid medium compared to control animals (Simmer *et al*., 2003). The knock-down *of icd-1* also resulted in slow growth, larval arrest, embryonic lethality as well as morphological and developmental defective in *C. elegans* (Simmer *et al*., 2003)

Hence, it is possible that the down-regulation of *Rs-icd-1* might have an adverse effect on nematode locomotion or its growth and development resulting in reduced capability to penetrate and establish within the plant roots.

The *in vitro* RNAi study showed that the *Rs-eng2* transcript level was not affected by dsRNA soaking and hence, the nematode could penetrate the root tissue and could establish well inside the roots.

A key challenge and essential step for the RNAi-based crop protection strategies is identification of the right targets. The optimal candidates of pathogenicity–related genes are involved in parasitism, detoxification or essential for development meaning that their knocking out leads to a lethal phenotype. Additionally these targets should be more specific to the target organism and should not be present in the host plant or consumer. Our soaking assay showed that infection of *R. similis* can be effectively controlled by the use of RNAi-mediated silencing of the genes involved in the parasitism or development of the nematodes. These targets are highly nematode specific and hence, *Rs-eng 1B* and *Rs-icd-1* can be potential target genes to control these nematodes.

As a control strategy against *R. similis,* we made an attempt to deliver dsRNA *in planta* to the nematodes. The number of blue foci per sample obtained in the transient *gus* assay was in agreement with the expected number for a 35S promoter driving the *gusA* gene (Sági *et al*., 1995; Arinaitwe *et al.*, 2004). This demonstrates that T-DNA transfer was highly successful during the transformation. Additionally, the PCR results also indicated the presence of the target genes in the plants and Southern hybridization results have shown the integration of the transformed cassette in some lines. However, the experiment could not be finalised due to the poor establishment of the plants.

Although different soil composition and growth conditions *in vivo* and *in vitro* were tested, the plants did not grow beyond 10 cm with very poor to no rooting in all the plants including transgenic and non-transformed control plants. Consequently, transformation itself was not the cause behind this improper development.

The *in vitro* plants as well as the greenhouse plants showed abnormality in leaf growth and root development indicative of somaclonal variation. Such a phentoype with erect narrow leaves is not new and can occur after *in vitro* culture, especially when the *in vitro* culture process has taken long time (Dhed'a *et al*., 1991; Cote *et al*., 1996; Vuylsteke *et al*., 1988, 1996; Stross *et al*., 2006). We therefore propose that this variant type is due to the *in vitro* culture and not due to the transformation events. Considering the long time period for developing a new cell suspension (approximately 1.5 years) and new transgenic lines, the experiment was not repeated. Nevertheless, the stable integration of the T-DNA in the transgenic lines was confirmed except in the case of *Mi-integ*. The PCR results for *Mi-integ* lines were sometimes inconsistent and an additional larger band besides the expected band was observed using the plasmid vector of *Mi-integ* as template which may indicate the instability of the RNAi vector.

Although the hpRNA expressing vectors based on the Gateway system have been used widely for constructing transgenic plant lines, an alternative high-throughput system for making hairpin RNA constructs has recently been developed (Xiao *et al*., 2006; Chen *et al*., 2009; Yan *et al*., 2009; Xu *et al*., 2010; Yan *et al*., 2012).

Yan *et al.* (2012) have demonstrated the utility of the RNAi constructs generated with the pRNAi-GG vector for the effective silencing of various genes individually as well as two genes simultaneously using one-tube restriction-ligation and one-step transformation. The application of the RNAi vectors such as pSAT-RNAi vector and pRNAi-GG vector for multiple gene silencing (Dafny-Yelin *et al*., 2007; Yan *et al*., 2012) may provide a novel and high-throughput platform for developing an efficient control strategy against nematodes as well as for large scale analysis of nematode functional genomics.

One of the concerns of using long dsRNA derived vectors for *in planta* RNAi can be non-specific (off-target) gene silencing (Scacheri *et al*., 2004; Lin *et al*., 2005; Ossowski *et al.,* 2008). In mammalian cells highly selective RNAi has been achieved by using siRNA expression vectors (Shi, 2003). The application of this approach may be an alternative control strategy against PPN by highly selective RNAi avoiding non-specific target silencing. However, the success of this approach among different species of PPN is still uncertain. Although a significant silencing effect has been observed in *M. incognita* by siRNA soaking against *Mi-CRT*, the effect was not persistent throughout the subsequent infection using the soaked nematodes (Arguel *et al*., 2012), whereas the migratory nematode *P. coffeae* was less susceptible to the siRNAs tested (*Pc-pat-10*, *Pc-eng-1*) (Chapter 5). However, the co-silencing of the RNAi inhibitor *eri-1* can enhance the persistence of siRNA-mediated silencing in *P. coffeae* (Chapter 5). An approach based on dual or multiple target genes RNAi against the nematodes can be an efficient system to restrict the reproduction of plant parasitic nematodes.

The efficacy of the RNAi approach as a control strategy may also depend upon the use of a promoter sufficiently strong to transcribe hairpin RNA that fulfils the requirement for a high dsRNA input to induce efficient silencing in the nematodes (Fairbairn *et al*., 2007). Zheng *et al.* (2004) have reported the development of a dual promoter siRNA expression system that allows facile *in vivo* transcription of multiple siRNAs in a high-throughput manner in mammalian cells. Adopting these approaches in plants can create a platform for efficient and stable expression of siRNA to induce a highly specific RNAi and hence the development of a high-throughput system for host-derived resistance against nematodes.

Transformation of the host plant remains a bottleneck in developing transgene resistance against nematodes and a quick means for testing gene functions without going through the lengthy process of producing stable transgenic plants is highly desirable. The production of a hairy root system for the crops which require a longer period of time for generating stable transgenic plants can be a feasible and faster approach in functional genomic study (Li *et al*., 2010b) although it has some disadvantages such as wide variation in root morphology and proliferation ability among different hairy root lines. This may result in variability in nematode infection consequently not allowing to accurately determining nematode resistance level in each transgenic line that is producing a specific dsRNA. By selecting lines with uniform size and vigour, the hairy root system can be used to evaluate the efficacy of dsRNA of nematode genes in suppressing nematode population (Klink *et al.,* 2009; Charlton *et al*., 2010).

However, further research is needed to evaluate the possibility of maintaining the RNAi-mediated nematode resistance in the target crop plant in the field at a sufficient level. The main objective to test the efficacy of RNAi-mediated transgenic resistance against *R. similis* in banana was not achieved in the present study and thus, a future attempt to generate transgenic resistance in banana against the migratory parasitic nematodes is necessary to deliver proof-of-concept. in a commercial crop

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Chapter seven

General discussion and future perspectives

The importance of using RNAi approaches as a versatile tool for gene discovery has been demonstrated in a wide variety of organisms (Grimm *et al.,* 2010; Lee *et al*., 2010; Miyoshi *et al*., 2010; Pontier *et al*., 2012; Avgousti *et al*., 2012). In addition, it has also found application as an effective control strategy against various crop pest including viruses, insects, parasitic plants as well as nematodes (Aly *et al*., 2009; Gu *et al*., 2013; Kumar and Sarin, 2013; Lilley *et al*., 2012; Qu *et al*., 2007). Although the use of RNAi has been demonstrated in a number of plant parasitic nematode (PPN) species, the feasibility of its application in other economically important nematodes species such as the migratory *P. coffeae* and *R. similis* had not been previously studied. This study was therefore initiated to assess the feasibility of RNAi in the root lesion nematode *P. coffeae*, a major pest in banana plantations together with *R. similis* and the feasibility of using RNAi as a control strategy in their management.

7.1 Selection of optimal RNAi target for a broader nematode resistance

In order to choose optimal candidates for developing RNAi mediated resistance in the host plants, it was necessary to perform a preliminary screen for the nematode genes, which are susceptible to RNAi as well as genes that impact on the parasitic success of the nematode. A set of target genes was screened in *P. coffeae* and *R. similis* by *in vitro* RNAi soaking of the mobile stages of nematodes in corresponding dsRNA solutions of the selected genes for 24h. During the initial stages of this study, the sequence information of the genes in *P. coffeae* was very limited. Hence profiting from the data extrapolated from the RNAi screens of *C. elegans,* two candidate genes, *Pc-pat-10* and *Pc-unc-87, P. coffeae* orthologs of *C. elegans* genes were cloned by PCR using degenerate primers based on conserved regions from different species of nematodes (Chapter 2).

The selected sequence for the experiment was found to be highly conserved among diverse groups of nematodes including the plant, animal and human parasitic nematodes as well as free-living and entomopathogenic nematodes and did not show any significant similarity in non-nematode species. This use of degenerate PCR primers can be useful approach to clone the candidate gene when the sequence information is limited in the target nematode. It is, however, necessary to have sufficient sequence information for the target gene from multiple species of nematodes and also have conserved region of adequate size to design the primers (Delgado *et al*., 2012; Haegeman *et al*., 2008).

Developing resistance against a single nematode species in plants that are host to multiple species such as banana, may not be an efficient control strategy as this will reduce inter specific competition and allow the remaining nematode pests to multiply exponentially. The selection of a dsRNA or siRNA from a nematode specific and highly conserved sequence among diverse group of parasitic nematode species can be a possible approach to develop resistant plants against multiple nematode species. The targets selected against *P. coffeae* in this study could thus be optimal candidates for host derived RNAi mediated resistance against multiple species associated with banana roots.

7.2 Factors influencing enhanced RNAi in *P. coffeae*

Although the results from the *in vitro* RNAi experiment have shown that the migratory endo-parasitic nematode, *P. coffeae* has a functional RNAi machinery, the rebound effect of *Pc-pat-10* and *Pc-unc-87* RNAi observed in *P. coffeae* was in contrast to *C. elegans* where the knocking down effect of *Pat-10* persists even in the progeny long after the removal of the dsRNA trigger (Chapter 2).

The results from the combinatorial RNAi experiments indicated an enhancement of the persistence of RNAi of *Pc-pat-10* when co-silenced with *Pc-eri-1,* although, this effect was not observed for all genes tested (Chapter 5).

The persistence or otherwise of the RNAi effect of *Pc-unc-87* was not influenced by the co- silencing with *Pc-eri-1*, and for *Pc-eng-1*, a clear conclusion could not be reached. This was because the silencing effect persisted after 48h of recovery even without the co-silencing of *Pc-eri-1*. Additionally, there is the possibility that the semi quantitive PCR method used might not have detected slight enhancement of the knock-down after the co-silencing with the *Pc-eri-1* or that the recovery *Pc-eng-1* occurs at a time later than the period observed in this study (> 48h). Further studies to assess the co silencing effect of *Pc-eri-1* on *Pc-eng-1* expression at different time points of recovery (durations over 48h) with additional cycles of PCR or Q-PCR may be necessary to determine more precisely if any reduction in the transcript level occurs.

Zhuang and Hunter, (2011b) showed that tissue-specific difference in RNAi sensitivity exists among the *eri* mutants of *C. elegans* and that RNAi can be a property of a particular cell type (Asikainen *et al.,* 2005; Calixto *et al.,* 2010). Hence it is important to evaluate the RNAi responses of different genes expressed in the same tissue as well as different tissues to confirm the cell to cell or gene to gene variation in RNAi response by targeting more genes along with the co- silencing of *Pc-eri-1* in *P. coffeae*.

The potency of RNAi in *C. elegans* is a result of transitive RNAi, which refers to the spread of RNAi silencing specificity *in cis* along a target mRNA to target sequences not in the original trigger dsRNA. This transitive RNAi is the product of secondary siRNAs produced by RNA directed RNA polymerase (RdRP) and these secondary siRNAs can target other mRNA transcripts *in trans* resulting in a robust RNAi (Pak *et al.,* 2012).

The long term persistence of knock-down effect in *Pc-eng-1* (RNAi) compared to *Pc-pat-10* and *Pc-unc-87* raises more questions regarding the RNAi mechanism in *P. coffeae.* While the gene encoding RdRP, *rrf-1*, required for the amplification step in somatic cells were also not recognized in the transcriptome of *P. coffeae* and *H. oryzae*, *ego-1* encoding the RdRP in germline was identified in these nematodes. However, RdRP homologs have never been found in insect species where robust RNAi has been reported (Tomoyasu *et al*., 2008). Hence it is also possible that *P. coffeae* may have RdRP-like activity via alternative enzymes as occurs in *Drosophila* cells or RdRP independent amplification may exist in *P. coffeae*. The presence of secondary siRNA in *P. coffeae* has yet to be confirmed. More insight into these factors can be obtained by complete genome sequencing of *P. coffeae* as well as by the sequencing of small RNAs in response to exogenous RNAi.

The competition model for enhanced RNAi phenotype proposes that the efficacy of exogenous RNAi is determined by the availability of enzymes utilized by both endogenous and exogenous RNAi pathways, such as DCR-1, SAGO-1, SAGO-2 and NRDE-3 (Yigit *et al.,* 2006; Zhuang *et al.*, 2013) in the *eri* mutants of *C. elegans*. The transcriptome analysis of *P. coffeae* did not reveal orthologs for SAGOs and NRDE-3.

Hence based on the current competition model of genes regulation and transcriptome analysis, it can be postulated that the silencing of *Pc-eri-1*, which is required for endogenous RNAi may result in a reduced flux through the endogenous pathway, this in turn allows for increased access to limiting resources, mainly, DCR-1 in P. coffeae resulting in an enhanced RNAi response. Additionally, Zhuang *et al*. (2013) discovered that PGL-1 is also another candidate for the limiting resources required for enhanced RNAi associated with *eri-1*.

The presence of PGL-1 and its influence in RNAi, however, is not yet known in *P. coffeae*. Zhuang *et al*. (2013) also reports that the genes that are NRDE-3 dependent are found to be PGL-1 independent and vice versa. It has also been found that when both *pgl-1* and *nrde-3* are lost simultaneously, it results in a depletion of RNAi enhancement even in the *eri-1* mutant background (Zhuang *et al*., 2013) suggesting that these two genes define the totality of *eri-1*. Consequently it is also possible that the presence or absence of nuclear RNAi components such as NRDE-3 and PGL-1 or other unknown effectors may have influence on the enhanced RNAi induced by the silencing of *Pc-eri-1* in *P. coffeae*. Additionally, the presence of these limiting resources required for enhanced RNAi can vary from tissue to tissue as observed in *C. elegans* and this might have been responsible for the variation in the response to the silencing of *Pc–eri-1* among the genes as observed in *P. coffeae*.

7.3 Nuclear effectors influencing RNAi in *P. coffeae*

Nuclear RNAi plays an important role in enhanced RNAi silencing phenomena in *C. elegans*. Although NRDE-3 (an essential component of transcriptional gene silencing which induces long term effect of RNAi in *C. Elegans*) was not recognized in the *P. coffeae* transcriptome, some of the nuclear RNAi effectors associated with transcriptional gene silencing such as *zfp-1, gfl-1, alg-1, tsn-1, mes-2* and *mes-6* were found in *P. coffeae* (Chapter 4). Additionally, the results from combinatorial RNAi (Chapter 5) have shown that the co-silencing of *Pc-gfl-l* attenuates the RNAi effect in *Pc-pat-10* (RNAi), which indicates the requirement of *Pc-gfl-1* in exogenous RNAi. However, the co-silencing of *Pc-gfl-1* did not influence the silencing of *Pc-unc-87*.

Thus there is the possibility that the expression of *Pc-pat-10* is sensitive to nuclear RNAi and dependent on *Pc-gfl-1* while *Pc-unc-87* and *Pc-eng-1* expression is independent of *Pc-gfl-1.* There is, however, the need for further exploration on the role of other nuclear RNAi effectors in RNAi of different genes to better understand the RNAi mechanism in *P. coffeae*.

7.4 Need for a careful characterization of RNAi effectors in *P. coffeae*

Considering gene specific RNAi response in *P. coffeae*, it is necessary to understand the cellular location of the proteins influencing RNAi in the parasitic nematodes. The comparative study on RNAi effectors in *P. coffeae* (Chapter 4) displays a greatly contracted suite of RNAi effector proteins in the nematode. For example, based on transcriptome analysis, *P. coffeae* seems to have only one RdRP, EGO-1, which is required for germline RNAi. Additionally, among the proteins, such as SMG-2, -5, and -6, which have an important role in the induction and maintenance of secondary amplification (Mango, 2001); SMG-2 and SMG-6 were well conserved in *P. coffeae* (Chapter 4). It is also possible that EGO-1 can act redundantly in somatic tissues like RRF-1 in these nematodes and hence, amplification of the silencing signal may exist to some extent enabling profound silencing of the genes. There is a need for a careful characterization of RNAi effectors in *P. coffeae* to understand their expression level and distribution to get more insight in RNAi regulation in *P. coffeae*.

7.5 Factors influencing the dsRNA dose required for efficient RNAi in *P. coffeae*

In the soaking assay with *P. coffeae*, it was observed that a higher dose of dsRNA (1µg/µl), which was found to be toxic in the case of other parasitic nematodes such as root-knot and cyst nematodes, did not cause any non specific action in *P. coffeae* (Chapter 2). In addition, the use of relatively higher dose of siRNA compared to the concentration used in *M. incognita* and *G. pallida* did not generate any inhibitory phenotype in *P. coffeae* (Chapter 5). A single soaking with 500ng/µl of dsRNA of *Pc-pat-10* did not show efficient RNAi in *P. coffeae* as compared to the single soaking with 1µg/µl of dsRNA (data not shown). Additionally, when dsRNA was removed from the solution, the knock-down effect was recovered within 24h. But in the case of combinatorial RNAi, the concentration of dsRNA of *Pc-pat-10* in the total solution was kept at 500ng/µl (50% of dsRNA dose used in single soaking) and the RNAi effect persisted even after 48h of recovery at this concentration. This may indicate that the co-silencing with *Pc-eri-1* enhances the RNAi potency in *P. coffeae* at lower dose of *Pc-pat-10* dsRNA. .

In *C. elegans* it has been shown that in RNAi defective mutants, a high concentration of dsRNA can compensate for the weak RNAi-defective phenotypes. For this reason, it is possible that the discrepancy among different nematodes in their response to the dsRNA concentration may be due to the differences in their inherent ability to take up dsRNA and process these introduced dsRNA which is in turn dependent on the presence of RNAi pathway components. In *C. elegans* it has been shown that RNAi phenotypic penetrance is sensitive to the dsRNA dose. Zhuang and Hunter (2011a) pointed out that RNAi penetrance versus dsRNA dose show a sigmoidal relationship.

In *C. elegans*, most nematodes do not respond to low dsRNA dose. Additionally, it is necessary to use the maximum possible dose to get high phenotypic penetrance during functional characterization of nematode genes.

Nevertheless, the optimal dose for dsRNA or siRNA should be determined based on the nematode and gene targeted as well as the use of appropriate controls to detect non-specific RNAi effects.

7.6 Possibility for alternative RNAi effectors or mechanism in *P. coffeae*

A search for the RNAi pathway components in *P. coffeae*, *M. incognita* and *H. oryzae* has shown that *P. coffeae* and *H. oryzae* possess the basic machinery required to facilitate an RNAi response as in *M. incognita* (Chapter 4) although, some of the core proteins in the RNAi pathway were missing from the transcriptome of these nematodes. *In vitro* RNAi studies have also demonstrated high susceptibility of *P. coffeae* to RNAi by dsRNA soaking (Chapter 2).

Although *P. coffeae* seems to lack systemic RNAi effectors, an efficient silencing of the genes expressed in different cells such as muscular and secretory gland cells has been observed in this study. A number of studies have also demonstrated the successful application of RNAi in *M. incognita*, and *Drosophila* S2 cells, which lack *sid-1*. This suggests that alternative uptake proteins or mechanisms might be involved in PPN. There is also the possibility that the transcription of the unidentified RNAi effectors might be too low to be detected by the analysis. However, upcoming genomic data of *P. coffeae* revealed that the RNAi pathway is partially conserved in the genome and it has reduced numbers in multi-gene families compared to *C. elegans* and other more specialized PPN such as *M. hapla* (personal communication, Opperman, C.H, 2013). Among the three RdRPs, only one RdRP, EGO-1 has been found in the

transcriptome of *P. coffeae*. Similarly *P. coffeae* possesses only a subset of putative AGO orthologs relative to *C. elegans*. Identification of genes based on gross sequence similarity may underestimate functional diversity of the gene. The function and expression profile of identified RdRP or AGOs in *P. coffeae* may be different from that of *C. elegans*. It is also possible that alternative proteins, which have similar functional role as unidentified *C. elegans* gene complement, may exist in *P. coffeae*.

7.7 Discrepancy in silencing efficiency in *R. similis*

The *in vitro* RNAi mediated by dsRNA soaking in *R. similis* was performed under the same soaking conditions as in the case of *P. coffeae*. Although the susceptibility to RNAi was not efficient in some of the genes targeted in *R. similis*, a significant level of knock-down effect was observed in some of genes tested in this study. While *Rs-eng 1B* showed a significant knock-down effect *Rs-eng2* did not. Similarly, *Rs-rpl-1* and *Rs-iff-2* did not show a significant silencing effect, while *Rs-icd-1* and *Rs-integ* were significantly downregulated following the dsRNA soaking. This is an indication that not all genes may be affected by RNAi or that the specific sequences were not suitable for efficient RNAi. This can be due to a number of factors including differing spatial expression patterns, level and turnover rate of the endogenous transcript, or length or sequence of the dsRNA. Additionally, some genes might be less susceptible due to the influence of antagonising factors of RNAi or other RNAi effectors as demonstrated in the *in vitro* RNAi study of *P. coffeae* (Chapter 5).

In this study, a significant RNAi effect was not noticed in the genes encoding ribosomal proteins such as *rpl-1.* Contrary to this, however, there are reports that have demonstrated successful knock-down of the ribosomal genes affecting nematode parasitic success in the plants (Alkharouf *et al*., 2007).

It might be possible to obtain an efficient knock-down of these target genes by testing different dsRNA sequences of the same gene or optimizing the dsRNA dose for each target. Nevertheless the present study results have demonstrated the possibility of using RNAi-mediated silencing of the genes involved in parasitism or nematode development in the control of *R. similis.*

7.8 Perspectives and recommendation for future study

In this study, the successful application of RNAi in the migratory nematodes, *P. coffeae* and *R. similis* has been demonstrated. Some of the potential targets suitable for parasitic control based on host-derived RNAi have also been identified in both nematodes. However, there is the need to explore the molecular data available for these nematodes and identify more target genes involved in parasitism, cell development or metabolism via *in vitro* RNAi screening.

This study has also shown variable RNAi efficacy among the target genes. This indicates the necessity to optimize various factors influencing RNAi efficacy depending on the target gene and nematode species. The identification of genes those are essential for RNAi or that modulate the RNAi process such as *Eri* genes have made it possible to manipulate the RNAi process in *C. elegans*. Mutations to these genes in other organisms such as mice have also been shown to have some endogenous defects, but assays in RNAi efficacy have not been thoroughly performed outside *C. elegans*. The present study has demonstrated that RNAi can also be manipulated in the plant-parasitic nematode, *P. coffeae*. Using the information on RNAi effectors in *P. coffeae* and the data documented in *C. elegans*, further experiments using combinatorial RNAi targeting different genes located in different cells should be conducted. As demonstrated in Chapter 4, EST analysis for detecting all genes used in parasitism or the RNAi pathway might be limited by the inadequate coverage of

transcriptome. Further search for unidentified genes should be performed based on the genomic sequence data. This was also illustrated in the case of *M. incognita* when novel cell wall modifying enzymes (not present in EST data) such as *arabinase* were discovered by the analysis of genomic data (Abad *et al*., 2008).

The comparative study of the migratory and sedentary nematodes based on available genomic data could provide useful insights on evolution of parasitism genes as well as RNAi effectors among the PPN. In addition to the identification of the RNAi effectors, the expression profile of these effectors will be useful for further manipulation of the RNAi response in these nematodes.

Although an attempt has been made in this study to generate transgenic banana with resistance against *R. similis*, this task could not be completed. It will be worthwhile to test the efficacy of host derived nematode resistance in banana using the selected targets. Based on the transcriptome analysis of *P. coffeae*, an efficient RNAi can be expected in germline cells due to the presence of RdRP required for siRNA multiplication in the germline. Therefore, it will also be interesting to test the effect of RNAi on the genes involved in germcell development and the impact of this knock-down on reproductive fitness of the nematode.

Despite the overall progress made in the management of nematodes using transgenic plants expressing nematode dsRNA, some hurdles are still to be overcome. One of the main challenges is to deliver a sufficient amount of siRNA in the right cells and at the right time to obtain durable and strong post transcriptional silencing. There is also a necessity for optimizing plant RNAi constructs for enhancing knock-down efficiency. Future strategies incorporating more features in construct design might help to develop a high throughput system for nematode control.

For example, incorporation of multiple gene fragments for a broad resistance using multiple shRNA expression methods, use of artificial microRNA constructs, which have been proven very efficient in generating silencing in animal RNAi research and plant research (Mcintyre *et al*., 2011a; Ossowski *et al*., 2008; Wu *et al*., 2013; Warthmann *et al*., 2013), can be some of the useful strategies to be adopted in PPN control based on RNAi.

In mammalian RNAi research, short hairpin RNAs (shRNAs) with short stem length of 19 or 21bp (<30bp) have also been used efficiently for silencing the target genes (Li *et al*., 2007; Mcintyre *et al*., 2011a; Zhou *et al*., 2005). Using short hairpin constructs which produce siRNAs more efficiently might also be an alternative strategy to obtain specific and broader resistance against nematodes. However, the properties such as stem length, the GC content and thermodynamic properties of the stem sequences are some of important factors that should be considered during the construction of shRNA for increasing the siRNA production (Hirari and Kodama, 2008; Mcintyre *et al.,* 2011b; Zhou *et al*., 2005). Additionally, a key feature for an efficient RNAi effect may be the amplification of the primary signal in the host plant. The nematodes which lack efficient secondary amplification may require a higher dose of siRNA input to induce an efficient silencing. When the dsRNA of a nematode gene is produced in plant cells, the amplification of the signal may not occur because the host lacks cognate target sequencing. Therefore, the co-transformation with native target genes may be helpful to trigger the RNAi amplification cascade (Valentine *et al*., 2007).

As discussed previously, the plant rhizosphere is colonized by different species of nematodes implying that species specific regulation of RNAi may not give complete protection for the host plant from other species of nematodes.

The selection of highly conserved sequences among diverse group of parasitic nematode species can be a possible approach to develop resistant plants against multiple species of nematodes.

Summary

RNA interference has extensively been applied as a tool to analyze the function of different genes in a wide range of organisms. In *C. elegans* this technique has been very succesful and the phenotypes resulting from the knockout of these genes have been well documented. Significant progress has been made in the development of novel control strategies against some species of plant-parasitic nematodes (PPN) based on RNAi. However, the feasibility of this approach in some economically important migratory endo-parasitic nematodes species has not been previously studied. The studies outlined in this thesis are thus focused on the application of RNAi in the root lesion nematode *Pratylenchus coffeae*, a major pest in banana plantations together with *Radopholus similis.* The aim was to study the function of some nematode genes and the feasibility of using RNAi as a control strategy in nematode management. Additionally, transcriptome analysis of *P. coffeae* was performed in order to get more insight into the genes involved in parasitic success, development and RNAi in *P. coffeae*.

In this thesis, *P. coffeae* orthologs for two *C. elegans* genes, *pat-10* and *unc-87* namely *Pc-pat-10* and *Pc-unc-87,* which encode body wall muscle proteins, were tested for their susceptibility to RNAi. Additionally, the gene encoding endo-1, 4-beta-glucanase (a plant cell wall degrading enzyme), *Pc-eng-1*, and some effectors involved in the RNAi pathway such as *Pc-eri-1* and *Pc-gfl-1* were also selected for the study. RNAi was performed by soaking mobile stages of *P. coffeae* in a solution containing dsRNA of the target gene for 24h. The persistence of the RNAi effect was checked by recovering the treated nematodes in water for 24h or 48h.

A significant down regulation of *Pc-pat-10* and *Pc-unc-87* in a sequence-specific manner was observed following soaking the nematodes with the dsRNA of the target

genes. The knocking down of these genes resulted in abnormal phenotypes: *Pc-pat-10* RNAi generated straight and rigid nematodes while *Pc-unc-87* RNAi resulted in a coiled phenotype in contrast to the regular sinusoidal movement of the control nematodes. Thus this study demonstrated the existence of the RNAi phenomenon in *P. coffeae* and indicates that the function of *Unc-87* and *Pat-10* genes has been evolutionarily conserved among free-living and plant-parasitic nematodes. The RNAi effect of *Pc-pat-10* and *Pc-unc-87* was found to be transient in *P. coffeae* contrary to its persistence in *C. elegans*. This transient RNAi effect was, however, not observed for all *P. coffeae* genes tested. The silencing effect of endo-1, 4-beta-glucanase (*Pc-eng-1*) and *Pc-gfl-1* was not recovered even 48h after dsRNA soaking showing that the transient effect of RNAi in *P. coffeae* might be gene specific.

In this study, the influence of *Pc-eri-1*, a negative regulator of exogenous RNAi, on the RNAi persistence was evaluated using the combinatorial RNAi approach by simultaneously targeting two genes. The dsRNA of *Pc-eri-1* or *Pc-gfl-1* was co-introduced with dsRNA of the target genes, *Pc-pat-10*, *Pc-unc-87* or *Pc-eng-1*. The results revealed that co-silencing of *Pc-eri-1* enhanced the persistence of RNAi of *Pc-pat-10,* however, the silencing of *Pc-eri-1* did not show a similar impact on the other genes tested. This may be an indication that the downregulation of *Pc-eri-1* only enhances the RNAi of some specific genes. Additionally, the soaking assays in *P. coffeae* showed that contrary to other parasitic nematodes such as root-knot and cyst nematodes, *P. coffeae* does not show an abnormal phenotype at a higher dose of dsRNA. This is an indication of a species-specific response to dsRNA dosage.

Another migratory nematode, *R. similis* was also used for RNAi studies. Some parasitism genes as well as genes that are required for development were targeted by *in vitro* RNAi. The results showed that the infection of *R. similis* can effectively be

controlled by RNAi-mediated silencing of nematode genes. The silencing effect was, however, not at the same level in all targets: *Rs-eng 1B* showed a significant RNAi effect by soaking. *Rs-eng2*, *Rs-rpl-1* and *Rs-iff-2* did not show a significant knock-down effect after dsRNA soaking while *Rs-icd-1* and *Rs-integ* transcripts were significantly decreased. When *M. truncatula* plants were infected with nematodes following the soaking with dsRNA of *Rs-eng-1B* and *Rs-icd-1*, a significant reduction in nematode invasion was observed. This result shows that *Rs-eng1B* and *Rs-icd-1* can be optimal candidates for host-delivered RNAi approach against *R. similis*. Vectors for dsRNA production in banana against *R. similis* were constructed for these genes using the gateway recombinational cloning system. Banana plants were transformed using the dsRNA vectors and the presence and integration of the target genes were confirmed by PCR and Southern hybridization. However screening of the transgenic banana plants for nematode resistance by infection test was not performed due to poor establishment of transgenic plants. Nevertheless, the present study results have demonstrated the possibility of using RNAi-mediated silencing of the genes involved in parasitism or nematode development in the control of *R. similis*.

The transcriptome analysis of *P. coffeae* identified several plant cell wall modifying proteins such as xylanase, pectate lyase, polygalacturonase and arabinogalactan galactosidase. In addition to this, other nematode secreted proteins with putative or proven functions in the plant such as 14-3-3b protein, acid phosphatase, calreticulin, chorismate mutase, ERp99, galectin, glutathione peroxidase, glutathione-S-transferase, peroxiredoxin, RBP-1, SEC-2 were also identified in the transcriptome data set. Additionally, the search for the RNAi pathway components in the *P. coffeae* transcriptome showed that *P. coffeae* possesses the basic machinery required to facilitate an RNAi response as in *M. Incognita*. However, the search revealed that some of the core proteins in the RNAi pathway such as RDE-1, RDE-4, PAZ-

PIWI/Argonaute protein were not identified in the nematode's transcriptome. Additionally, none of the effectors involved in systemic RNAi were found in the *P. coffeae* data set. The genes encoding RdRps such as *rrf-1* and *rrf-3*, required for the siRNA amplification step in somatic cells were also not recognized in the transcriptome of *P. coffeae.* The information obtained from the transcriptome analysis can be useful for comparative and evolutionary studies as well as to select interesting genes for functional studies.

In summary, this study has shown the feasibility of RNAi as a functional tool and a promising control strategy in two migratory nematodes. Elucidation of RNAi pathway components using transcriptomic/genomic data can be useful to understand the factors influencing the RNAi response in parasitic nematodes. Although the complex RNAi pathway is not fully understood in plant-parasitic nematodes, it is possible to manipulate the RNAi response in PPN based on the information obtained from RNAi screens in *C. elegans* and insight from the nematode genome. The results from the study also point out that there is a clear potential for engineering host derived RNAi-mediated nematode resistance against migratory nematodes by targeting optimal candidate genes. However, it is necessary to gain more insight into the approaches for further improvement of the technology in order to achieve broader and durable nematode resistance.

Nederlandse samenvatting

RNA-interferentie is een heel belangrijke methode voor de functionele analyse van verschillende genen in een brede waaier van organismen. Bovendien is er significante voor tuitgang geboekt in het gebruik van RNAi voor de controle van bepaalde pathogenen zoals planten-parasitaire nematoden (PPN).

Bij de aanvang van dit doctoraat was er echter geen kennis over de bruikbaarheid van RNAi voor de studie en controle van migratorische endoparasitaire nematoden, en daarom vormde dit de belangrijkste doelstelling van dit onderzoek. Bovendien werd ook een transcriptoomanalyse uitgevoerd op *Pratylenchus coffeae* om meer inzicht te krijgen in de genen die belangrijk zijn voor ontwikkeling, parasitisme en RNAi.

In deze thesis werden twee orthologen van *C. elegans* genen geïsoleerd uit *P. coffeae*, namelijk *Pc-pat-10* en *Pc-unc-87*, die coderen voor spiereiwitten van de lichaamswand. Deze genen werden getest op hun RNAi-gevoeligheid. Daarnaast werden ook nog een aantal andere genen uitgetest, namelijk een gen voor een celwandafbrekend enzyme, endo-1, 4-beta-glucanase (*Pc-eng-1*), en genen voor eiwitten die een rol spelen in het RNAi-proces, *Pc-eri-1* and *Pc-gfl-1.* RNAi werd uitgevoerd door incubatie van gemengde *P. coffeae* stadia in dsRNA. Een specifieke en significante neerregulatie van RNA van *Pc-pat-10* en *Pc-unc-87* werd geobserveerd, samen met de typische abnormale fenotypes van verlamming die ook in *C. elegans* voorkomen. *Pc-pat-10* RNAi veroorzaakte gestrekte wormen terwijl *Pc-unc-87* RNAi resulteerde in gekrulde wormen.

In tegenstelling tot een langdurig RNAi effect in *C. elegans* was het effect in *P. coffeae* 48 uur later al veel verminderd. RNAi op endo-1, 4-beta-glucanase (*Pc-eng-1*) en *Pc-gfl-1* bleek na 48 uur wel nog effect te hebben wat erop wijst dat de duur van het RNAi-effect genafhankelijk is. In een gecombineerde RNAi waarbij tegelijk *Pc-eri-1*, een negatieve regulator van RNAi uitgeschakeld werd, bleek RNAi of *Pc-pat-10* langer te werken. Op silencing van de andere genen was er echter geen effect.

RNAi werd ook uitgetest in *Radopholus similis* door incubatie van de nematoden in dsRNA. Silencing werd waargenomen maar het effect was genafhankelijk. RNAi werkte beter op *Rs-eng 1B* dan op *Rs-eng2*. *Rs-rpl-1* en *Rs-iff-2* vertoonden weinig verschil, terwijl *Rs-icd-1* en *Rs-integ* significant neergereguleerd werden op RNA-niveau. Wanneer *M. truncatula* planst geïnfecteerd met nematoden na inweken met dsRNA of *Rs-eng-1B* en *Rs-icd-1*, werd een significante vermindering van nematode invasie waargenomen. Dit resultaat toont aan dat de *Rs-eng1B* en *Rs-icd-1* kan optimaal zijn kandidaten voor host-geleverde RNAi aanpak tegen *R. similis*. Vectoren voor dsRNA productie banaan tegen *R. similis* werden geconstrueerd voor deze genen met de gateway recombinatorische kloneringssysteem. Banaan planten werden getransformeerd met de vectoren dsRNA en de aanwezigheid en integratie van de doelgenen werd bevestigd door PCR en Southern hybridisatie. Echter screening van de transgene bananenplanten voor nematode resistentie door infectie test werd niet uitgevoerd vanwege de slechte instelling van transgene planten. Niettemin hebben de onderhavige studieresultaten heeft de mogelijkheid om RNAi-gemedieerde silencing van genen betrokken bij parasitisme of nematode ontwikkeling in de controle van *R. similis* aangetoond.

De transcriptoomanalyse van *Pratylenchus coffeae* identificeerde meerdere plantencelwandmodificerende eiwitten zoals xylanase, pectaatlyase, polygalacturonase en arabinogalactangalactosidase. Verschillende eiwitten met hypothetische functies in de plant werden ook gevonden zoals 14-3-3b protein, acid phosphatase, calreticulin, chorismate mutase, ERp99, galectin, glutathion peroxidase, glutathion-S-transferase, peroxiredoxin, RBP-1 en SEC-2. Ook werden componenten van de RNAi-pathway in *P. coffeae* gevonden alhoewel er in vergelijking met *M. incognita* een aantal basiscomponenten lijken te ontbreken zoals RDE-1, RDE-4, PAZ-PIWI/Argonaute. Geen enkele van de factoren belangrijk voor systemische RNAi werden gevonden in *P. coffeae*. De genen die coderen voor RdRps zoals *rrf-1* en *rrf-3*, belangrijk voor siRNA-amplificatie werden ook niet gevonden in het transcriptoom van *P. coffeae.* De informatie bekomen via de transcriptoomanalyse zal ook nuttig zijn voor vergelijkende en evolutionaire studies.

Samenvattend kunnen we besluiten dat deze studie de haalbaarheid van RNAi aantoonde als een analysemiddel van, en voor de controle van, migratorische nematoden. Opheldering van de RNAi-pathwaycomponenten zal nuttig zijn om de werking van RNAi in deze nematoden beter te begrijpen en te optimaliseren.

References

Abad, P., Favery, B., Rosso, M.N., Castagnone-Sereno, P. (2003). Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. Molecular Plant Pathology, 4, 217-224.

Abad, P., Gouzy, J., Aury, J.M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E. *et al*. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nat Biotechnol 26, 909-915.

Aboobaker, A.A. and Blaxter, M. (2003) Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*. Mol. Biochem. Parasitol. 129, 41–51.

Agrios, G.N. (1997). Plant diseases caused by nematodes. In: Agrios G.N. (Ed.), Plant Pathology.Academic Press, San Diego, USA, pp. 565-597.

Alder, M. N., Dames, S., Gaudet, J., Mango, S. E. (2003). Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. RNA 9, 25–32.

Alkharouf, N.W., Klink, V.P., Matthews, B.F. (2007). Identification of *Heterodera glycines* (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of *Caenorhabditis elegans* having lethal mutant or RNAi phenotypes. Exp. Parasitol. 115, 247-258.

Alvarez-Saavedra, E and Horvitz, H.R. (2010) Many families of *C. elegans* microRNAs are not essential for development or viability. Curr Biol 20, 367-373.

Aly, R., Cholakh, H., Joel, D.M., Leibman, D., Steinitz, B., Zelcer, A. *et al*. (2009). Gene silencing of mannose 6-phosphate reductase in the parasitic weed *Orobanche aegyptiaca* through the production of homologous dsRNA sequences in the host plant. Plant Biotechnol J 7, 487-98.

Ambros, V. (2001). microRNAs: tiny regulators with great potential. Cell. 107, 823-826.

Ansel, K.M., Pastor, W.A., Rath, N. *et al.* (2008). Mouse *Eri-1* interacts with the ribosome and catalyzes 5.8S rRNA processing. Nat Struct Mol Biol. 15, 523-530.

Anwar, S.A and McKenry, M.V. (2012). Incidence and population density of plant-parasitic nematodes infecting vegetable crops and associated yield losses. Pakistan J. Zool. 44, 327-333.

Arguel, M.J., Jaouannet, M., Magliano, M., Abad, P., Rosso, M.N. (2012). siRNAs Trigger Efficient Silencing of a Parasitism Gene in Plant Parasitic Root-Knot Nematodes. Genes, 3, 391-408.

Arinaitwe, G., Remy, S., Strosse, H., Swennen, R., Sa´gi, L. (2004). *Agrobacterium*—and particle bombardment-mediated transformation of a wide range of banana cultivars. In: Mohan Jain S, Swennen R (eds) Banana improvement: cellular, molecular biology, and induced mutations. Science Publishers, Inc.,Enfield, pp 351–357.

Arvanitoyannis, I.S., Mavromatis, A.G., Avgeli, G.G., Sakellariou, M. (2008). Banana: cultivars, biotechnological approaches and genetic transformation. International Journal of Food Science and Technology 43, 1871–1879.

Asikainen, S., Storvik, M., Lakso, M., Wong, G. (2007). Whole genome microarray analysis of *C. elegans rrf-3* and *eri-1* mutants. FEBS Letters 581, 5050–5054.

Asikainen, S., Vartiainen, S., Lakso, M., Nass, R., Wong, G. (2005). Selective sensitivity of *Caenorhabditis elegans* neurons to RNA interference. Neuroreport.16, 1995-1999.

Atkinson, H.J., Grimwood, S., Johnston, K., Green, J. (2004) Prototype demonstration of transgenic resistance to the nematode *Radopholus similis* conferred on banana by a cystatin. Transgenic Res. 13, 135–142.

Atkinson, L. E., Stevenson, M., McCoy C.J. *et al*. (2013). *flp-32* Ligand/Receptor Silencing Phenocopy Faster Plant Pathogenic Nematodes." PLoS Pathog 9(2), e1003169.

Avgousti, D.C., Palani, S., Sherman, Y., Grishok, A. (2012). CSR-1 RNAi pathway positively regulates histone expression in *C. elegans.* The EMBO Journal 31, 3821 – 3832.

Bairu, M.W., Aremu, A.O., Van Staden, J. (2011). Somaclonal variation in plants: causes and detection methods Plant Growth Regul 63,147–173.

Bairu, M.W., Fennell, C.W., van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (Musa AAA cv. 'Zelig'). Sci Hortic 108, 347–351.

Bakhetia, M., Urwin, P.E., Atkinson, H.J. (2008). Characterisation by RNAi of pioneer genes expressed in the dorsal pharyngeal gland cell of *Heterodera glycines* and the effects of combinatorial RNAi. Int. J. Parasitol. 38, 1589–1597.

Baldwin, J.G., Nadler, S. A., Adams, B.J. (2004). Evolution of plant parasitism among nematodes. Annual Review of Phytopathology 42, 83–105.

Barcala, M., García, A., Cabrera, J., Casson, S., Lindsey, K., Favery, B., García-Casado, G., Solano, R., Fenoll, C. and Escobar, C. (2010), Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. The Plant Journal, 61, 698–712.

Barret, P., Brinkman, M., Beckert, M. (2006) A sequence related to rice Pong transposable element displays transcriptional activation by *in vitro* culture and reveals somaclonal variations in maize.*g*enome 49, 1399–1407.

Baulcombe, D. (2004). RNA silencing in plants. Nature. 431, 356-363.

Bauters, L., Haegeman, A., Kyndt, T., Gheysen, G. (2012). Analysis of the transcriptome of *Hirschmanniella oryzae* to explore potential survival strategies and host-nematode interactions. Submitted to Mol. Plant. Pathol.

Bére-Maileet, C., Arthaud, L., Abad, P., Rosso, M.N. (2000). Biochemical characterization of MI-ENG1, a family 5 endoglucanase secreted by the root-knot nematode *Meloidogyne incognita*. Eur. J. Biochem. 267, 3255–3263.

Becker, D.K., Dugdale, B., Smith, M.K., Harding, R.M., Dale, J.L. (2000) Genetic transformation of Cavendish banana (Musa spp. AAA group) cv. Grand Nain via microprojectile bombardment. Plant Cell Rep 19, 229–234.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 340, 783-795.

Bert, W., Leliaert, F., Vierstraete, A.R.,Vanfleteren, J.R., Borgonie, G. (2008). Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Namatoda: Rhabditida). Mol. Phylogenet. Evol. 48, 728-744.

Bettencourt, R., Pinheiro, M., Egas, C., Gomesm P., Afonso, M., Shank, T. *et al.* (2010). High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*. BMC Genomics, 11. 559.

Billy, E., Brondani, V., Zhang, H., Muller, U., Filipowicz, W. (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc Natl Acad Sci U S A, 98, 14428-14433.

Bird, D. M and Koltai, H. (2000). Plant parasitic nematodes: Habitats, hormones and horizontally-acquired genes. Journal of Plant Growth Regulation 19, 183-194.

Braun, A.C (1959). A demonstration of the recovery of the crown-gall tumour cell with the use of complex tumours of single-cell origin. Proc Natl Acad Sci USA 45, 932–938.

Bridge, J., Fogain, R. and Speijer, P. (1997). The root lesion nematodes of banana. Musa Pest Fact Sheet, INIBAP, Montpellier, France.

Bridge, J. (1996). Nematode management in sustainable and subsistence agriculture. Annual Review of Phytopathology 34, 201-225.

Britton, C., and Murray, L. (2006). Using Caenorhabditis elegans for functional analysis of genes of parasitic nematodes. Int. J. Parasitol. 36, 651–659.

Brody, J.R. and Kern, S.E. (2004) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. Biotechniques, 36, 214-216.

Buckley, B. A., Burkhart, K. B., Gu, S.G., Spracklin, G., Kershner, A *et al.* (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature. 489, 447–451

Burton, N. O., Burkhart, K. B., S. Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 108, 19683-19688.

Byrd, D.W., Kirkpatrick, T., Barker, K.R. (1983). An improved technique for clearing and staining plant-tissues for detection of nematodes. Journal of Nematology 14, 142-143.

Cai, D., Thurau, T., Tian, Y., Lange, T., Yeh, K.W., Jung, C. (2003) Sporamin-mediated resistance to beet cyst nematodes (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgaris* L.) hairy roots. Plant Mol. Biol. 51, 839–849.

Calixto, A., Chelur, D., Topalidou, I., Chen, X., Chalfie, M. (2010). Enhanced neuronal RNAi in C. elegans using SID-1. Nat Methods. 7, 554-559.

Cantacessi, C., Campbell, B.E, Young, N.D., Jex, A.R., Hall, R.S. *et al*. (2010a). Differences in transcription between free-living and CO2-activated third-stage larvae of *Haemonchus contortus*. BMC Genomics 11, 266.

Cantacessi, C., Mitreva, M., Campbell, B.E, Hall, R.S., Young, N.D. *et al*.(2010b) First transcriptomic analysis of the economically important parasitic nematode, *Trichostrongylus colubriformis*, using a next-generation sequencing approach. Infect Genet Evol 10,1199-207.

Cantacessi. C., Mitreva, M., Jex, A.R., Young, N.D., Campbell, B.E. *et al.* (2010c). Massively parallel sequencing and analysis of the *Necator americanus* transcriptome. PLoS Negl Trop Dis, e684.

Carine Dochez, C., Speijer, P.R., De Schutter, B., Dubois, T., Tenkouano, A., De Waele, D., Ortiz, R. (2009). Host plant resistance and tolerance of *Musa* land races and hybrids to nematode infestation. JARTS Supplement 92.

Carthew, R.W., Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell. 136, 642-655.

Castanotto, D., Sakurai, K., Lingeman, R., Li, H., Shively, L., Aagaard, L. *et al.* (2007). Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. Nucleic Acids Res 35, 5154-64.

Castillo, P and Vovlas, N. (2007). *Pratylenchus* (Nematoda: Pratylenchidae): Diagnosis, biology, pathogenicity and management. Nematology monographs and perspectives No. 6. Brill, Leiden, The Netherlands. 529 pp.

Caudy, A.A, Ketting, R.F, Hammond, S.M, Denli, A.M, Bathoorn, A.M. *et al*. (2003) A micrococcal nuclease homolog in RNAi effector complexes. Nature 425:411–414.

Ceron, J., Rual, J.F., Chandra, A., Dupuy, D., Vidal, M., van den Heuvel, S. (2007). Large-scale RNAi screens identify novel genes that interact with the C. elegans retinoblastoma pathway as well as splicing-related components with synMuv B activity BMC Dev Biol. 7, 30.

Cerutti, H. (2003). RNA interference: Traveling in the cell and gaining functions? Trends Genet 19, 39-46.

Chabrier, C. and Quénéhervé, P. (2008) Preventing nematodes from spreading: a case study with *Radopholus similis* (Cobb) Thorne in a banana field. Crop Prot. 27, 1237–1243.

Chabrier, C., Tixier, P., Duyck, P.F., Carles, C., Quénéhervé, P. (2010). Factors influencing the survivorship of the burrowing nematode, *Radopholus similis* (Cobb.) Thorne in two types of soil from banana plantations in Martinique. Applied soil ecology, 44, 116-123.

Chan, S.P., Ramaswamy, G., Choi, E.Y., Slack, F.J. (2008). Identification of specific let-7 microRNA binding complexes in *Caenorhabditis elegans*. RNA 14, 2104–2114.

Chandler, S. (1995). The nutritional value of bananas. Bananas and Plantains. S. Gowen, Springer Netherlands: 468-480.

Chapman, E.J. and Carrington, J.C. (2007) Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. 8, 884–896.

Charlton, W.L, Harel, H.Y., Bakhetia, M., Hibbard, J.K., Atkinson, H.J.F, *et al*. (2010) Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. Int J Parasitol 40, 855-864.

Chen, C.C., Simard, M.J., Tabara, H., Brownell, D.R., McCollough, J.A., Mello, C.C. (2005a). A member of the polymerase beta nucleotidyltransferase Superfamily is required for RNA interference in *C.elegans*. Curr Biol.15, 378-383.

Chen, Q., Rehman, S., Smant, G., Jones, J.T. (2005b). Functional Analysis of Pathogenicity Proteins of the Potato Cyst Nematode *Globodera rostochiensis* using RNAi. Mol. Plant Microbe Interact. 18, 621-625..

Chen, S., Songkumarn, P., Liu, J., Wang, G.L. (2009). A versatile zero background T-vector system for gene cloning and functional genomics. Plant Physiol 150, 1111–1121.

Cheng, X.Y., Dai, S.M., Xiao, L., Xie, B.Y. (2010). Influence of cellulase gene knock-down by dsRNA interference on the development and reproduction of the pinewood nematode, *Bursaphelenchus xylophilus*. Nematology 12, 225-33.

Chitwood, B.G and Chitwood, M.B. (1974). Introduction to Nematology, Baltimore Univ. Park Press, pp:334

Chuang, C.F and Meyerowitz, E. M. (2000). Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana* PNAS 97, 4985-4990.

Ciche, T.A. and Sternberg, P.W. (2007) Postembryonic RNAi in *Heterorhabditis bacteriophora*: a nematode insect parasite and host for insect pathogenic symbionts. BMC Dev. Biol. 7, 101.

Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J. *et al*. (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. Cell 139, 123–134.

Cobb, N.A. (1983). Nematodes, mostly Australian and Fijian. MacLeay Mem. Vol., Linn. Soc. N. S. Wales, 252-308.

Cogoni, C. and G. Macino (1997). Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi. Trends in Plant Science 11, 438-443.

Collingborn, F.M.B., Gowen, S.R. (1997). Screening of banana cultivars for resistance to *Radopholus similis* and *Pratylenchus coffeae*. International Network for Improvement in Bananas and Plantains: Montpellier, France. INFOMUSA 6, 3.

Cote, F.X., Domergue, R., Monmarson, S., Schwendiman, J., Teisson, C., Escalant, J.V. (1996). Embryogenic cell suspensions from male (*Musa* spp.) flower of *Musa* AAA cv. Grand Nain. Physiol. Plant. 97, 285–290.

Couzin, J. (2002). Small RNAs make big splash.Science. 298, 2296-2297.

Coyne, D. (2009). Pre-Empting Plant-Parasitic Nematode Losses on Banana in Africa: Which Species Do We Target? Acta Hort. 828, 227-235.

Crombach, A and Hogeweg, P. (2011). Is RNA-dependent RNA polymerase essential for transposon control? BMC Syst Biol. 5, 104.

Cutter, A.D., Payseur, B.A., Salcedo, T., Estes, A.M., Good, J.M. *et al*. (2003). Molecular correlates of gene exhibiting RNAi phenotypes in *C. elegans.* Genome. Res. 13, 2651-2657.

Czech, B and Hannon, G.J. (2011). Small RNA sorting: matchmaking for Argonautes. Nature Reviews Genetics 12, 19-31.

Dafny-Yelin, M., Chung, S.M., Frankman, E.L., Tzfira, T. (2007). pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. Plant Physiol 145, 1272–1281.

Dalzell, J.J., McMaster, S., Fleming, C.C., Maule, A.G. (2010a). Short interfering RNA-mediated gene silencing in *Globodera pallida* and *Meloidogyne incognita* infective stage juveniles. International Journal for Parasitology 40, 91–100.

Dalzell, J. J., Warnock, N.D., Stevenson, M.A., Mousley, A., Fleming, C.C., Maule, A. G. (2010b). Short interfering RNA-mediated knock-down of *drosha* and *pasha* in undifferentiated *Meloidogyne incognita* eggs leads to irregular growth and embryonic lethality. Int J Parasitol. 40, 1303–1310.

Dalzell, J.J., McMaster, S., Johnston, M.J., Kerr, R., Fleming, C.C., Maule, A.G. (2009). Non-nematode-derived double-stranded RNAs induce profound phenotypic changes in *Meloidogyne incognita* and *Globodera pallida* infective juveniles. Int JParasitol 39, 1503-1516.

Dalzell, J.J., McVeigh, P., Warnock, N.D. *et al*. (2011). RNAi effector diversity in nematodes. PLoS Negl Trop. 5(6), e1176.

Danchin, *E.G*.J., Rosso, M.N., Vieira, P., de Almeida-Engler, J., Coutinho, P.M., Henrissat, B. *et al*. (2010) Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes. PNAS. 107, 17651-6.

Dautova, M., Rosso, M.N., Abad, P., Gommers, F.J., Bakker, J., Smant, G. (2001). Single pass cDNA sequencing - a powerful tool to analyse gene expression in preparasitic juveniles of the southern root-knot nematode *Meloidogyne incognita*. Nematology 3, 129-139.

Davis, E.L., Hussey, R.S., Mitchum, M.G., Baum, T.J. (2008). Parasitism proteins in nematode-plant interactions. Curr. Opin. Plant Biol. 11, 360-366.

De Ley, P., Blaxter, M. (2002). Systematic position and phylogeny. In: Lee DL, ed. The Biology of Nematodes. London, UK: Taylor & Francis, pp. 1–30.

De Lima de Souza, D.S., de Souza, J.D.A., Grossi-de-Sá, M., Rocha, T.L. *et al.* (2011). Ectopic expression of a *Meloidogyne incognita* dorsal gland protein in tobacco accelerates the formation of the nematode feeding site. Plant Sci 180, 276-282.

De Schutter, B., Speijer, P.R., Dochez, C., Tenkouano, A., De Waele, D. (2001). Evaluating host plant reaction of *Musa* germplasm to *R. similis* by inoculation of single primary roots. Nematropica. 31, 295-299.

De Waele, D. and Elsen, A. (2002). Migratory endoparasites: *Pratylenchus* and *Radopholus* species. pp. 175-206. *In:* Starr, J. L., R. Cook, and J. Bridge. (eds.) Plant Resistance to Parasitic Nematodes. CABI Publishing, Wallingford, Oxon,UK .pp. 258.

Debondt, A., Eggermont, K., Druart, P., Devil, M., Goderis, I., Vanderleyden, J., Broekert, W.E. (1994). *Agrobacterium*-mediated transformation of apple (*Malus X domestica Borkh*): an assessment of factors affecting gene-transfer efficiency during early transformation steps. Plant Cell Reports 13, 587-593.

Decraemer, W. and D. J. Hunt. (2006). Structure and classification. pp. 3-32. *In*: Perry, R. N., and M. Moens (eds.). Plant Nematology. CAB International, Oxfordshire, UK. pp. 447.

Delgado, J.A., Meinhardt, S., Markell, S.G., Goswami, R.S. (2012). Gene cloning using degenerate primers and genome walking. In Melvin D. Bolton and Bart P.H.J. Thomma (eds.), Plant Fungal Pathogens: Methods and Protocols, Methods in Molecular Biology, vol. 835, p. 611. DOI 10.1007/978-1-61779-501-5_39.

Deliopoulos, T., Devine, K.J., Haydock, P.P.J., Jones, P.W. (2007). Studies on the effect of mycorrhization of potato roots on the hatching activity of potato root leachate towards the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis.* Nematology.9, 719–729.Dellaporta, S.L., Wood, J., Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Molecular Biology Report, 1, 19-21.

DeLuca, F., Troccoli, A., Duncan, L.W., Subbotin, S.A., Waeyenberge, L., Coyne, D.L., Brentu, F.C., Inserra, R.N. (2012). *Pratylenchus speijeri* n. sp. (Nematoda: Pratylenchidae), a new root-lesion nematode pest of plantain in West Africa Nematology, 00(0), 1-18.

Dhed'a, D., Francoise Dumortier, F., Panis, B., Vuylsteke, D.R., De. Langhe, E.A. (1991). Plant regeneration in cell suspension cultures of the cooking banana cv. 'Bluggoe' (*Musa* spp. ABB group). Fruit, 46, 125-135.

Dieterich, C., Clifton, S.W., Schuster, L.N., Chinwalla, A., Delehaunty, K., Dinkelacker, I. *et al*. (2008). The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. Nat Genet 40, 1193-1198.

Ding, L., Spencer, A., Morita, K., Han, M. (2005). The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell 19, 437–447.

Ding, X., Shields, J., Allen, R., Hussey, R.S. (2000). Molecular cloning and characterisation of a venom allergen AG5-like cDNA from *Meloidogyne incognita*. Int J Parasitol 30, 77-81.

Dochez, C., Dusabe, J., Tenkouano, A., Ortiz, R., Whyte, J., De Waele, D. (2009). Host plant resistance to *Radopholus similis* in a diploid banana hybrid population. Nematology, 11, 329-335.

Dochez, C., Dusabe, J., Whyte, J., Tenkouano, A., Ortiz, R., De Waele, D. (2006). New sources of resistance to *Radopholus similis* in *Musa* germplasm from Asia. Australasian Plant Pathology. 35, 481–485.

Duan, G., Saint, R.B., Helliwell, C.A. *et al*. (2012). *C. elegans* RNA-dependent RNA polymerases *rrf-1* and *ego-1* silence *Drosophila* transgenes by differing mechanisms. Cellular and Molecular Life Sciences 70, 1469-1481

Dubois, T and Coyne, D.L. (2009). Integrated Pest Management of Banana Pests. In. M. Pillay and A. Tenkouano (Eds), Banana breeding constraints and Progress, CRC Press, Boca Raton, USA,121-144.

Dubreuil, G., Magliano, M., Deleury, E., Abad, P., Rosso, M.N. (2007). Transcriptome analysis of root-knot nematode functions induced in the early stages of parasitism. New Phytol. 176, 426-36.

Duchaine, T. F., Wohlschlegel, J. A., Kennedy, S. *et al*. (2006). Functional proteomics reveals the biochemical niche of C. elegans DCR-1 in multiple small-RNAmediated pathways. Cell 124, 343–354.

Dudley, N.R., Labbe, J.C., Goldstein, B. (2002). Using RNA interference to identify genes required for RNA interference. Proc. Natl. Acad. Sci. 99, 4191–4196.

Duncan, L.W. and Moens, M. (2006). Migratory endoparasitic nematodes. In: *Plant Nematology* (Perry, R.N. and Moens, M., eds), Oxfordshire: CABI Publishing. pp. 123–152.

Dunoyer, P., Schott, G., Himber, C., Meyer, D. *et al*. (2010). Small RNA Duplexes Function as Mobile Silencing Signals Between Plant Cells *Science* 328, 912.

Echeverri, C.J., Beachy, P.A., Baum, B. *et al*. (2006). Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods. 3, 777-779.

Elbadri, G.A.A., De Ley, P., Waeyenberge, L., Vierstraete, A., Moens, M. and Vanfleteren, J. (2002). Intraspecific variation in *Radopholus similis* isolates assessed with restriction fragment length polymorphism and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron. Int. J. Parasitol. 32, 199–205.

Elbashir, S.M., Lendeckel, W., Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev.15, 188-200.

Elling, A.A., Mitreva, M., Gai, X.W., Martin, J., Recknor, J., Davis, E.L *et al*. (2009). Sequence mining and transcript profiling to explore cyst nematode parasitism. BMC Genomics 10, 58.

Elsen, A., Declerck, S., De Waele, D. (2000). Reproduction of the burrowing nematode (*Radopholus similis*) on Ri T-DNA transformed carrot roots. Nematology 2, 247–250.

Evans, D.A., Sharp, W.R., Medina-Filho, H.P. (1984). Somaclonal and gametoclonal variation. Am J Bot 77, 759–774.

Fairbairn, D., Cavallaro, A., Bernard, M., Mahalinga-Iyer, J., Graham, M., Botella, J. (2007) Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes. Planta, 226:1525-33.

Fallas, G. and Sarah, J.L. (1995). Effect of temperature on the *in vitro* multiplication of seven *Radopholus similis* isolates from different banana producing zones of the world. Fundam. Appl. Nematol. 18, 445–449.

FAOSTAT. (2011). Website for data on world crop production [online]. Available from http://faostat.fao.org/site/567/default.aspx [accessed in December 2012]

Ferguson, E.L., Horvitz, H.R.(1989) The multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. Genetics 123, 109-121.

Filipjev, I.N and Schuurmans Stekhoven, J.H. (1941). A manual of agricultural helminthology. Leiden, E.J.Brill, 878p.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998). Potent and specific genetic interference by doublestranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Fischer, S.E. (2010). Small RNA-mediated gene silencing pathways in *C. elegans*. Int J Biochem Cell Biol. 42, 1306-15

Fischer, S.E., Butler, M.D., Pan, Q., Ruvkun, G. (2008). Trans-splicing in *C. elegans* generates the negative RNAi regulator ERI-6/7. Nature. 455, 491-496.

Fogain, R. (2000) Effect of *Radopholus similis* on plant growth and yield of plantains (*Musa*, AAB). Nematology, 2, 129–133.

Ford, L. *et al*. (2009) Functional analysis of the cathepsin-like cysteine protease genes in adult *Brugia malayi* using RNA interference. PLoS Negl. Trop. Dis. 3, e377.

Frison, E. and Sharrock, S. (1999). The economic, social and nutritional importance of banana in the world. In: Picq, C., Foure, E., Frison, E.A (Eds). Banana and Food Security International Symposium, Douala, Cameroon, 10-14 November, 1998.

Furlanetto, C., Cardle, L., Brown, D.J.F., Jones, J.T. (2005). Analysis of expressed sequence tags from the ectoparasitic nematode *Xiphinema index*. Nematology 7, 95-104.

Gabel, H.W., Ruvkun, G. (2008). The exonuclease ERI-1 has a conserved dual role in 5.8S rRNA processing and RNAi. Nat Struct Mol Biol. 15, 531-533.

Gaidashova, S, V., Vanasten, P.J.A., Dochez, C, Delvaux, B., De Walele, D. (2010). Impact of the root-lesion nematode *Pratylenchus goodeyi* and mulch on the East African Highland banana crop performance in Kibuye, Western Rwanda. Nematology.12, 349-356.

Ganapathi, T.R., Higgs, N.S., Balint-Kurti, P.J., Arntzen, C.J., May, G.D., Vaneck, J.M. (2001). *Agrobacterium*- mediated transformation of embryogenic cell suspension of the banana cultivar Rasthali (AAB). Plant Cell Reports 20, 157-162.

Gao, B., Allen, R., Davis, E.L., Baum, T.J., Hussey, R.S. (2004). Molecular characterization and developmental expression of a cellulose-binding protein gene in the soybean cyst nematode *Heterodera glycines*. Int. J. Parasitol. 34, 1377-1383.

Gao, B.L., Allen, R., Maier, T., Davis, E.L, Baum, T.J., Hussey, R.S. (2003). The parasitome of the phytonematode *Heterodera glycines*. Mol Plant Microbe Interact 16, 720-726.

Gao, B.L., Allen, R., Maier, T., McDermott, J.P., Davis, E.L., Baum, T.J. *et al*. (2002). Characterisation and developmental expression of a *chitinase* gene in *Heterodera glycines*. Int J Parasitol 32, 1293-300.

Geldhof, P. *et al*. (2006a) Testing the efficacy of RNA interference in *Haemonchus contortus*. Int. J. Parasitol. 36, 801–810.

Geldhof, P., Molloy, C., Knox, D.P. (2006b). Combinatorial RNAi on intestinal cathepsin B-like proteinases in *Caenorhabditis elegans* questions the perception of their role in nematode biology. Mol. Biochem. Parasitol. 145, 128-132.

Geldhof, P., Visser, A., Clark, D., Saunders, G., Britton, C., Gilleard, J., Berriman, M., Knox, D. (2007). RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. Parasitology.134, 609-619.

Gelvin, S.B. (2003). *Agrobacterium*-Mediated Plant Transformation: the Biology behind the “Gene-Jockeying” Tool Microbiol. Mol. Biol. Rev. March 67, 16-37.

Gent, J. I., Lamm, A. T., Pavelec, D. M., Maniar, J. M., Parameswaran, P., Tao, L., Kennedy, S. and Fire, A. Z. (2010). Distinct phases of siRNA synthesis in an endogenous RNAi pathway in *C. elegans* soma. Molecular Cell 37, 679–689.

Gent, J. I., Schvarzstein, M., Villeneuve, A. M. *et al*. (2009). A *Caenorhabditis elegans* RNA directed RNA polymerase in sperm development and endogenous RNA interference. Genetics 183, 1297–1314.

Ghedin, E., Wang, S., Spiro, D., Caler, E., Zhao, Q., Crabtree, J. *et al*. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. Science 317, 1756-1760.

Gheysen G and Fenoll C. (2002). Gene expression in nematode feeding sites.Annu. Rev. Phytopathol.40191–219.

Gheysen G, Angenon G, Montague MV (1998). *Agrobacterium* mediated plant transformation: a scientifically intriguing story with significant application. In: Lindsey K (ed) Transgenic plant research.Harwood Academic Press, Netherlands. pp. 1-33.

Ghildiyal, M and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nat Rev Genet.10, 94-108.

Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C. *et al*. (2008). Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. Science. 320, 1077-1081.

Goellner, M., G. Smant, G., de Boer,J.M., Baum, T., Davis, E.L. (2000). Isolation of *β-1,4-endoglucanase* genes *Globodera tabacum* and their expression during parasitism. Journal of Nematology 32, 154–165.

Goetinck, S.D., Waterston, R.H. (1994). The *Caenorhabditis elegans* UNC-87 protein is essential for maintenance, but not assembly, of body wall muscle. J Cell Biol 127, 71-78.

González-González, E., López-Casas, P.P., Del Mazo, J. (2008). Gene silencing by RNAi in mouse Sertoli cells. Reprod Biol Endocrinol. 6, 29.

Gottschalk, A., Almedom, R.B., Schedletzky, T., Anderson, S.D., Yates, J.R., Schafer, W.R. (2005). Identification and characterization of novel nicotinic receptor-associated proteins in *Caenorhabditis elegans*. EMBO J 24, 2566-78.

Gowen, S. and Quénéhervé, P. (1990). Nematodes parasites of bananas, plantains and abaca. pp. 431-460. In: Luc, M., R.A. Sikora, and J. Bridge (eds.). Plant parasitic nematodes in subtropical and tropical agriculture. CAB International, Wallingford, UK.

Gowen, S.R. (2000). Root-lesion nematodes. In: Jones D.R. (Ed.), Diseases of Banana, Abaca and Enset. CAB International, Wallingford, UK, pp. 303-307

Gowen, S.R., Quénéhervé, P., Fogain, R. (2005). Nematode parasites of bananas and plantains. *In*: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (Luc M, Sikora RA, Bridge J, eds). CAB International, Wallingford, UK. pp. 611-643.

Grimm, D., Streetz, K.L., Jopling, C.L., Storm, T.A., Pandey, K., Davis, C.R *et al*. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441, 537-541.

Grimm, D., Wang, L., Lee, J.S. *et al*. (2010). Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. J Clin Invest. 120, 3106-3119.

Grishok, A. (2012). Endogenous RNAi and adaptation to environment in *C. elegans*. Worm 1, 129–133.

Grishok, A., Hoersch, S., Sharp, P.A. (2008). RNA interference and retinoblastoma-related genes are required for repression of endogenous siRNA targets in *Caenorhabditis elegans*. Proc Natl Acad Sci.U S.A. 105, 20386-91.

Grishok, A., Sinskey, J.L., Sharp, P.A. (2005). Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. Genes Dev. 19, 683-696.

Gu, L and Knipple, D.C. (2013). Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. Crop Protection 45, 36-40

Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S *et al*. (2012) Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. Nat Genet 44, 157-164.

Gu, W., Shirayama, M., Conte, D., Vasale, J., Batista, P.J. *et al*. (2009) Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the *C. elegans* germline. Mol Cell 36, 231–244.

Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M. *et al.* (2010) Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature. 465, 1097-1101.

Gupta, B.P., Sternberg, P.W. (2003) The draft genome sequence of the nematode *Caenorhabditis briggsae*, a companion to *C. elegans*.Genome Biol., 4, 238

Habig, J. W., Aruscavage, P. J. and Bass, B. L. (2008). In *C. elegans*, high levels of dsRNA allow RNAi in the absence of RDE-4. PLoS One 3, e4052.

Haegeman, A., Elsen, A., De Waele, D., Gheysen, G. (2010). Emerging molecular knowledge on *Radopholus similis*, an important nematode pest of banana. Mol. Plant Pathol. 11, 315-323.

Haegeman, A., Jacob, J., Vanholme, B., Kyndt, T., Gheysen, G. (2008). A family of GHF5 endo-1,4-beta-glucanases in the migratory plant-parasitic nematode *Radopholus similis*. Plant Pathology 57, 581-590.

Haegeman, A., Jacob, J., Vanholme, B., Kyndt, T., Mitreva, M., Gheysen, G. (2009a). Expressed sequence tags of the peanut pod nematode *Ditylenchus africanus*: The first transcriptome analysis of an Anguinid nematode. Mol Biochem Parasitol 167, 32-40.

Haegeman, A., Vanholme, B., Gheysen, G. (2009b). Characterization of a putative endoxylanase in the migratory plant-parasitic nematode *Radopholus similis*. Mol Plant Pathol 10, 389-401.

Haegeman, A., Joseph, S., Gheysen, G. (2011). Analysis of the transcriptome of the root lesion nematode *Pratylenchus coffeae* generated by 454 sequencing technology. Mol Biochem Parasitol 178, 7-14.

Haegeman, A., Kyndt, T., Gheysen, G. (2010). The role of pseudo-endoglucanases in the evolution of nematode cell wall modifying proteins. J Mol Evol.70, 441-52.

Hamilton, A.J and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science, 286, 950-952.

Hammond, S.M. (2005). Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 579, 5822-5829.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293-296.

Hannon, G.J. (2002). RNA interference. Nature 418, 244-251.

Hannon, G.J., Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. Nature, 431, 371-378.

Hansen, G and Wright, M.S (1999). Recent advances in transformation of plants. Trends Plant Sci. 4, 226-231.

Hao, Y.J and Deng, X.X. (2002) Occurrence of chromosomal variations and plant regeneration from long-term-cultured citrus callus. In Vitro Cell Dev Biol Plant 38, 472–476.

Hartman, J.B., Vuylsteke, D., Speijer, P.R., Ssango, F., Coyne, D.L., De Waele, D. (2010). Measurement of the field response of *Musa* genotypes to *Radopholus simili*s and *Helicotylenchus Multicinctus* and the implications for nematode resistance breeding. Euphytica 172, 139–148.

Hershman, D.E., Heinz, R.D. and Kennedy, B.S. (2008) Soybean cyst nematode, *Heterodera glycines*, populations adapting to resistant soybean cultivars in Kentucky. Plant Dis. 92, 1475.

Hewezi, T., Howe, P.J., Maier, T.R., Hussey, R.S., Mitchum, M.G., Davis, E.L. *et al*. (2010). *Arabidopsis* spermidine synthase Is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. Plant Physiol 152, 968-84.

Hiei, Y., Komari, T., Ishida, Y., Saito, H. (2000) Development of *Agrobacterium*-mediated transformation method for monocotyledonous plants. Breed Res 2, 205–213.

Hirai, S., Kodama, H. (2008). RNAi vectors for manipulation of gene expression in higher plants. The Open Plant Science Journal 2, 21-30.

Hirochika, H (1993) Activation of tobacco transposons during tissue culture. EMBO J 12, 2521–2528.

Holterman, M., Holovachov, O., Van Den Elsen, S., Megen, H., Bongers, T., Bakker, J., Helder, J., (2008). Small subunit ribosomal DNA-based phylogeny of basal Chromadoria (Nematoda) suggests that transitions from marine to terrestrial habitats (and *vice versa*) require relatively simple adaptations. Mol Phylogenet Evol 48, 758-763.

Hong, J., Qian Z., Shen, S., Min, T., Tan, C., Xu, J., Zhao, Y., Huang, W. (2005). High doses of siRNAs induce *eri-1* and *adar-1* gene expression and reduce the efficiency of RNA interference in mouse. Biochem. J. 390, 627–631.

Hooks, C.R.R., Wang, K.H., Ploeg, A., McSorley, R. (2010).Using marigold (*Tagetes* spp.) as a cover crop to protect crops from plant-parasitic nematodes Appl. Soil Ecol. 46, pp. 307–320

Huang, G.Z., Dong, R.H., Allen, R., Davis, E.L, Baum, T.J., Hussey, R.S. (2006). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. Mol Plant Microbe Interact 19, 463-470.

Huang, G.Z., Gao, B.L,, Maier, T., Allen, R., Davis, E.L, Baum, T.J, *et al*. (2003) A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. Mol Plant Microbe Interact 16, 376-81.

Huettel, R.N., Dickson, D.W., Kaplan, D.T. (1984). *Radopholus citrophilus* sp. n. (Nematoda), a sibling species of *Radopholus similis*. Proc.Helm. Soc. Wash. 51, 35.

Hussein, A.S., Kichenin, K., Selkirk, M.E. (2002). Suppression of secreted *acetylcholinesterase* expression in *Nippostrongylus brasiliensis* by RNA interference. Mol Biochem Parasitol 122, 91-94.

Hussey, R. S and Janssen, G.J.W. (2002). Root-knot nematodes: *Meloidogyne* species. In J. L. Starr, R. Cook, and J. Bridge, eds. Plant resistance to parasitic nematodes. Wallingford, UK: CABI Publishing. Pp. 43–70.

Hwang, S.; Tang, C. Y. (1996). Somaclonal variation and its use for improving Cavendish (AAA dessert) bananas in Taiwan. In: Frison, E. A.; Horry, J. P.; deWaele, D., eds. New frontiers in resistance breeding for nematode, *Fusarium* and Sigatoka. Kuala Lumpuro 173–181.

Hwang, S.C and. Ko, W.H. (1987). Somaclonal variation of bananas and screening for resistance to Fusarium wilt, in: G.J. Persley, E.A. De Langhe (Eds.), Banana and Plantain Breeding Strategies. Proceedings of an International Workshop, Australian Centre for Int. Agric. Res. (ACIAR), Canberra, Proceedings No. 21, 13–17 October 1986, at Cairns, Australia, pp. 151–156.

Israeli, Y., Reuveni, O., Lahav, E. (1991). Qualitative aspects of somaclonal variation in banana propagated by *in vitro* techniques. Scientia Hort.48, 71–88.

Issa, Z., Grant, W.N., Stasiuk, S., Shoemaker, C.B. (2005). Developement of methods for RNA interference in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis.* Int. J. Parasitol. 35, 935-940.

Jacob, J., Mitreva, M., Vanholme, B., Gheysen, G. (2008). Exploring the transcriptome of the burrowing nematode *Radopholus similis*. Mol Genet Genomics. 280, 1-17.

Jacob, J., Vanholme, B., Haegeman, A., Gheysen, G. (2007). Four transthyretin-like genes of the migratory plant-parasitic nematode *Radopholus similis*: members of an extensive nematode-specific family. Gene 402, 9-19.

Jaouannet, M., Perfus-Barbeoch, L., Deleury, E., Magliano, M., Engler, G., Vieira, P. *et al*. (2012). A root-knot nematode-secreted protein is injected into giant cells and targeted to the nuclei.New Phytol.19, 4924–931

Jaubert, S., Ledger, T.N., Laffaire, J.B., Piotte, C., Abad, P., Rosso, M.N. (2002). Direct identification of stylet secreted proteins from root-knot nematodes by a proteomic approach. Mol Biochem Parasitol 121, 205-211.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W (1987). GUS fusions:*1,3-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907.

Jones, D. R. (2009). Diseases and pest constraints to banana production. *Acta Horticulturae* 828, 21-36.

Jones, D.R. (2000). Diseases of Banana, Abaca and Enset. CABI Publishing, Wallingford, UK. Pp 544.

Jones, J.T., Furlanetto, C., Kikuchi, T. (2005). Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes. Nematology 7, 41-6.

Jones, J.T., Furlanetto, C., Phillips, M.S. (2007). The role of flavonoids produced in response to cyst nematode infection of *Arabidopsis thaliana*. Nematology 9, 671-677.

Jones, J.T., Reavy, B., Smant, G., Prior, A.E. (2004). *Glutathione peroxidases* of the potato cyst nematode *Globodera rostochiensis*. Gene 324, 47-54.

Jones, J.T., Smant, G., Blok, V.C. (2000). SXP/RAL-2 proteins of the potato cyst nematode *Globodera rostochiensis*: secreted proteins of the hypodermis and amphids. Nematology 2, 887-93.

Jose, A.M., Smith, J.J., Hunter, C.P. (2009). Export of RNA silencing from *C. elegans* tissues does not require the RNA channel SID-1. Proc Natl Acad Sci U S A. 106, 2283-22888.

Kagawa, H., Takuwa, K., Sakube, Y. (1997). Mutations and expressions of the tropomyosin gene and the troponin C gene of *Caenorhabditis elegans.* Cell Struct Funct 22, 213-18.

Kang, J.S., Lee, D.W., Koh, Y.H., Lee, S.H. (2011). A soluble *acetylcholinesterase* provides chemical defense against xenobiotics in the pinewood nematode. PLoS One 6(4), e19063.

Kaplan, D.T. and Opperman, C.H. (2000). Reproductive strategies and karyotype of the burrowing nematode, *Radopholus similis*. J. Nematol.32, 126–133.

Kaplan, D.T. and Oppermann, C.H. (1997). Genome similarity implies that citrus-parasitic burrowing nematodes do not represent a unique species. J. Nematol. 29, 430–440.

Kaplan, D.T., Thomas, W.K., Frisse, L.M., Sarah, J.L., Stanton, J.M., Speijer, P.R., Marin, D.H. and Opperman, C.H. (2000). Phylogenetic analysis of geographically diverse *Radopholus similis* via rDNA sequence reveals a monomorphic motif. J. Nematol. 32, 134–142.

Kaplan, D.T., Vanderspool, M.C., Opperman, C.H. (1997). Sequence tag site and host range assays demonstrate that *Radopholus similis* and *R. citrophilus* are not reproductively isolated. J. Nematol. 29, 421–429.

Karim, N., Jones, J.T., Okada, H., Kikuchi, T. (2009). Analysis of expressed sequence tags and identification of genes encoding cell-wall-degrading enzymes from the fungivorous nematode *Aphelenchus avenae.* BMC Genomics 10, 525.

Karimi, M., Depicker, A., Hilson, P. (2007). Recombinational Cloning with Plant Gateway Vectors. Plant Physiology. 145, 1144-1154.

Kaya, E and Doudna, J. A. (2012). Biochemistry. Guided tour to the heart of RISC. Science.336, 985-986.

Kennedy, S., Wang, D. and Ruvkun, G. (2004). A conserved siRNAdegrading RNase negatively regulates RNA interference in *C. elegans*. Nature 427, 645–649.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G., Plasterk, R.H. (1999). *Mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell. 99, 133-141.

Khan, M and Hasan, M. (2010). Nematode diversity in banana rhizosphere from West Bengal, India. Journal of Plant Protection Research 50, 264-267.

Khanna, H., Becker, D., Kleidon, J., Dale, J. (2004). Centrifugation Assisted *Agrobacterium tumefaciens*-mediated Transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). Mol. Breed. 14, 239–252.

Khayat, E., Duvdevani, A., Lahav, E., Ballesteros, B.A. (2004). Somaclonal variation in banana (*Musa acuminata* cv. „Grande Naine"). Genetic mechanism, frequency, and application as a tool for clonal selection. In: Jain, S. M. & Swennen. R. (Eds). Banana improvement: Cellular, Molecular Biology, and Induced Mutations. Science Publishers Inc., Plymouth, UK. pp. 97-109.

Kikuchi, T., Aikawa, T., Kosaka, H., Pritchard, L., Ogura, N., Jones, J.T (2007). Expressed sequence tag (EST) analysis of the pine wood nematode *Bursaphelenchus xylophilus* and B. mucronatus. Mol Biochem Parasitol 155,b9-17.

Kikuchi, T., Cotton, J.A., Dalzell, J.J., Hasegawa, K., Kanzaki, N., McVeigh, P., Takanashi, T., Tsai, I.J., Assefa, S.A., Cock, P.J.A. (2011) Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. PLoS Pathogens 7: e1002219.

Kikuchi, T., Jones, J.T., Aikawa, T., Kosaka, H *et al*. (2004). A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*. FEBS Lett. 572, 201-205.

Kikuchi, T., Shibuya, H., and Jones, J.T. (2005). Molecular and biochemical characterization of an *endo-β-1,3-glucanase* from the pinewood nematode *Bursaphelenchus xylophilus* acquired by horizontal gene transfer from bacteria. Biochem. J. 389, 117-125.

Kimber, M. J.,McKinney, S.,McMaster, S., Day, T. A., Fleming, C. C. and Maule, A. G. (2007). flp gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference. FASEB Journal 21, 1233–1243.

Klink, V.P., Kim, K.H., Martins, V., MacDonald, M.H., Beard, H.S., *et al*. (2009) A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female *Heterodera glycines* cyst formation during infection of *Glycine max*. Planta 230, 53-71.

Knight, S.W and Bass, B.L. (2002). The role of RNA editing by ADARs in RNAi. Mol Cell 10, 809–817.

Koffi, M.C., Vos, C., Draye, X., Declerck, S (2012) Effects of *Rhizophagus irregularis* MUCL 41833 on the reproduction of *Radopholus similis* in banana plantlets grown under *in vitro* culture conditions. Mycorrhiza. doi:10.1007/s00572-012-0467-6.

Koller, E., Propp, S., Murray, H., Lima, W., Bhat, B., Prakash, T.P *et al*. (2006). Competition for RISC binding predicts *in vitro* potency of siRNA. Nucleic Acids Res 34, 4467-4476.

Kosky, R.G., Silva, M.D.F., Perez, L.P., Gilliard, T., Martinez, F.B., Vega, M.R., Milian, R.C., Mendoza, E.Q. (2002). Somatic embryogenesis of banana hybrid cultivar FHIA-18 (AAAB) in liquid medium and scaled-up in a bioreactor. Plant Cell Tiss. Org. Cult. 68, 21–26.

Kranewitter, W.J., Ylanne, J., Gimona, M. (2001). UNC-87 is an actin-bundling protein. J Biol Chem 276, 6306-12.

Kumar, A and Sarin, N.B. (2013). RNAi: A Promising approach to develop transgenic plants against Geminiviruses and Insects. J Plant Physiol Pathol 1, 1

Kyndt, T., Haegeman, A., Gheysen, G. (2008). Evolution of GHF5 *endoglucanase* gene structure in plant-parasitic nematodes: no evidence for an early domain shuffling event. BMC Evol Biol 8, 305.

Lambert, K.N., Allen, K.D., Sussex, I.M. (1999). Cloning and characterization of an esophageal-gland-specific *chorismate mutase* from the phytoparasitic nematode *Meloidogyne javanica*. Mol Plant Microbe Interact 12, 328-336.Larkin, P.J and Scowcroft, W.R. (1981). Somaclonal variation — a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics. 60, 197-214.

Lau, N.C., Robine, N., Martin, R. Chung, W.J., Niki, Y., Berezikov, E., Lai, E.C. (2009). Abundant primary piRNAs, endo-siRNAs, and microRNAs in a *Drosophila* ovary cell line. Genome Res. 19, 1776–1785.

Lee, C.T., Risom, T., Strauss, W.M (2007). Evolutionary conservation of microRNA regulatory circuits: an examination of microRNA gene complexity and conserved microRNA-target interactions through metazoan phylogeny. DNA Cell Biol. 26, 209–218

Lee, H.C., Li, L., Gu, W. *et al.* (2010). Diverse Pathways Generate MicroRNA-like RNAs and Dicer-Independent Small Interfering RNAs in Fungi. Mol. Cell. 38, 803-814.

Lee, R.C., Hammell, C.M., Ambros, V. (2006). Interacting endogenous and exogenous RNAi pathways in *Caenorhabditis elegans.* RNA 12, 589–597.

Lehner, B., Calixto, A., Crombie, C., Tischler, J., Fortunato, A., Chalfie, M., *et al*. (2006). Loss of LIN-35, the *Caenorhabditis elegans* ortholog of the tumor suppressor p105Rb, results in enhanced RNA interference.*g*enome Biol 7, R4.

Lendner, M., Doligalska, M., Lucius, R., Hartmann, S. (2008). Attempts to establish RNA interference in the parasitic nematode *Heligmosomoides polygyrus*. Mol. Biochem. Parasitol. 161, 21–31.

Li, J., Todd, T.C., Oakley, T.R., Lee, J. and Trick, H.N. (2010a) Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of *Heterodera glycines* Ichinohe. Planta, 232, 775–785.

Li, J., Todd, T.C. and Trick, H.N. (2010b) Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants. Plant Cell Rep. 29, 113–123.

Li, L.M., Lin, X.Y., Khvorova, A., Fesik, S.W., Shen, Y. (2007). Defining the optimal parameters for hairpin-based knock-down constructs. RNA 13, 1765-1774.

Li, X., Zhuo, K., Luo, M., Sun, L., Liao, J. (2011). Molecular cloning and characterization of a *calreticulin* cDNA from the pinewood nematode *Bursaphelenchus xylophilus*. Exp Parasitol 128, 121-6.

Lilley, C.J., Davies, L.J, Urwin, P.E. (2012). RNA interference in plant parasitic nematodes: a summary of the current status. Parasitology 139, 630-40

Lin, X., *et al*. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Res. 33, 4527-35.

Lipardi, C., Paterson, B.M. (2009).Identification of an RNA-dependent RNA polymerase in *Drosophila* involved in RNAi and transposon suppression PNAS. 106, 15645–15650

Lipardi,C., Wei, Q., Paterson, B.M. (2001). RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell. 107, 297-307.

Long, H., Peng, H., Huang, W., Wang, G., Gao, B., Moens, M., Peng, D. (2012). Identification and molecular characterization of a new *β-1,4-endoglucanase* gene (*Ha-eng-1a*) in the cereal cyst nematode *Heterodera avenae*. European journal of plant pathology, 134, 391-400.

Loof, P.A.A (1991*)* The family Pratylenchidae Thorne, 1949*.* In: Manual of Agricultural Nematology (Ed. *NickleWR*), Marcel Dekker, Inc., New York (US) pp*.* 363–421*.*

Loos C.A. (1962). Studies on the life-history and habits of the burrowing nematode, *Radopholus similis*, the cause of blackhead disease of banana. Proceedings of the Helminthological Society of Washington: 29, 43-52.

Louvet-Vallee, S., Kolotuev, I., Podbilewicz, B., and Felix, M. A. (2003) Control of vulval competence and centering in the nematode *Oscheius* sp. 1 CEW1. Genetics 163, 133–146.

Lu, X., Horvitz, H.R. (1998*). lin-35* and *lin-53*, two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell 95, 981-91.

Luc, M., Hunt, D.J., Machon, J.E. (1990). Morphology, anatomy and biology of plant parasitic nematodes – a synopsis. In: Luc M., Sikora R.A. and Bridge J. (Eds), Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. CAB International, Wallingford, UK, pp. 1-44.

Luc, M., R.A. Sikora and J. Bridge, (2005). Plant Parasitic Nematodes in Tropic and Subtropical Agriculture. 2nd Edn., CAB International, Wallingford, Oxford, UK, pp: 871s

MacGowan, J.B. (1977). The burrowing nematode *Radopholus similis* (C, 1893) T 1949. Nematology Circ. Fla. Dpt. Agric. 27.

Macrae, I. J., Zhou, K., Li, F. *et al*. (2006). Structural basis for doublestranded RNA processing by Dicer. Science 311, 195–198.

Maier, T.R., Hewezi, T., Peng, J., Baum, T.J. (2013). Isolation of whole esophageal gland cells from Plant-Parasitic Nematodes for transcriptome analyses and effector identification. Mol. Plant Microbe Interact. 26, 31-35.

Maine EM, Hauth J, Ratliff T, Vought VE, She X, *et al*. (2005) EGO-1, a putative RNA-dependent RNA polymerase, is required for heterochromatin assembly on unpaired DNA during *C. elegans* meiosis. Curr Biol 15: 1972–1978.

Mango SE (2001) Stop making nonsense: the *C. elegans smg* genes. Trends Genet17: 646–653.

Martin, K., Pachathundikandi, S., Zhang, C., Slater, A., Madassery, J. (2006). RAPD analysis of a variant of banana (Musa sp.) cv. grande naine and its propagation via shoot tip culture. In Vitro Cell Dev Biol Plant 42, 188–192.

Massirer, K.B and Pasquinelli, A.E. (2013). MicroRNAs that interfere with RNAi. Worm 2, 1-6.

Massirer, K.B., Perez, S.G., Mondol, V., Pasquinelli, A.E. (2012). The miR-35-41 family of microRNAs regulates RNAi sensitivity in *Caenorhabditis elegans*. PLoS Genet 8, e1002536.

Matthews, B.F., Pilitt, K.L., Klink, V. (2004). Molecular characterization of a Soybean Cyst Nematode (*Heterodera glycines*) Homolog of unc-87. J Nematol 36, 57-65.

Maule A.J., McVeigh, P., Dalzell, J.J., Atkinson, L., Mousley, A., Marks, N.J. (2011). An eye on RNAi in nematodes. Trends. Parasitol. 27, 505-513.

May, G.D., Afza, R., Mason, H.S., Wiecko, A., Novak, F.J., Artzen, C.J. (1995). Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium* mediated transformation. Nature Biotechnology 13, 486-492.

Mbega, E.R and Nzogela, Y.B. (2012). Strategies used by plant parasitic nematodes to conquer the host. Journal of animal and plant sciences. 14, 1848-1854.

McCarter, J.P., Mitreva, M.D., Martin, J., Dante, M., Wylie, T., Rao, U *et al*. (2003). Analysis and functional classification of transcripts from the nematode *Meloidogyne incognita*. Genome Biol 4, R26.

McEwan, D.L., Weisman, A.S., Hunter, C.P. (2012). Uptake of Extracellular Double-Stranded RNA by SID-2. Mol.Cell. 47, 746-754.

Mcintyre, G.J., Arndt, A.J., Gillespie, K.M., Mak, W.M., Fanning, G.C. (2011a). A comparison of multiple shRNA expression methods for combinatorial RNAi. Genet Vaccines Ther. Apr 17;9(1), 9. doi: 10.1186/1479-0556-9-9.

Mcintyre, G.J., Yu, Y.H., Lomas, M., Fanning, G.C (2011b). The effects of stem length and core placement on shRNA activity. BMC Mol Biol 12, 34.

McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J., Sharp, P.A. (2002). Gene silencing using micro-RNA designed hairpins RNA, 8, 842–850

Mehta, Y.R and Angra, D.C. (2000). Somaclonal variation for disease resistance in wheat and production of dihaploids through wheat 9 maize hybrids. Genet Mol Biol 23, 617–622.

Meister G, and Tuschl T. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature, 431, 343-349.

Mendel, R.R., Muller, B., Schulze, J. *et al*. (1989) Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Applied Genetics 78:31-34.

Mikuma, T., Kawasaki, H., Yamamoto, Y. and Taira, K. (2004). Overexpression of Dicer enhances RNAi-mediated gene silencing by shorthairpin RNAs (shRNAs) in human cells. Nucleic Acids Symposium Series (Oxford Journals) 48, 191–192.

Min, K., Kang, J., Lee, J. (2010). A modified feeding RNAi method for simultaneous knock-down of more than one gene in Caenorhabditis elegans. Biotechniques 48, 229-232.

Min, X.J., Butler, G., Storms, R., Tsang, A. (2005). OrfPredictor: predicting protein-coding regions in EST-derived sequences. Nucleic Acids Res. 33, W677-W680.

Mitchum, M.G., Wrather, J.A., Heinz, R.D., Shannon, J.G. and Danekas, G. (2007) Variability in distribution and virulence phenotypes of *Heterodera glycines* in Missouri during 2005. Plant Dis. 91, 1473–1476.

Mitreva, M., Elling, A.A., Dante, M., Kloek, A.P., Kalyanaraman, A., Aluru, S. *et al*. (2004). A survey of SL1-spliced transcripts from the root-lesion nematode *Pratylenchus* penetrans. Mol Genet Genomics 272, 138-148.

Mitreva, M., Jasmer, D.P., Zarlenga, D.S., *et al*. (2011) The draft genome of the parasitic nematode Trichinella spiralis. Nat Genet 43: 228–235.

Miyoshi, K., Miyoshi, T., Hartig, J.V., Siomi, H., Siomi, M.C. (2010). Molecular mechanisms that funnel RNA precursors into endogenous small-interfering RNA and microRNA biogenesis pathways in *Drosophila*. RNA. 16, 506-15.

Montgomery, M.K. (2004). RNA interference: historical overview and significance. *Methods Mol Biol* 265: 3–21.

Moody, E.H., Lownsberry, B.F., Ahmed, J.M. (1973). Culture of root-lesion nematode *Pratylenchus vulnus* on carrot discs. Journal of Nematology 5, 225-226.

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A.C., Kanehisa, M. (2007). KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res. 35, W182-W185.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon. *et al.* (2000).*Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101, 533–542.

Mujib, A., Banerjee, S., Dev Ghosh, P (2007). Callus induction, somatic embryogenesis and chromosomal instability in tissue culture raised hippeastrum (*Hippeastrum hybridum* cv. United Nations).Propag Ornam Plants 7, 169–174.

Múnera-Uribe G.E., Bert, W., Vierstraete, A.R., Peña. E.D. L., Moens, M., Decraemer, W. (2010). Nematology*,* 12, 619 - 629.

Moxon, S., Jing, R., Szittya, G., Schwach, F., Rusholme Pilcher, R.L., Moulton, V., Dalmay, T (2008). Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Res. 18, 1602–1609

Nakae, H., Obinata, T. (1993). Immunocytochemical localization of troponin I and C in the muscles of *Caenorhabditis elegans*. Zool Sci 10, 375-79.

Nicol, P., Gill, R., Fosu-Nyarko, J., Jones, M.G.K. (2012). de novo analysis and functional classification of the transcriptome of the root lesion nematode, *Pratylenchus thornei*, after 454 GS FLX sequencing. International Journal for Parasitology. 42, 225-237.

Nuez, I and Félix, M.A. (2012). Evolution of susceptibility to ingested double-stranded RNAs in *Caenorhabditis* nematodes. PLoS One. 7(1), e29811.

O'Bannon, J.H. (1977). Worldwide dissemination of *Radopholus similis* and its importance in crop protection. Journal of nematology, 9, 16-25.

O'Bannon, J.H and Tomerlin, A.T. (1973) Citrus Tree Decline Caused by *Pratylenchus coffeae,* The journal of Nematology, 5, 311-316.

Opperman, C.H., Bird, D.M., Williamson, V.M., *et al*. (2008). Sequence and genetic map of *Meloidogyne hapla*: a compact nematode genome for plant parasitism. Proc. Natl Acad. Sci. USA.105,14802-14807.

Ossowski, S., Schwab, R., Weigel, D (2008). Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J 53, 674–690.

Pak, J. and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. Science 315, 241–244.

Pak, j., Maniar, J.M., Mello, C.C., Fire, A. (2012). Protection from Feed-Forward Amplification in an Amplified RNAi Mechanism. Cell. 151, 885-899.

Parchman, T.L., Geist, K.S., Grahnen, J.A., Benkman, C.W., Buerkle, C.A. (2010). Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery. BMC Genomics 11, 180.

Park, J.E., Lee, K.Y., Lee, S.J., Oh, W.S., Jeong, P.Y., Woo, T. *et al*. (2008). The efficiency of RNA interference in *Bursaphelenchus xylophilus*. Mol. Cells 26, 81-86.

Parrish, S. and Fire, A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*. RNA 7, 1397–1402.

Patel, N., Hamamouch, N., Li, C. Y., Hewezi, T., Hussey, R. S., Baum, T., Mitchum, M. and Davis, E. L. (2010). A nematode effector protein similar to annexins in host plants. Journal of Experimental Botany 61, 235–248.

Patel, N., Hamamouch, N., Li, C. Y., Hussey, R. S., Mitchum, M., Baum, T., Wang, X. and Davis, E. L. (2008). Similarity and functional analyses of expressed parasitism genes in *Heterodera schachtii* and *Heterodera glycines*. Journal of Nematology 40, 299–310.

Patel, N., Hamamouch, N., Li, C.Y., Hewezi, T., Hussey, R.S., Baum, T.J. *et al*. (2010). A nematode effector protein similar to annexins in host plants. J Exp Bot 61, 235-48.

Patil, P. V and Navale, P. A. (2000). Somaclonal variation in fruit crops: a review.J. Maharashtra Agric. Univ. 25(1):8–14.

Pavelec, D. M., Lachowiec, J., Duchaine, T. F., Smith, H. E. and Kennedy, S. (2009). Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in *Caenorhabditis elegans*. Genetics 183, 1283–1295.

Pérez Hernandez, J.B., Remy, S., Swennen, R., Sagi, L. (2006a). Banana (*Musa* sp.). Wang K. (ed.). *Agrobacterium* Protocols 2/e, volume 2. Humana Press Inc., Totowa, NJ. Methods in Molecular Biology, vol. 344,167-175.

Pérez-Hernández, J.B., Swennen, R., Sagi, L (2006b). Number and Accuracy of T-DNA Insertions in Transgenic Banana (*Musa* spp.) Plants Characterized by an Improved Anchored PCR Technique. Transgenic Research 15, 139-150.

Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C, *et al*. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nat. Biotechnol.26, 909-915.

Perrimon, N., Ni, J.Q., Perkins, L. (2010). In vivo RNAi: today and tomorrow. Cold Spring Harbor Perspectives in Biology 2, 1–11.

Perry, R. N., and Moens, M. (eds.). (2006). Plant Nematology. CABI Publishing, Wallingford, United Kingdom. 447 pp.

Phillips, C.M., Montgomery, T.A., Breen, P.C., Ruvkun, G. (2012). MUT-16 promotes formation of perinuclear mutator foci required for RNA silencing in the *C. elegans* germline. Genes Dev. 26, 1433-1444.

Pillay, M., and L. Tripathi. (2007). Banana. In Genome mapping and molecular breeding in plants. Vol. 4, fruits and nuts, edited by C. Kole, 281–301. Berlin, Germany: Springer-Verlag.

Pires da Silva A (2006) *Pristionchus pacificus* genetic protocols. WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.114.1.

Plasterk, R.H.A. (2002). RNA Silencing: The Genome's Immune System. *Science* 296 (5571), 1263.

Pontier, D., Picart, C., Roudier, F.*et al.* (2012). NERD, a Plant-Specific GW Protein, Defines an Additional RNAi-Dependent Chromatin-Based Pathway in *Arabidopsis.* Mol. Cell*.* 48, 121-132*.*

Popeijus, M., Blok, V.C., Cardle, L., Bakker, E., Phillips, M.S., Helder, J. *et al*. (2000). Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *Globodera. pallida* using the expressed sequence tag approach. Nematology 2, 567-74.

Predieri, S (2001) Mutation induction and tissue culture in improving fruits. Plant Cell Tissue Organ Cult 64, 185–210.

Price, N.S. (2006). The banana burrowing nematode, *Radopholus similis* (Cobb) Thorne, in the Lake Victoria region of East Africa: its introduction spread and impact. Nematology, 8, 801–817.

Prior, A., Jones, J.T., Blok, V.C., Beauchamp, J., McDermott, L., Cooper, A. *et al*. (2001). A surface-associated retinol- and fatty acid-binding protein (Gp-FAR-1) from the potato cyst nematode *Globodera pallida*: lipid binding activities, structural analysis and expression pattern. Biochem J 356, 387-394.

Qu, J., Ye, J., Fang, R. (2007). Artificial MicroRNA-Mediated Virus Resistance in Plants. J Virol 2007; 81, 6690-91.

Quénéhervé, P., Marie-Luce, S., Barout, B., Grosdemange, F. (2006).Une technique de criblage variétal précoce des bananiers envers les nématodes phytoparasites. Nematology, 8, 147–152

Quénéhervé, P., Valette, C., Topart, P., Montcel, H.T., Salmon, F. (2009) Nematode resistance in bananas: screening results on some wild and cultivated accessions of *Musa* spp. Euphytica, 165, 123–136.

Radewald, J., O'Bannon, H., Tomerlin, A. (1971).Temperature effects on reproduction and pathogenicity of *Pratylenchus coffeae* and *P. brachyurus* and survival of *P. coffeae* in roots of *Citrus jambhiri,* Journal of Nematology, 3, 390–394

Ramana, K.V. and Eapen, S.J. (2000). Nematode induces disease of black pepper. pp. 269-295. In: Kavindran, P.N. (ed.). Black pepper, Piper nigrum. Harwood Academic, Amsterdam, The Netherlands.

Rehman, S., Postma, W., Tytgat, T., Prins, P., Qin, L., Overmars, H. *et al*. (2009). A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. Mol Plant Microbe Interact 22, 330-340.

Remeeus, P.M., van Bezooijen, J., Wijbrandi, J. and van Bezooijen, J. (1998) *In vitro* testing is a reliable way to screen the temperature sensitivity of resistant tomatoes against *Meloidogyne incognita*. In Proceedings of 5th International Symposium on Crop Protection, 63. pp. 635–640. Belgium: Universiteit Gent.

Remy, S., Thiry, E., Coemans, B., Windelinckx, S., Swennen, R., Sagi, L. (2005). Improved T-DNA vector for tagging promoters via high-throughput luciferase screening. BioTechniques, 38:763-770.

Reuveni O and Israeli Y (1990) Measures to reduce somaclonal variation in *in vitro* propagated bananas. Acta Hortic 275, 307–313.

Robertson, L., Robertson, W.M., Sobczak, M., Helder, J., Tetaud, E., Ariyanayagam, M.R. *et al*. (2000). Cloning, expression and functional characterisation of a *peroxiredoxin* from the potato cyst nematode *Globodera rostochiensis*. Mol Biochem Parasitol 111, 41-9.

Rosso, M. N., Dubrana, M. P., Cimbolini, N., Jaubert, S. and Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. Molecular Plant-Microbe Interactions 18, 615–620.

Rosso, M.N., Jones, J.T., Abad, P. (2009). RNAi and functional genomics in plant parasitic nematodes. Annu Rev Phytopathol 47, 207-232.

Roux, N.S (2004). Mutation induction in Musa-review. In: Jain SM, Swennen R (eds) Banana improvement: cellular, molecular biology, and induced mutation. Science Publishers, Inc., Enfield, pp 23–32.

Ruiz, M.T., Voinnet, O., Baulcombe, D.C (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell*, 10, 937–946.

Sacco, M.A., Koropacka, K., Grenier, E., Jaubert, M.J., Blanchard, A., Goverse, A. *et al*. (2009). The cyst nematode SPRYSEC protein RBP-1 elicits Gpa2-and RanGAP2-dependent plant cell death. Plos Pathogens 5, e1000564.

Sagi, L., Remy, S., Verelst, T, B., Panis, B., Cammue, B.P.A., Volckaert, G., Swennen, R. (1995). Transient gene expression in transformed banana (*Musa* cv. 'Bluggoe') protoplasts and embryogenic cell suspensions. Euphytica 85, 89-95.

Sahijram, L., Soneji, J.R., Bollamma, K.T. (2003). Analyzing somaclonal variation in micropropagated bananas (*Musa* spp).In Vitro Cell. Dev. Biol.—Plant 39, 551–556.

Saleh, M.C., van Rij, R.P., Hekele, A., Gillis, A., Foley, E. *et al*. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat Cell Biol 8, 793–802.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular Cloning, a Laboratory Manual, ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Samson, J.A. (1992). Tropical Fruits. 2nd Edition. Tropical Agriculture series. Longmans, London, UK.

Sasser J. N., Freckman D. W. (1987). A world perspective on nematology: the role of the society. p. 28-34. In: "Vistas on Nematology" (J. A. Veech, D. W. Dickson, eds.). Hyattville, MD, Society of Nematologists, 509 pp.

Scacheri, P.C., *et al*. (2004).Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. PNAS 101, 1892-97.

Schmid, A., Schindelholz, B., Zinn, K. (2002). Combinatorial RNAi: a method for evaluating the functions of gene families in *Drosophila*. Trends. Neurosci. 25, 71-74.

Scholl, E.H., Thorne, J.L., McCarter, J.P., Bird, D.M. (2003). Horizontally transferred genes in plant-parasitic nematodes: a high-throughput genomic approach. Genome Biol 4, R39.

Semblat, J.P., Rosso, M.N., Hussey, R.S., Abad, P., Castagnone-Sereno, P. (2001). Molecular cloning of a cDNA encoding an amphid-secreted putative avirulence protein from the root-knot nematode *Meloidogyne incognita*. Mol Plant Microbe Interact 14, 72-79.

Shannon, A.J., Tyson, T., Dix, I., Boyd, J., Burnell, A.M. (2008). Systemic RNAi mediated gene silencing in the anhydrobiotic nematode *Panagrolaimus superbus.* BMC Molecular Biology 9:58.

She, X., Xu, X., Fedotov, A., Kelly, W.G., Maine, E.M (2009) Regulation of heterochromatin assembly on unpaired chromosomes during *Caenorhabditis elegans* meiosis by components of a small RNA-mediated pathway. PLoS Genet 5:e1000624.

Shepherd, K.; Souza, F.V.D.; Da Silva, K.M. (1996). Mitotic instability in banana varieties. IV. BAP concentration and effects of number of subcultures. Fruits 51, 211–216.

Shi, Y. (2003). Mammalian RNAi for the masses. Tren. in Gen. 19, 9-12.

Shi, Z., Montgomery, T.A., Qi, Y., Ruvkun, G. (2013). High-throughput sequencing reveals extraordinary fluidity of miRNA, piRNA, and siRNA pathways in nematodes. Genome Res. 23, 497-508

Shibata, D. and Liu, Y.G (2000). *Agrobacterium*-mediated plant transformation with large DNA fragments. Trends plant Sci. 5: 354-357.

Siddiqi, M.R. (2000). Tylenchida parasites of plants and insects, 2nd edition. Wallingford, UK, CABI Publishing, pp 833.

Sijen, T. and Plasterk, R.H. (2003) Transposon silencing in the *C. elegans* germ line by natural RNAi. Nature 426, 310–314.

Sijen, T., Fleenor, J., Simmer, F. *et al*. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell, 107, 465-476.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107, 465–476.

Sijen, T., Steiner, F.A., Thijssen, K.L., Plasterk, R.H. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science 315, 244-247.

Sijmons, P.C., Atkinson, H.J., and Wyss, U. (1994). Parasitic strategies of root nematodes and associated host cell responses. Annu.Rev. Phytopathol. 32, 235-259.

Silva, J., Chang, K., Hannon, G.J. and Rivas, F.V. (2004). RNA interference-based functional genomics in mammalian cells: reverse genetics coming of age. Oncogene. 23, 8401–8409.

Silvarolla M B (1992). Plant genomic alternations due to tissue culture. J. Brazil. Assoc. Adv. Sci. 44, 329-335.

Simmer, F., Moorman, C., Linden, A.M., Kuijk, E., Berghe, P.V.E., Kamath R.S. *et al*. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. PLoS Biol 1, 77-84.

Simmer, F., Tijsterman, M., Parrish, S. *et al*. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. Current Biology 12, 1317–1319

Simmonds, N.W. (1962). The evolution of the bananas. Longman, London, UK.

Sindhu, A. S., Maier, T. R., Mitchum, M. G., Hussey, R. S., Davis, E. L. and Baum, T. J. (2009). Effective and specific *in planta* RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. Journal of Experimental Botany 60, 315–324.

Sioud, M. (2011). Promises and challenges in developing RNAi as a research tool and therapy. Methods in Molecular Biology 703, 173–187.

Sipen, P., Chubo, J.K., King, P.J.H., Huat, O.K., Davey, M.R. (2011). Genetic improvement of banana using conventional and *In Vitro* technologies. Journal of Crop Improvement, 25, 697–727.

Smant, G., Stokkermans, J., Yan, Y., de Boer, J. M., Baum, T. *et al*. (1998). Endogenous cellulases in animals: cloning of expressed ß-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes.

Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N.and Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Current Biology 10, 169–178.

Song, J.J., Smith, S.K., Hannon, G.J., Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science, 305, 1434-1437.

Speijer, P.R., De Waele, D. (1997).Screening of *Musa* germplasm for resistance and tolerance to nematodes. INIBAP technical guidelines 1 ed Montpellier France: INIBAP.

Speijer, P.R., Napala, P.M., Elsen, A., Ekwamu, A., De Waele, D. (2001). Reinfestation by nematodes and performance of hot-water-treated East African Highland cooking bananas as perceived by farmers in Ikulwe, Iganga district, Uganda. African Plant Protection, 7, 85-89.

Steeves, R.M., Todd, T.C., Essig, J.S. and Trick, H.N. (2006) Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. Funct. Plant Biol. 33, 991–999.

Stein, P., Svoboda, P., Anger, M., Schultz, R.M. (2003). RNAi: mammalian oocytes do it without RNA-dependent RNA polymerase. RNA.9, 187-92.

Steiner, F. A., Okihara, K. L., Hoogstrate, S. W., Sijen, T. and Ketting, R. F. (2009). RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in *Caenorhabditis elegans*. Nature Structural and Molecular Biology 16, 207–211.

Strosse, H., Schoofs, H., Panis, B., Andre, E., Reyniers, K., Swennen, R. (2006). Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa* spp.). Plant Science 170, 104-112.

Subbotin, S.A., Sturhan, D., Chizhov, V.N., Vovlas, N., Baldwin, J.G. (2006). Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. Nematology 8, 455–74.

Subramanyam, K., Subramanyam, K., Sailaja, K.V., Srinivasulu, M., Lakshmidevi, K. (2011). Highly efficient *Agrobacterium*-mediated transformation of banana cv. Rasthali (AAB) via sonication and vacuum infiltration

Sundararaju, P., Shanthi, A., Sathiamoorthy, S. (2005). Status report on *Musa* nematode problems and their management in India. In: Dela Cruz FS, Van den Bergh I, De Waele D, Hautea DM, Molina AB, editors. Towards management of *Musa* nematodes in Asia and the Pacific. International Plant Genetic Resources Institute.

Tabara, H., Sarkissian, M., Kelly, W.G., *et al*. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans.* Cell. 99, 123-132.

Tabara, H., Yigit, E., Siomi, H., Mello, C.C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1,and a DExH-box helicase to direct RNAi in *C. elegans*. Cell 109, 861–871.

Tan, M., Cobon, J., Aitken, E., Cook. L.G. (2010). Support for the "out-of-Southeast Asia" hypothesis for the origin of Australian populations of *Radopholus similis* (Cobb, 1893) Nematoda: Pratylenchidae. Syst. Parasitol. 77, 175-183.

Terami, H., Williams, B.D., Kitamura, S., Sakube, Y., Matsumoto, S., Doi, S. *et al*. (1999). Genomic organization, expression, and analysis of the troponin C gene *pat-10* of *Caenorhabditis elegans*. J Cell Biol 146, 193-202.

Thorne, G. (1949). On the classification of *Tylenchida,* new order (Nematoda, Phasmidia). Proc. helminth. Soc. Wash., 16, 37-73.

Thorne, G. (1961). Principles of nematology. McGraw-Hill Book Company, New York, NY.

Timmons, L. (2004). Endogenous inhibitors of RNA interference in Caenorhabditis elegans. Bioessays 26, 715-718.

Timmons, L., Court, D.L., Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263, 103-112.

Tijsterman, M., Ketting, R.F., Okihara, K.L., Sijen, T., Plasterk, R.H. (2002) RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. Science 295, 694–697.

Tijsterman, M., May, R.C., Simmer, F., Okihara, K.L., Plasterk, R.H. (2004) Genes required for systemic RNA interference in *C. elegans*. Curr. Biol. 14, 111–116.

Tijsterman, M., Okihara, K. L., Thijssen, K. and Plasterk, R. H. A. (2002). PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. Current Biology 12, 1535–1540.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. Nature 395, 854.

Tomari, Y., Matranga, C., Haley, B., Martinez, N. and Zamore, P. D. (2004). A protein sensor for siRNA asymmetry. Science 306, 1377–1380.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biology. 9, R10 (doi:10.1186/gb-2008-9-1-r10).

Tops, B.B., Tabara, H., Sijen, T *et al*. (2005). RDE-2 interacts with MUT-7 to mediate RNA interference in *Caenorhabditis elegans.*Nucleic Acids Res, 33, 347-355.

Trinh, P.Q., Nguyen, C.N., Waeyenberge, L., Subbotin, S.A., Karssen, G., Moens, M. (2004). *Radopholus arabocoffeae* sp.n. (Nematoda: Pratylenchidae), a nematode pathogenic to *Coffea arabica* in Vietnam, and additional data on *R. duriophilus*. *Nematology* 6, 681–93.

Trinh, P.Q., Waeyenberge, L., Nguyen, C.N., Baldwin, J.G., Karssen, G., Moens, M. (2009). *A pratylenchus vietnamensis* gen. n., sp n. and *A. binhi* gen. n., sp n., sedentary Pratylenchidae (Nematoda: Tylenchida) from coffee in Vietnam, with proposal of A pratylenchinae subfam. n. *Nematology*, 11, 565–581.

Tripathi, L., Tripathi, J.N., Oso, R.T., Hughes, Jd.'A, Keese, P (2003). Regeneration and transient gene expression of African *Musa* species with diverse genomic constitution and ploidy levels. Tropical Agr.80, 182-187.

Tripathi, L., Tripathi, J.N., Roderick, H., Atkinson, H.J. (2013). Engineering nematode resistant plantains for sub-saharan Africa. Acta Hort. (ISHS) 974:99-107.

Tyson, T., Zamora, G.O., Wong, S *et al*. (2012). A molecular analysis of desiccation tolerance mechanisms in the anhydrobiotic nematode *Panagrolaimus superbus* using expressed sequenced tags. BMC Research Notes. 5, 68.

Uehara, T., Kushida, A., Momota, Y. (2001). PCR-based cloning of two beta-1,4-endoglucanases from the root-lesion nematode *Pratylenchus penetrans*. Nematology 3, 335-341.

Uma, S., Selvarajan, R., Saraswathi, M. S., Rameshkumar, A., Sathiamoorthy, S. (2002). Production of quality planting material in banana. Global Conf. on Banana and Plantain, October 28–31, Bangalore, India. Souvenir, 24–30.

Unai, E., Iselen, T., de Garcia, E. (2004). Comparison of characteristics of bananas (*Musa* sp.) from the somaclone CIEN BTA-03 and its parental clone Williams. Fruits 59:257–263.

Updike, D.L and Strome, S. (2009). A genomewide RNAi screen for genes that affect the stability, distribution and function of P granules in *Caenorhabditis elegans*.Genetics 183, 1397–1419.

Urwin, P.E., Lilley, C.J., Atkinson, H.J. (2002). Ingestion of double-stranded RNA by pre-parasitic juvenile cyst nematodes leads to RNA interference. Molecular Plant-Microbe Interactions, 15, 747-752.

Vaast, P., Caswell-Chen, E.P., Zasoski, R.J. (1998). Influences of a root-lesion nematode, *Pratylenchus coffeae*, and two arbuscular mycorrhizal fungi, *Acaulospora mellea* and *Glomus clarum* on coffee *(Coffea arabica L.).* Biology and Fertility of Soils. 26,130–135.

Valentine, T., Randall, E., Wypijewski, K., Chapman, S., Jones J, Oparka K. (2007). Delivery of macromolecules to plant parasitic nematodes using a tobacco rattle virus vector.Plant Biotech J 5, 827-34

Valette, C., Mounport, D., Nicole, M., Sarah, J.L., Baujard, P. (1998). Scanning electron microscope study of two African populations of *Radopholus similis* (Nematoda: Pratylenchidae) and proposal of *R. citrophilus* as a junior synonym of *R. similis*. *Fundam. Appl.* Nematol. 21, 139–146.

Van Megen, H., Van den E., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., *et al* (2009). A phylogenetic tree of nematodes based on about 1,200 full length small subunit ribosomal DNA sequences. Nematology ;11:927-50.

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. Gene, 332, 13-27.

Vanholme, B., Haegeman, A., Jacob, J., Cannoot, B., Gheysen, G. (2009). Arabinogalactan endo-1,4-beta-galactosidase: a putative plant cell wall-degrading enzyme of plant-parasitic nematodes. Nematology 11, 739-47.

Vanholme, B., Kast, P., Haegeman, A., Jacob, J., Grunewald, W., Gheysen, G. (2009). Structural and functional investigation of a secreted *chorismate mutase* from the plant-parasitic nematode *Heterodera schachtii* in the context of related enzymes from diverse origins. Mol Plant Pathol 10, 189-200.

Vanholme, B., Van Thuyne, W., Vanhouteghem, K., De Meutter, J., Cannoot, B. and Gheysen, G. (2007). Molecular characterization and functional importance of pectate lyase secreted by the cyst nematode *Heterodera schachtii*. Molecular Plant Pathology 8, 267–278.

Vasale, J.J., Gu, W., Thivierge, C. *et al*. (2010). Sequential rounds of RNA-dependent RNA transcription drive endogenous small-RNA biogenesis in the ERGO-1/Argonaute pathway. Proceedings of the National Academy of Sciences, USA 107, 3582–3587.

Vastenhouw, N. L., Brunschwig, K., Okihara, K. L., Müller, F., Tijsterman, M., Plasterk, R. H. A. (2006). Long-term gene silencing by RNAi. Nature 442, 882.

Vastenhouw, N.L., Fischer, S.E., Robert, V.J., Thijssen, K.L., Fraser, A. G., Kamath, R. S., Ahringer, J., Plasterk, R.H. (2003). A genome-wide screen identifies 27 genes involved in transposon silencing in *C. elegans*. Curr. Biol. 13, 1311–1316.

Verdejo, S., Jaffee, B.A., Mankau, R. (1988). Reproduction of *Meloidogyne javanica* on plant roots genetically transformed by *Agrobacterium rhizogenes*. J Nematol 20, 599–604.

Vidhya, R and Ashalatha, S.N. (2002). *In-vitro* culture, pseudostem pigmentation and genetic characterization of *Musa acuminata* cv. Red. Global Conf. on Banana and Plantain, October 28-31, Bangalore, India. Abstracts, 65.

Viney, M.E., Thompson, F.J. (2008). Two hypotheses to explain why RNA interference does not work in animal parasitic nematodes. Int. J.Parasitol.38, 43–47.

Visser, A., Geldhof, P., DeMaere, V., Knox, D.P., Vercruysse, J., Claerebout, E. (2006). Efficacy and specificity of RNA interference in larval life-stages of *Ostertagia ostertagi.* Parasitology 133, 777–783.

Voinnet, O (2001) RNA silencing as a plant immune system against viruses. Trends Genet, 17, 449–459.

Vos, C., Claerhout,S., Mkandawire,R., Panis,B.,De Waele, D., Elsen, A. (2012). Arbuscular mycorrhizal fungi reduce root–knot nematode penetration through altered root exudation of their host. Plant and Soil, 354, 335–345.

Vought, V.E, Ohmachi, M., Lee, M.H., Maine, E.M. (2005). EGO-1, a putative RNA directed RNA polymerase, promotes germline proliferation in parallel with GLP-1/notch signaling and regulates the spatial organization of nuclear pore complexes and germline P granules in *Caenorhabditis elegans*. Genetics 170, 1121–1132.

Vuylsteke, D., Swennen, R., De Langhe, E. (1991). Somaclonal variation in plantains (Musa spp., AAB group) derived from shoot-tip culture.Fruits 46, 429–439.

Vuylsteke, D., Swennen, R., De Langhe, E.A. (1996). Field performance of somaclonal variants of plantain (*Musa* spp., AAB Group). J. Amer. Soc. Hort, 121, 42-46.

Vuylsteke, D., Swennen, R., Wilson, G.F., De Langhe, E.A. (1988). Phenotypic variation among *in vitro* propagated plantain (Musa spp- cv_ AAB). Scientia Hort. 36, 79-88.

Wang, D., Kennedy, S., Conte, D., Kim, J.K., Gabel, H.W., Kamath, R.S., Mello, C.C., Ruvkun, G. (2005), Somatic misexpression of germline P granules and enhanced RNA interference in retinoblastoma pathway mutants. Nature 436, 593–597.

Wang, W., Wang, Y.J., Zhang, Q., Qi, Y., Guo, D.J. (2009). Global characterization of Artemisia annua glandular trichome transcriptome using 454 pyrosequencing. BMC Genomics 10, 465.

Wang, X.H., Allen, R., Ding, X.F., Goellner, M., Maier, T., de Boer, J.M. *et al*. (2001). Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. Mol Plant Microbe Interact. 14, 536-44.

Warthmann, N., Ossowski, S., Schwab, R., Weigel, D. (2013). Artificial microRNAs for specific gene silencing in rice. Methods in Molecular BiologyVolume 956, 131-149

Waterhouse, P. M., Graham, M.W., Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences 95(23): 13959-13964.

Wheeler, D., Darby, B.J., Todd, T.C., Herman, M.A. (2012). Several grassland soil nematode species are insensitive to RNA-Mediated Interference. J Nematol. 44, 92–101.

Williams, A.E (2008). Functional aspects of animal microRNAs. Cell. Mol. Life Sci. 65, 545–62.

Williams, B.D., Waterston, R.H. (1994). Genes critical for muscle development and function in *Caenorhabditis elegans* identified through lethal mutations. J Cell Biol 124, 474-490.

Winston, W.M., Olodowitch, C., Hunter, C.P (2002). Systemic RNA in *C.elegans* requires the putative transmembrane protein SID-1. Science, 295, 2456–2459.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H., Hunter, C.P. (2007). *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. Proc. Natl Acad. Sci. USA. 104, 10565–10570.

Wu, P., Wilmarth, M.A., Zhang, F., Du, G. (2013). miRNA and shRNA expression vectors based on mRNA and miRNA processing. Methods in Molecular Biology. 936, 195-207

Wubben, M.J., Callahan, F.E., Scheffler, B.S. (2010a). Transcript analysis of parasitic females of the sedentary semi-endoparasitic nematode *Rotylenchulus reniformis*. Mol Biochem Parasitol 172, 31-40.

Wubben, M.J., Ganji, S., Callahan, F.E. (2010b). Identification and molecular characterization of a *β-1,4-endoglucanase* gene (*Rr-eng-1*) from *Rotylenchulus reniformis*. J Nematol, 42, 342-351.

Wyss, U. (1997). Root parasitic nematodes In: Fenoll C, Grundler FMW and Ohl S, eds. An overview. Cellular and molecular aspect of plant-nematodes interactions. Dordrecht, The Netherlands: Kluwer Academic Press, pp 233–259.

Wyss, U. (2002). Feeding behavior of plant parasitic nematodes. In: Lee, D.L. (ed) The biology of nematodes. Taylor and Francis, New York, pp.233-259.

Xiao, Y.H., Yin, M.H., Hou, L., Pei, Y. (2006). Direct amplification of intron containing hairpin RNA construct from genomic DNA. Biotechniques 41, 548, 550, 552.

Xu, G., Sui, N., Tang, Y., Xie, K., Lai, Y., *et al*. (2010). One-step, zero-background ligation-independent cloning intron-containing hairpin RNA constructs for RNAi in plants. New Phytol 187, 240–250.

Xue, B., Hamamouch, N., Li, C., Huang, G., Hussey,R.S., Baum, T.J., Davis, E.L. (2013). The 8D05 parasitism gene of *Meloidogyne incognita* is required for successful infection of host roots. Phytopathology 2, 175-181.

Yadav, B. C., Veluthambi, K. and Subramaniam, K. (2006). Hostgenerated double strandedRNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Mol. Bio Parasitol 148, 219–222.

Yan, P., Shen, W., Gao, X., Duan, J., Zhou, P. (2009). Rapid one-step construction of hairpin RNA. Biochem Biophys Res Commun 383, 464–468.

Yan, P., Shen, W., Gao, X., Li, X., Zhou, P., Duan, J. (2012). High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. PLoS ONE 7, e38186.

Yang, H., Zhang, Y., Vallandingham, J., Li, H., Florens. L., Mak, H.Y. (2012). The RDE-10/RDE-11 complex triggers RNAi-induced mRNA degradation by association with target mRNA in *C. elegans*. Genes Dev. 26, 846-56.

Yeates, G.W., Ferris, H., Moens, T., Putten, W.H.V. (2009). The role of nematodes in ecosystem. In (Wilson, M.J. and Kakouli-Duarte, T) Edn. Nematodes as environmental indicators, CAB International, Wallingford, Oxford, UK, pp, 1-44.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H. *et al*. (2006). Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell 127, 747-57.

Zamore, P.D., Tuschl, T., Sharp, P.A., Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell.101, 25-33

Zhang, L., Ding, L., Cheung, T.H., Dong, M.Q., Chen, J. *et al*. (2007). Systematic identification of *C. elegans* miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell 28, 598–613.

Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S., Schultz, P.G. (2004). An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. PNAS. 101, 135-140.

Zhou, H., Xu Gang Xia, X.G., Xu, Z. (2005). An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. Nucleic Acids Research, 33, e62.

Zhou, M.Y., Xue, D., Gomez-Sanchez, E.P., Gomez-Sanchez, C.E. (1994) Improved downward capillary transfer for blotting of DNA and RNA. BioTechniques 16, 58-59.

Zhuang, J.J., Banse, S.A., Hunter, C.P. (2013). The nuclear Argonaute NRDE-3 contributes to transitive RNAi in *Caenorhabditis elegans*. In press.Genetics doi:10.1534/genetics.113.149765.

Zhuang, J.J and Hunter, C.P. (2012).The Influence of competition among *C. elegans* small RNA pathways on development. Genes. 3(4), 671-685.

Zhuang, J.J. and Hunter, C.P. (2011a) RNA Interference in *Caenorhabditis elegans*: Uptake, Mechanism, and Regulation. Parasitology 1-14.

Zhuang, J.J and Hunter, C.P. (2011b) Tissue Specificity of *Caenorhabditis elegans* Enhanced RNA Interference mutants. Genetics 188, 235–237.

Zimmermann, A. (1898) De nematoden der Koffiewortels. Deel I. Mededeel.'s Lands Plantentuin (Buitenzorg), 27, 64.

Zunke, U. (1991). Observations on the invasion and endoparasitic behavior of the root lesion nematode *Pratylenchus penetrans*. Journal of Nematology 22, 309-320.

Curriculum vitae

Personal Information

Soumi Joseph

Birth date/place: 29th July 1980 / Nedumkunnam

Nationality: India

Personal e-mail: soumi80@yahoo.com

Ghent University, Faculty of Bioscience Engineering
Department of Molecular Biotechnology
Coupure links 653
9000 Ghent, Belgium
e-mail: Soumi.joseph@ugent.be
tel: 003292645967
fax: 003292646219

Education

Oct 2007-April 2013 — **PhD training**

The Doctoral School of Bioscience Engineering, Ghent University, Belgium

Oct 2005-Oct 2006 — **Master of Science** in Nematology
Ghent University, Belgium.

Sept 1999-Aug 2003 — **Bachelor of Science** in Agriculture
University of Agricultural Sciences, Bangalore, India

Professional career

2007-2012:	**VLIR-ICP- PhD scholarship**
Project:	Analysis of RNAi strategies against migratory parasitic nematodes of banana. Ghent University, Belgium Katholik university of Leuven Indian Institute of technology, Kanpur, India
Promoter:	Prof. dr.G. Gheysen
Co-promoters:	Prof. R. Swennen Dr. K. Subramaniam Dr. Ir. Serge. Remy
2005-2006	**VLIR-UOS scholarship (2005-2006*)*: for training** and master programmes in Ghent University, Belgium
Project:	Application of RNAi in parasitic nematodes
Promoter:	Prof. dr. G. Gheysen

Publications with peer reviewing

Joseph, S., Gheysen, G. and Subramaniam, K. (2012). RNA interference in *Pratylenchus coffeae*: Knock-down of *Pc-pat*-10 and *Pc-unc-87* impedes migration. *Molecular and Biochemical parasitology* 186:51-59.

Haegeman, A., **Joseph, S**., and Gheysen, G. (2011). Analysis of the transcriptome of the root lesion nematode *Pratylenchus* coffeae generated by 454 sequencing technology. *Molecular and Biochemical parasitology* 178(1-2):7-14.

Conference and symposia oral presentations

Joseph, S., Gheysen, G. and Subramaniam, K. (2010). RNAi mediated knock-down of *pat-10* and *unc-87* disrupts movement of root lesion nematode, *Pratylenchus coffeae*. *Proceedings of 30th International symposium of the European Society of Nematologists*, September 19-23, 2010, Vienna, Austria.

Joseph, S., Gheysen, G. and Subramaniam, K. (2010). RNAi of *pat-10* and *unc-87* impedes movement of the migratory nematode, *Pratylenchus coffeae*. *Proceedings of 62nd International Symposium on Crop protection*, May 18, 2010, Gent, Belgium.