Silencing parasitism effectors of the root lesion nematode, *Pratylenchus thornei*.

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by

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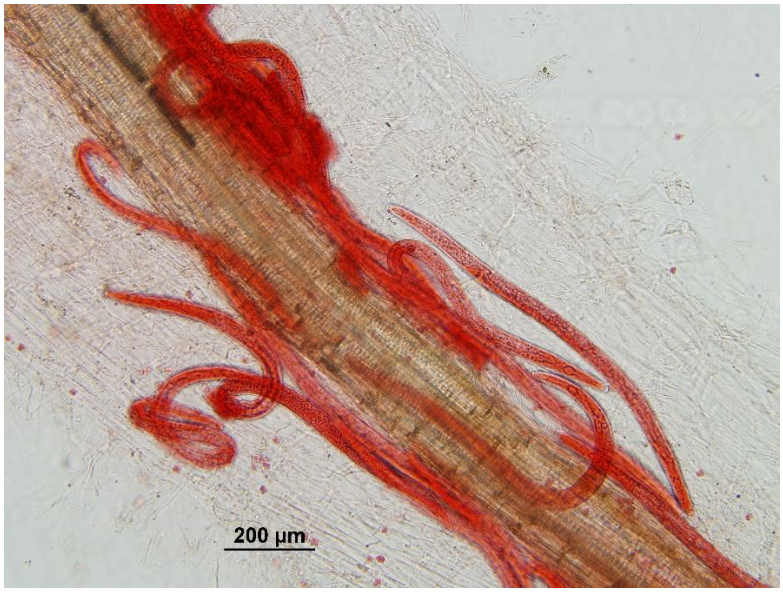

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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ABSTRACT

The root lesion nematode (RLN), *Pratylenchus thornei*, is a biotrophic migratory pest of plant roots and its infestation causes losses in many economically important crops. RNA interference (RNAi) is a naturally occurring eukaryotic phenomenon and can be used to silence parasitism effector genes of *P. thornei* using host-mediated RNAi. This may be developed as an environmentally friendly and a cost-effective control strategy. The overall aims of this research were to investigate the effects of *in vitro* and *in planta* RNAi silencing of putative *P. thornei* parasitism effector genes, and their nematicidal effects in two host plants.

Five putative target parasitism genes vital for nematode entry into roots (*Pt-Eng-1*, *Pt-PL*), feeding (*Pt-CLP*) and suppressing host defence responses (*Pt-UEP, Pt-GST*) were identified, validated *in silico* using comparative bioinformatics, cloned into suitable *in vitro* transcription and binary vectors, and advanced to RNAi studies. Partial sequences for four of these target effector genes (*Pt-Eng-1*, *Pt-PL*, *Pt-CLP*, *Pt-GST*) were identified using Rapid Amplification of cDNA (RACE) PCRs and annotated *in silico*. Protein families, conserved domains, taxonomic and phylogenetic relationships for all four effectors were studied. This sequence information will help inform future investigations involving gene expression and proteomics of the selected putative effectors.

In vitro RNAi was used for functional characterisation of the five effector sequences. Effects on nematode phenotype, behaviour, gene expression, and longer-term effects on reproduction were assessed after soaking nematodes in dsRNA through infection of healthy wild type soybean and alfalfa roots. Soaking of mixed stage *P. thornei* in 1mg/mL dsRNA of target genes for 16 h did not cause phenotypic changes except for *Pt-PL,* which exhibited straight or slightly curved phenotypes after soaking compared to the normal sigmoid body movement, also evident for green fluorescent protein (*gfp*) and no dsRNA treated controls. Semi-quantitative PCRs and densitometry analysis revealed a significant reduction of transcript accumulation for all five putative parasitism effector genes. Longer-term effects assessed at 21 dpi reduced nematode reproduction by 40 to 70% for all target genes compared to respective control treatments suggesting that the effectors studied were required for nematode infectivity, survival or reproduction.

In planta RNAi involved *Agrobacterium*-mediated plant transformations to develop axenic transgenic hairy root events of soybean (*Glycine max* var. Williams 82) and alfalfa

(*Medicago sativa*), and non-axenic hairy roots (composite plants) of soybean. Both hosts were amenable to *Agrobacterium*-mediated transformation, but hairy root induction was faster in alfalfa than soybean. However, more events were generated for soybean than alfalfa. Transgenic hairy roots confirmed by molecular analyses were challenged with *P. thornei* and their presence confirmed after 14 dpi. After 21 dpi, nematode numbers and transcript abundance was assessed using semi-quantitative PCRs and densitometry analysis. Host-mediated silencing of the five putative parasitism effector genes using transgenic soybean and alfalfa hairy roots showed a significant reduction in target transcript accumulation and approximately 38 to 75% reduction in *P. thornei* numbers compared to untransformed wild-type controls. For some events, there was a positive correlation between reduced transcripts and nematode numbers.

Based on percent reduction in transcript accumulation of the target genes relative to 18S rRNA as assessed by densitometry, the extent of gene knockdown measured (from most to least) was: *Pt-Eng-1*, *Pt-PL*, *Pt-CLP*, *Pt-UEP*, and *Pt-GST*. Similarly, *Pt-Eng-1*, *Pt-PL* and *Pt-CLP* were ranked in the same order, from the lowest to highest reproduction on soybean and alfalfa, indicating a positive correlation between the level of knockdown and reduced reproduction. In soybean, these genes were followed by *Pt-GST* and *Pt-UEP* for the percentage of reproduction recorded, whereas, in alfalfa, reduction in reproduction for these two target genes did not differ significantly.

Composite soybean with wild-type shoots and transgenic hairy roots expressing *Pt-Eng-1* and *Pt-PL* genes were developed and provided an opportunity to test the effectiveness of silencing target genes *in planta* and on nematode numbers in conditions that mimicked natural host infections. For both *Pt-Eng-1* and *Pt-PL* genes, there was a significant reduction in percentage of transcript accumulation relative to 18S rRNA, which correlated with a reduction in nematode numbers by 53.4% and 48.5% for *Pt-Eng-1* and *Pt-PL*, respectively. The amenability of *P. thornei* to host-mediated RNAi using effector gene sequences, and the overall results of this study, point towards the potential use of this technology to control *P. thornei* and related RLN species effectively in different host crops.

PUBLICATIONS AND PRESENTATIONS

Poster Presentations

Khot, S., Berryman, D. and Jones, M. G. K. 2015. Bioinformatics, identification and functional characterisation of parasitism effectors of the root lesion nematode, *Pratylenchus thornei.* VLS Poster Day, Murdoch University, November 11, 2015.

Khot, S., Berryman, D. and Jones, M. G. K. 2015. RNAi silencing of parasitism effectors in the root lesion nematode, *Pratylenchus thornei*. Murdoch Agricultural Research Symposium, Murdoch University, September 01, 2017.

Khot, S., Berryman, D. and Jones, M. G. K. 2015. Host-mediated RNAi of *Pratylenchus thornei* effectors reduces its population. Murdoch University Annual Research Symposium, Murdoch University, November 08, 2017.

Oral Presentation

Khot, S., Berryman, D. and Jones, M. G. K. 2017. Silencing parasitism effectors of the root lesion nematode, *Pratylenchus thornei*. VLS Pre-Submission Presentation, Murdoch University, December 08, 2017.

Additional presentations

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LIST OF ABBREVIATIONS

35S	35S RNA transcriptional promoter of CaMV
aa	amino acid
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BLASTP	Protein BLAST
bp	base pair
cDNA	complementary DNA
COBALT	constraint-based multiple alignment
cv	cultivar
CN	cyst nematode
DNA	deoxyribonucleic acid
dNTPs	nucleotide mix
dsRNA	double-stranded DNA
EDTA	ethylenediaminetetra-acetate acid disodium salt
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GSP	gene specific primer
HSP	high-scoring segment pair
kb	kilobase
LB broth	Luria Bertani broth
NCBI	National Center for Biotechnology Information
ng	nanogram
nt	nucleotide
PCR	polymerase chain reaction
pDo	prefix for pDoubler clones
PPN	plant parasitic nematode
RACE	Rapid Amplification of cDNA ends
RKN	root knot nematode
RNAi	RNA interference
RNase	ribonuclease
RT	reverse transcription
siRNA	small interfering RNA
TAE	tris-acetate-EDTA
TBLASTX	search translated nucleotide database using a translated nucleotide query
TSA	transcriptome shotgun assembly

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DEDICATION

To

my beloved wife,

Vineeta.

CHAPTER 01

General Introduction and Literature Review

1.1 General Introduction

Nematodes belong to the phylum Nematoda, a large and diverse group of invertebrates with about 25,000 described species (Abad and Williamson, 2010; Wylie et al., 2004). Nematodes occupy a broad spectrum of habitats around the globe adapting to diverse environmental conditions (Parkinson et al., 2004b). They are found as free-living marine and terrestrial microbivores, predators of meiofauna, herbivores, and as plant and animal parasites (Abad and Williamson, 2010; Parkinson et al., 2004b). Of these, the animal and plant parasitic nematodes (PPNs) important because of their impact on human health and agricultural production, respectively (Abad and Williamson, 2010). There are over 4100 PPN spp. described to date (Decraemer and Hunt, 2006). A range of PPN spp. are responsible for annual crop losses estimated at 120 billion USD worldwide, with 10 billion USD worth of losses just in the USA (Chitwood, 2003).

The top three PPNs responsible for the majority of reported crop losses are root-knot nematodes (RKN) (*Meloidogyne* spp.), cyst-nematodes (CN) (*Heterodera* and *Globodera* spp.) and root lesion nematodes (RLNs) (*Pratylenchus* spp.) (Castillo and Vovlas, 2007; Jones et al., 2013). Unlike the sedentary RKNs or CNs, the migratory endoparasitic RLNs do not form any elaborate modification of plant cells during feeding. RLNs use a 'hit-and-run' strategy during their polyphagous feeding which causes considerable tissue damage due to depletion of vital plant nutrients, reduced water uptake, and infection by secondary soil pathogens. The success of these pests can be attributed to their ability to evade or suppress host defence mechanisms much like their sedentary counterparts (Castillo and Vovlas, 2007; Hussey et al., 2002). Losses worth millions of dollars have been attributed to RLNs globally, whilst in Australia substantial losses in many important crops such as wheat, barley and sugarcane have been well documented (Blair and Stirling, 2007; Hodda and Nobbs, 2008; Nicol et al., 1999).

Much of the damage caused by RLNs is caused by their migratory behaviour whilst feeding from cells of plant roots, especially in the cortex. RLNs, like other PPNs, use their hollow mouth stylet to mechanically puncture cells and deliver secretions that modify cell walls during their migration and feeding whilst the RLNs parasitise their host. These secretions that are delivered into the host tissue during invasion, feeding and salivation contain proteins known as 'effectors' (Davis et al., 2000; Davis et al., 2008; Vanholme et al., 2004). Effectors play a crucial role in

nematode-plant parasitism and can act by alteration of host-cell structures, as well as suppressing host defences and modifying cell metabolism (Davis et al., 2008; Hogenhout et al., 2009). The effector-containing secretions in RLNs and other PPNs originate in different parts of the nematodes secretory and excretory systems. Amongst these sources, the dorsal oesophageal and the two sub-ventral gland cells along with the stylet are two essential adaptions that make most RLNs and PPNs successful plant parasites (Vanholme et al., 2004).

The PPN threat to crop production can be mitigated to a limited extent by integrating control measures which can involve the use of chemical nematicides, nematode resistant crop varieties, and application of several cultural practices. Most of the generic control measures that fall into the above categories have high monetary or environmental costs associated with them (Atkinson et al., 2012). Natural sources of genetic resistance against RLNs are limited or unavailable for many crops-based approaches. Development of nematode resistant crop varieties using classical breeding and using race-specific resistance might be good options but have their limitations (Jung and Wyss, 1999; Noel, 2007). Lack of adequate RLN management options and a demand for economically and environmentally-friendly alternatives are leading to exploring the use of modern genetic tools and technologies to control RLNs more efficiently. Transgenic maize and cotton expressing *Bacillus thuringiensis* (*Bt*) toxin against certain destructive insect pests are good examples of deployment of such genetic engineering tools (Cannon, 2000). Use of a similar approach to develop novel resistance against nematodes by targeting vital or important nematode biology may be possible (Mitchum et al., 2012). New molecular strategies involving the use of RNA interference have recently been shown to be promising for nematode control (Gheysen and Vanholme, 2007; Yadav et al., 2006).

RNAi mediated genetic interference (RNAi) first reported in *Caenorhabditis elegans*, the free-living nematode and is a natural phenomenon in which presence of double-stranded RNA (dsRNA) triggers the destruction of mRNA transcripts with homologous sequence leading to the depletion of the encoded protein (Fire et al., 1998). Exploring nematode effectors and their molecular function during host invasion is an emerging paradigm that may help in identification of molecular targets for developing novel RLN control strategies using RNAi (Joseph et al., 2012; Mitchum et al., 2013; Sindhu et al., 2009). RNAi has been used successfully for functional genomic studies of several sedentary PPNs, such as *G. pallida*, *H. glycines*, *M. incognita*,

(Gheysen and Vanholme, 2007; Jacob et al., 2007; Rosso et al., 2005; Roze et al., 2008; Urwin et al., 2002; Vanholme et al., 2007) and migratory PPNs like *Radopholus similis*, *Bursaphelenchus xylophilus*, *P. coffeae*, *P. thornei* and *P. zeae* (Haegeman et al., 2009; Li et al., 2011b; Park et al., 2008; Tan et al., 2013). More than 40 PPN genes from five genera and nine species have so far been targeted using RNAi (Lilley et al., 2012; Maule et al., 2011).

Whilst the majority of the successful RNAi studies on PPNs have been undertaken using *in vitro* delivery methods, such as feeding artificial diets, injection, soaking, an increasing number of experiments have demonstrated the efficacy of *in planta* RNAi in PPNs (Dutta et al., 2014; Lilley et al., 2012). *In planta* RNAi as a crop protection strategy involves generating plants engineered to express dsRNA molecules by cloning the target gene, sense and antisense cDNA sequences including an intronic and spacer region into a suitable binary vector with a plant promoter, for example the constitutive 35S RNA transcriptional promoter of CaMV (Lilley et al., 2012; Patel, 2008; Steeves et al., 2006). Plant-mediated RNAi has been shown to give a reasonable level of PPN control (for example, *Rs*-CRT of *R. similis*, MSP dsRNA and *Pv-eng-1* in *P. vulnus,* and on *H. glycines* egg number) under controlled environment experiments, paving a path for control of PPNs using RNAi (Dutta et al., 2014; Fanelli et al., 2014; Li et al., 2015; Steeves et al., 2006).

Molecular characterisation and effects of RNAi on PPN effectors both *in vitro* and *in planta* is a common topic of study. There are several factors that govern the efficacy of RNAi in nematodes: these include factors such as the choice of target gene and sequence, amount of dsRNA, turn-over of targeted gene, method and timing of dsRNA delivery and the targeted region (Fanelli et al., 2014; Fire et al., 1998; Haegeman et al., 2009; Lilley et al., 2012; Sukno et al., 2007). There are also a limited number of studies that describe the cloning and functional characterisation of full-length effector sequences of PPNs to study their role in pathogenicity. One of the aims of this work was to identify potential essential pathogenicity-related genes of *Pratylenchus thornei* through comparative bioinformatics and to investigate molecular and functional characters using RNAi as a tool. This research also includes plant-mediated RNAi of potential pathogenicity effectors of *P. thornei* the results of which could provide environmentally and economically cost-effective means of crop protection against not only *P. thornei* but other migratory nematodes as well (Fanelli et al., 2014).

1.2 *Pratylenchus thornei*: A plant parasitic root lesion nematode (RLN)

There are about 68 known *Pratylenchus* spp., of which only a dozen (*P. brachyurus*, *P. coffeae*, *P. goodeyi, P. loosi, P. neglectus, P. penetrans, P. scribneri, P. fallax, P. thornei, P. zeae, P. crenatus, P. vulnus, P. goodeyi*) are known to cause substantial yield losses in a wide range of agricultural and horticultural crops world-wide (Blair and Stirling, 2007; Blair et al., 1999; Castillo and Vovlas, 2007; Hodda and Nobbs, 2008). In Australia, a few *Pratylenchus* spp. such as *P. neglectus*, *P. penetrans*, *P. thornei* and *P. zeae* (Sher and Allen, 1953) contribute in yield losses accounting for millions of dollars in important crops such as wheat, barley, legumes and sugarcane (Blair et al., 1999; Nicol et al., 1999; Thompson et al., 1999; Thompson et al., 2008). The cereal and legume RLN, *P. thornei* is a polyphagous, vermiform, migratory, endoparasitic PPN that parasitises many cereals and legumes globally, mainly feeding and reproducing in the cortex of host crops. Under favourable conditions it can complete its life cycle several times in one growing season (Castillo and Vovlas, 2007; Nicol et al., 1999; Thompson et al., 2008).

High populations of *P. thornei* result in root damage, leading to loss of root function and overall appearance of a nutrient deficient and water-stressed plants, especially in areas where soil moisture is a limiting factor towards the end of crop growing season or in dry years (Glazer and Orion, 1983; Thompson et al., 1999). Population densities of 2 to 48 nematodes/100 mL of soil with 6 to 70 nematodes per gram of root have been reported in Spain on chickpea (Castillo et al., 1995).

Infected plants appear stunted with fewer tillers and yellowing of lower leaves (Doyle et al., 1987; Van Gundy et al., 1974). Susceptible hosts exhibit root lesions and browning as a result of cell lysis, cavities, necrosis and finally degradation of the cortical tissue. The nematode infection also encourages attack by fungi and bacteria that invade and cause further damaged to plant tissues (Baxter and Blake, 1968; Baxter and Blake, 1967; Castillo et al., 1995; Larson, 1953; Nicol et al., 1999). Above ground symptoms are non-specific, with infected plants exhibiting stunting and weak canopy structure, there is a reduction in the number of tillers in grain crops and yellowing of lower leaves observed in most infected host crops (Nicol et al., 2011).

1.2.1 Taxonomy and general morphology

Pratylenchus spp. can be sorted readily into their respective genus, but at species level they are morphologically very similar and can be differentiated only at high magnifications (Castillo and Vovlas, 2007). Hence, there is no satisfactory key for identification of their widely diverging species synonymies due to continued revisions of the genus. *P. thornei* according to classification scheme by Siddiqi (2000) would be under Phylum Nematoda, Class Secernentea, Subclass Tylenchia, Order Tylenchida, Suborder Tylenchina, Superfamily Hoplolaimoidea, Family Pratylenchidae, Subfamily Pratylenchinae, Genus *Pratylenchus*, Species *thornei* (Castillo and Vovlas, 2007; Sher and Allen, 1953). *P. thornei* has its own characteristic labial region with three annuli that are not offset from the body, with the outer margin of the sclerotised labial framework extending conspicuously approximately two annuli into its body and one annulus into its labial region. Its body exhibits lateral fields with four conspicuous lines, the outer ones being straight or weakly crenate, a medium sized stylet (17-19 µm long), it is difficult to see the spermatheca, which does not contain spermatozoa, and males are very rare. The shape of the tail and above-mentioned characters help in distinguishing this nematode from its closely related species such as *P. mediterraneus*, *P. fallax*, *P. penetrans*, *P. pseudopratensis* and *P. sudanensis* (Castillo and Vovlas, 2007).

Nematodes have a well-conserved body plan despite such a diversity in lifestyle. Their body is made up of an external cylinder or body wall that encloses an internal cylindrical digestive system both of which are separated by a pseudocoelomic cavity filled with fluid which is under pressure. This cavity contains a number of cells, the reproductive tract, secretory –excretory system and nervous system (Decraemer and Hunt, 2006).

1.2.2 Life cycle

P. thornei has a simple life cycle composed of six life stages like all other PPNs, *i.e.*, egg, four juvenile stages (J1 to J4) and finally the adult stage. The female lay eggs, singly or in small groups in host roots or in the soil proximal to root surface. Male *P. thornei* are rarely found, and reproduction is mainly by mitotic parthenogenesis. Both embryogenesis and the first moult (from first stage juvenile (J1) to second stage juvenile (J2) take place within the eggshell, followed by hatching of infective J2 from the eggs (Figure 1.1). The J2-J4 and adult stages are vermiform and mobile, and can infect host plants, primarily the roots, although above ground

plant parts are also known to be parasitised (Castillo and Vovlas, 2007). *In vitro* studies show that the life cycle of *Pratylenchus* spp. is three to nine weeks depending on environmental conditions (Chitambar and Raski, 1985; Turner and Chapman, 1972). In general, RLNs can complete three to six generations in a single growing season depending on host susceptibility (Vanstone et al., 2008). They can survive long periods of unfavourable conditions at the egg stage or through anhydrobiosis and can be wind dispersed at these stages (Glazer and Orion, 1983; Vanstone et al., 2008).

1.2.3 Feeding behaviour

Feeding of RLNs involves the motile stages locating, penetrating and migrating through plant tissues. J2s may feed from epidermal cells, but at later stages individual nematodes are mainly found in the cortical cells where they use their sclerotised stylet to deliver secretions and to feed from host cells. Evidence from studies conducted on sedentary PPNs points to the parasitic potential of the nematode secretions containing multiple disease-inducing peptides or proteins (Davis et al., 2000; Vanholme et al., 2004). PPN secretions come from different sources like the pharyngeal gland cells, the hypodermis, the amphids, the cuticle and the secretory/excretory system.

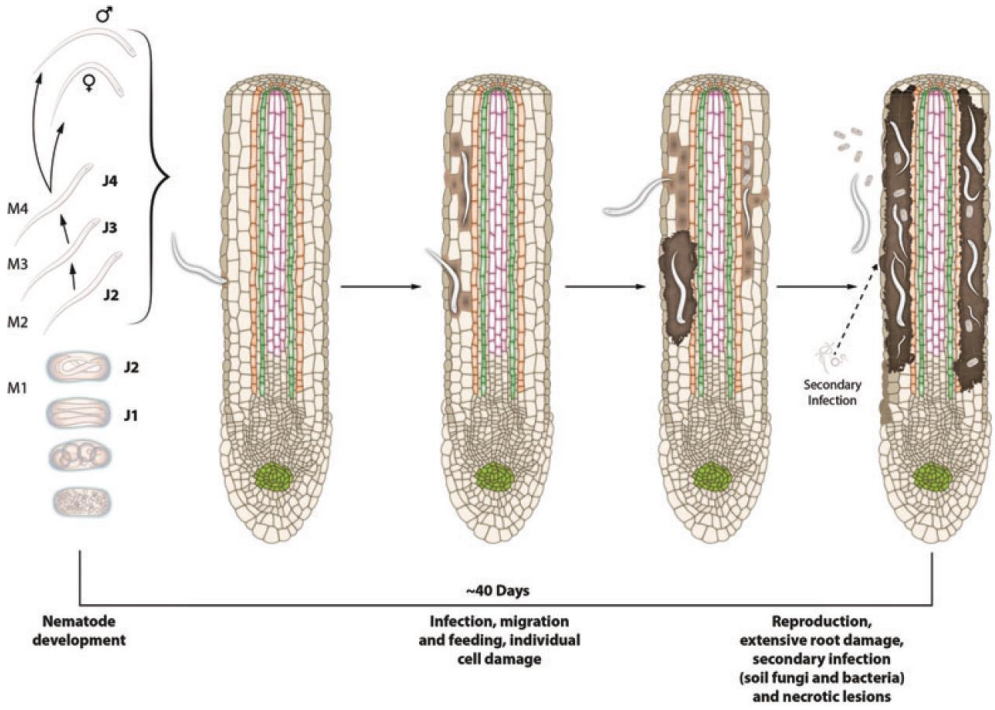


Figure 1.1. Life cycle of root lesion nematodes (Jones and Fosu-Nyarko, 2014).

The protrusible hollow stylet and specialised secretory oesophageal gland cells are two important and essential adaptations for parasitism (Vanholme et al., 2004). Secretions of the oesophageal glands are synthesised and sequestered within the secretory granules present in the gland cells (Hussey, 1989). The single dorsal oesophageal gland and the two sub-ventral gland cells extend anteriorly through different passages or ducts: the dorsal oesophageal gland releases secretions into the oesophageal lumen at the base of the stylet and the sub-ventral glands release secretion at the metacarpal chamber basal to the metacorpus of the alimentary canal (Figure 1.2). Studies from sedentary and migratory PPNs show that sub-ventral glands secretions contain enzymes capable of modifying plant cell walls, and other granules possibly important during root invasion, migration through plant tissues and induction of specialised feeding cells. On the other hand, secretions from the granule-rich single dorsal oesophageal gland cell possibly help in the manipulation of plant response during nematode infection and prolonged feeding within the host (Gheysen and Mitchum, 2011). The sub-ventral glands of endoparasitic nematodes do not markedly expand during feeding, whereas the dorso-ventral gland becomes larger and is characterised by the presence of many granules. This suggests a role in feeding and long-term maintenance of the feeding cell(s) (giant cells or syncytia). The importance of the presence of these secretory granules, which are only in the single dorsal oesophageal gland cell of *Pratylenchus* spp. is yet to be elucidated. Changes in oesophageal gland cell activity during RLN life cycle and importance of specific gland secretions during different stages of parasitism also need to be studied.

1.2.4 Economic importance of root lesion nematodes

The socioeconomic impact caused by all PPNs has a global significance with a major global challenge of providing food security to ever-growing human population. Hence, crop damage due to PPNs can have disastrous consequences in developing countries lacking proper biosecurity and food security measures (Jones et al., 2011). Like the RKNs and CNs, RLNs of the genus *Pratylenchus* are responsible for substantial crop losses on a global scale. *Pratylenchus* spp. are a pest of almost every important food crop, including maize (*Zea mays*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), oats (*Avena sativa*), rye (*Secale cereal*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*), and wheat (*Triticum aestivum*). The species also parasitises important oil crops like soybean (*Glycine max*) and other high-value food and fruit crops (Nicol et al., 2011).

P. thornei is of a special economic importance because of the losses it causes in staple food crops. This nematode is associated with yield losses in cereal crops worldwide: losses of up to 85% in wheat have been reported when the nematode population is high (Nicol et al., 1999; Nicol et al., 2011; Thompson et al., 1993; Zwart et al., 2005). *P. thornei* is found throughout the Australian grain growing regions and is responsible for substantial crop damage in southern Queensland, northern New South Wales, Victoria, parts of South Australia and Western Australia (Thompson et al., 1999). Losses of up to A$36 million in Australian wheat production have been associated to *P. thornei* in reports published in 1998 (Brennan and Murray, 1998).

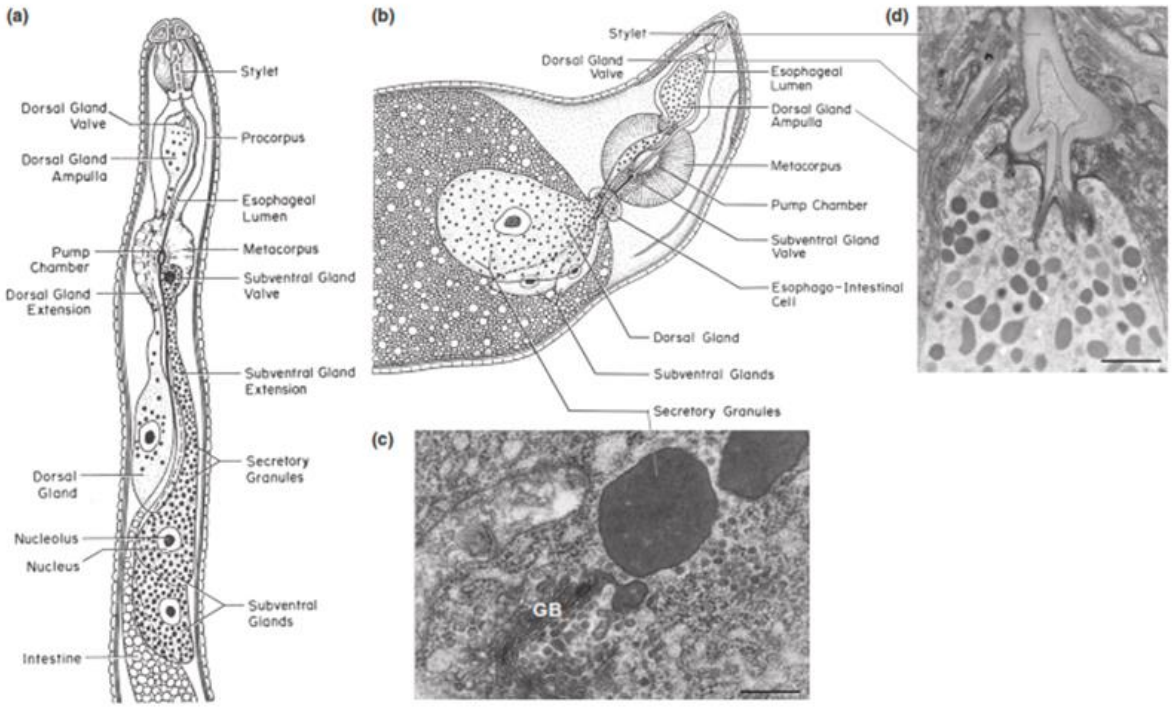


Figure 1.2. Illustrations and images of the oesophageal glands and secretory cells that produce effector proteins from the anterior parts of the migratory J2 (a) and sedentary (b, c, d) stages of root-knot nematodes exhibiting more secretory granules within the enlarged dorsal glands during the sedentary stage. (a) Illustration of anterior region of a migratory infective second-stage juvenile with two sub-ventral oesophagal gland cells containing secretory granules. (b) Sedentary swollen female within the infected roots exhibiting reduced sub-ventral glands and enlarged dorsal gland cell containing secretory granules probably related to its role during the sedentary stages. (c) Image shows the formation of new secretory granules by the process of budding from the trans-Golgi network alongside a large mature secretory granule. (d) Dorsal

gland ampulla containing secretory granules near the dorsal gland valve seen in the longitudinal section. GB, Golgi bundles. Bars, 0.24 µm. Source Mitchum et al. (2013).

Overall wheat yield reduction of approximately 8% in 70% of the infested fields suggest even higher losses of up to A$69 million/year, with additional losses due to use of intolerant cultivars of many other alternate crops such as chickpea and mungbean (Thompson et al., 2008). *P. thornei* population densities vary from 20 to 160 nematodes/g soil depending on the crops grown, plant growth stage, soil type, and climate. The nematode seems to be in abundance in regions where a susceptible or a tolerant host is planted continuously for many years (Thompson et al., 2008).

1.3 Current nematode control strategies and their limitations

Although use of chemical nematicides is one important nematode management tactic, the high price of nematicides, unwanted resistance development and ban on many of the chemical nematicides from the world markets due to their adverse effects on the environment, has shifted PPN control to the use of combination of alternative control strategies. Damage due to PPNs including *Pratylenchus* spp. can be mitigated using cultural practices such as crop rotation, host plant resistance, cover crops, irrigation management, fallow periods, and soil amendments. Most efforts to control *Pratylenchus* nematodes is by the use of prophylactic measures since once present they are difficult to control. All factors governing the choice of nematode management depend upon the timely and accurate diagnosis of the species and population levels from the soil and plant roots in infested areas. The damage threshold amongst *Pratylenchus* spp. and host crops varies greatly as it depends on geographic location, the value of the crop and possible potential disease complexes.

Unfortunately, most of the available nematode control strategies fail to control *Pratylenchus* nematodes, for several reasons. As available resistance to *Pratylenchus* is only moderate and limited to a few cultivated crops, this makes breeding for resistance difficult for this nematode species. The wide host range in both the dicots and monocots limits the use of crop rotation using non-host crops. Biological control using antagonistic microbes and some soil fungi can provide good control in laboratory tests but may not be a viable option in field conditions. The

microscopic nature of the nematodes can defeat some effective management practices such as proper sanitation and prevention of introduction into fields and planting materials (Castillo and Vovlas, 2007; Jones et al., 2011). Additionally, although nematodes can be controlled using a combination of cultural practices these integrated approaches are labour and time intensive and may not be economically viable. Therefore, the best strategy for achieving successful nematode control would be to have nematode resistance introduced in crops using genetic engineering. This could be in the form of enabling control of multiple species of nematodes and other pests of a crop (Dutta et al., 2014; Jones and Fosu-Nyarko, 2014).

1.4 Genetic engineering as a potential crop protection strategy against PPNs

There is an increasing need of food for human and livestock, and it is already a challenge to feed the rising human population, against a background of plant pests and diseases, climate change and uncertain economic trends. The application of agricultural biotechnology and genetic engineering are already helping to address these issues by improving production efficiency, better disease resistance, increasing market focus and enhancing environmental conservation. Genetic engineering can increase the diversity of genes and germplasm available for incorporation into crops and also shorten the time required to produce new improved varieties and hybrids. Genetically modified (GM) crops can have required agronomic traits, enhanced and well-balanced nutrition, and herbicide tolerance for a better weed control, high yields, pest and diseased resistance (Gasser and Fraley, 1989; James, 2010).

GM crops are now being grown in 28 countries with around 180 million hectares planted globally. Indeed, they now account for more than 10% of all crops grown (ISAAA). Crops such as tomato, cotton, soybean, wheat, rice, eggplant, maize, canola, potato, squash, papaya, alfalfa, sugarbeet, sweet pepper, poplar, petunia, sweet pepper, carnation occupy most of the GM area with many other crops awaiting release (Beyer et al., 2002; Gasser and Fraley, 1989; James, 2010). Every year new traits are being introduced in new GM crops since they were first grown economically in 1996, traits such as RR® herbicide tolerance in sugar beet, soybean, *B. thuringiensis* (*Bt*) toxin expressing cotton, maize, eggplant and poplar, enhanced beta-carotene or vitamin A production in golden rice and non-browning Arctic® apples are some examples. There have been tremendous economic gains from the use of GM crops globally

since 1996 to present date, with reductions in production costs, pesticide usage, land usage, and CO_2 emissions (Carpenter, 2010; Gasser and Fraley, 1989; James, 2010).

Genes can be introduced into plant chromosomes using several methods, such as *Agrobacterium*-mediated plant transformation, bombardment of DNA-coated microparticles, electroporation, chemical-assisted introduction into protoplasts, gene transfer into pollen, direct injection into reproductive organs, microinjection into immature embryo cells, and rehydration of desiccated embryos (Gasser and Fraley, 1989). Of these, *Agrobacterium*-mediated plant transformation is the most widely used.

Therefore, the introduction of nematode resistance in crops using 'synthetic' resistance genes may provide a better alternative when conventional means of nematode control are not effective, too expensive or not available at all (Atkinson et al., 2003; Grundler, 1996). Nematode resistant transgenic development involves cloning and transfer of naturally occurring plant resistance genes, toxins, or RNAi triggers. It also involves the study of regulation and expression of the transferred gene(s) together with considerations of cost of development and stability under intensive conditions and any effects on the environment. The *Mi* gene confers commercially available resistance against important RKN species, *M. incognita*, *M. javanica*, and *M. arenaria* in tomato. Similar to the *Mi* gene other single dominant genes conferring resistant against potato cyst nematodes (*G. rostochiensis* and *G. pallida*) and sugar beet cyst nematode (*H. schachtii*) have been mapped in potato and sugar beet, respectively (Grundler, 1996).

As co-evolved natural resistance against PPNs tends to be on a 'gene-for-gene' basis it does not offer complete protection against all isolates or races of pest or diseases. The introduction of 'synthetic' gene products from outside the usual plant-nematode interaction may prove to be a more effective generic approach against PPNs (Grundler, 1996). Introduction of genes encoding certain proteins such as *B. thuringiensis* (*Bt*) toxins and protease inhibitors that may be lethal to PPNs after ingestion is one such approach. Similarly, use of transgenics with genes encoding substances that can cause lethal effects in nematodes without their ingestion could be very important in PPN control. Such gene products for example, collagenase can be used to target nematode cuticle collagen *via* transgenic plants to impair its development or

components that disturb the amphids so that nematodes cannot find their way. Nematode cuticle and stylet secretions are highly immunogenic at very low concentrations and hence can be used for developing plants expressing genes of secretion-specific monoclonal antibodies (plantibodies) thus inhibiting nematode invasion (Grundler, 1996; Schots et al., 1992). However, at present, the best transgenic approach would to use RNAi, a naturally occurring phenomena with a good pest management potential, this approach has shown promise in conferring RKN resistance in transgenic tobacco and *Arabidopsis thaliana* plants producing dsRNA (Gheysen and Vanholme, 2007; Huang et al., 2006a; Yadav et al., 2006).

1.5 RNA interference (RNAi)

RNAi is a naturally-occurring highly conserved gene regulatory mechanism that regulates the transcript levels of gene(s) through sequence-specific RNA degradation/posttranscriptional gene silencing (PTGS). Ground-breaking RNAi/PTGS events were first observed in plants and later reported in almost all eukaryotic organisms such as invertebrates, vertebrates, algae, fungi and in some protozoa. RNAi has three phenotypically different but mechanistically similar forms known as co-suppression or PTGS in plants, quelling in fungi and RNAi in the animal kingdom (Agrawal et al., 2003). This well-studied mechanism of gene regulation or silencing has been used as a powerful functional genomics tool to investigate gene function via loss of function studies in many organisms such as *C. elegans* (Fire et al., 1998; Ketting and Plasterk, 2000), *Drosophila melanogaster* (Elbashir et al., 2001b; Hammond et al., 2000) and *A. thaliana* (Xie et al., 2004).

1.5.1 History of RNAi

The history of RNAi is an account of scientists interpreting unexpected results of their experiments in a new and imaginative way. The phenomenon of RNAi was first reported by Napoli and Jorgensen in 1990, whilst investigating whether chalcone synthase (CHS), one of the key enzymes in flavonoid biosynthesis pathway, was a rate-limiting enzyme in the anthocyanin biosynthesis pathway in Petunia flowers (Napoli et al., 1990). This anthocyanin biosynthesis pathway was responsible for the deep violet colour in petunias; therefore to generate violet petunias, the scientists over-expressed the chalcone synthase gene only to get unexpected white petunias instead of deep violet. They observed highly reduced levels (50-

fold) of endogenous as well as introduced CHS in comparison to wild-type petunias, which led to the hypothesis that the introduced transgene causes co-suppression of the endogenous CHS gene (Napoli et al., 1990).

Later in 1995, Guo and Kemphues during their study of *par-1* a gene required for establishing the polarity in *C. elegans* embryos reported some confusing observations during the silencing of the gene using antisense RNA. Injection of *C. elegans* with antisense RNA reduced the function of *par-1*, whilst injecting with sense RNA resulted in blocking the *par-1* expression, suggesting its role in base pairing with endogenous target mRNA thereby leading to inhibition of translation (Guo and Kemphues, 1995). These observations were better explained by Fire et al. in 1998 during their study of locomotion and muscle regulatory genes such as *unc-22* in *C. elegans* (Fire et al., 1998). In this study, the authors observed that injecting sense and antisense single-stranded RNA molecules could disrupt the expression of target endogenous mRNA. They also observed that only a few molecules of dsRNA were required for a greater impact on gene silencing. Their findings indicated that interference is not triggered by dsRNA corresponding to the intronic regions or sequences, dsRNA injection resulted in significant silencing of the corresponding endogeneous gene, this silencing effect due to interference was systemic and could spread from gut to the other body parts and also through the germ-line into many progenies. This process of interference was also observed after transcription in the cytoplasm (Fire et al., 1998).

Since the discovery of RNAi in *C. elegans* RNAi has been used successfully as a tool for functional genomics (Fire et al., 1998) including its application to study the function of nematode genes and its potential as a strategy to control nematodes and other crop pests (Bakhetia et al., 2005b; Lilley et al., 2007).

1.5.2 Molecular mechanisms of RNAi

RNAi is a natural mechanism by which eukaryote cells regulate gene expression (Fire et al., 1998). RNAi is initiated within a eukaryotic cell when a dsRNA is detected and consequently cleaved by RNase III enzyme, Dicer into 21 to 24 bp RNA duplexes called small interfering RNAs (siRNAs). SiRNAs/primary siRNAs are guided to the multicomponent RNA induced silencing complex (RISC). The RISC participates in strand separation and after incorporation of the

antisense strand, is guided to the target mRNA which is degraded *via* endonucleolytic cleavage by Argonaute family proteins (Figure 1.3) (Lilley et al., 2012). In plants and nematodes, RNA silencing is further amplified by ATP-dependent synthesis of a distinct class of secondary siRNAs involving RNA-dependent RNA polymerases (RdRPs) using the target mRNA as a template (Lilley et al., 2012). High silencing efficiency has been attributed to the higher number of secondary siRNAs (compared to primary siRNAs) and their association with CSR-1, a specific class of Argonaute proteins with a prominent slicer activity (Aoki et al., 2007; Lilley et al., 2012).

The RNAi process is initiated upon the introduction of double-stranded RNA (> 300 bp) into the cell by microinjection or feeding, followed by its recognition by the dicer (Step 1 in Figure 1.3). Dicer uses an ATP-dependent process to cleave the dsRNA into the 21 to 24 bp duplexes siRNAs. These short duplexes display overhangs of two nucleotides (nt) at the 3' ends (Step 2 in Figure 1.3). This siRNA can be transmitted systemically by an interaction with the SID protein, a product of *sid-1* (Step II in Figure 1.3). The siRNA duplex forms a complex with the RISC a nuclease protein (Step 3 in Figure 1.3). This duplex is unwound in a further ATP-dependent step to give single-stranded (ss) siRNA and leads to activation of the RISC (Step 4 in Figure 1.3). At this stage, the release of ss siRNA may result in RNAi effect amplification (Step II in Figure 1.3). By using a base-paring mechanism, the activated RISC searches for homologous mRNA transcripts and cleaves the mRNA ~12-nt from the 3'-end of the siRNA (Step 5 in Figure 1.3) leading to mRNA degradation (Step 6 in Figure 1.3). In *C. elegans*, there is an amplification cycle carried out by RNA-dependent RNA polymerase, RdRP, a product of *ego-1* (Step I in Figure 1.3). During this process, ss siRNA molecules originating from the dissociated dsRNA or released from the activated RISC act as primers on the complementary mRNA and leads to the production of new dsRNA molecules which become a substrate for dicer (Step 1 in Figure 1.3) (Bakhetia et al., 2005b).

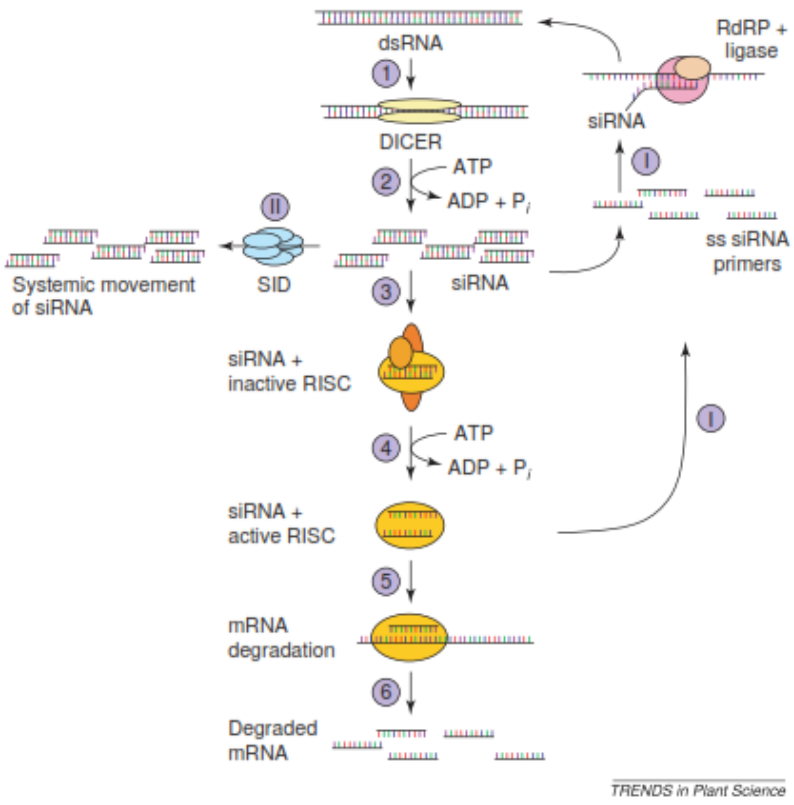


Figure 1.3. Diagrammatic representation of RNAi mechanism in *C. elegans*. Source: Bakhetia, et al. 2005.

1.5.3 Small RNA pathways

Small RNAs are classified as short interfering RNAs (siRNAs), micro RNAs (miRNAs) and piwi-interacting RNAs (piRNAs) based on their precursors. The length of siRNA and miRNA is mostly in the range of ~21 to 25 nt whereas, piRNA is ~24 to 31 nt long, with reports of 21 nt long piRNAs called 21U-RNAs found in *C. elegans* (Xue et al., 2012). These small RNAs have different production pathways but share a common mode during the RNAi processes and their effects across vertebrates, insects, nematodes, fungi and plants (Carthew and Sontheimer, 2009; Tang, 2005; Xue et al., 2012). A diagrammatic representation of the small RNA pathways in *C. elegans* is shown in Figure 1.4 (Hoogstrate et al., 2014).

The RNAi mechanism can decribed as two stages; initiation and effector. The initiation step is characterised by type III endonuclease Dicer-mediated siRNA and miRNA generation. SiRNA is synthesised from an exogenous long dsRNA of natural or artificial origin whilst miRNA is

synthesised from short hairpin precursor miRNA (pre-miRNA). During this process, pre-miRNA and dsRNA are cleaved into short RNA duplexes ranging from 21 to 25 nt by dicers which are large multidomain RNase III endonuclease enzymes (Agrawal et al., 2003; Das et al., 2011; Hannon, 2002). Dicer proteins play a key role of dsRNA recognition and processing; dicer cleaves long dsRNA into small RNA duplexes that have 3' 2-nt overhangs, bearing 5' phosphate and 3' hydroxyl termini (Elbashir et al., 2001a). Dicers exist in different forms or classes; in plants there are four classes of dicers, in nematodes (*C. elegans*), mouse and humans one class of dicer, whilst *Drosophila* has two dicers, *dcr-1* and *dcr-2* (Bernstein et al., 2001).

Dcr-1 and *dcr-2* share a structural homology, despite differences in ATP requirements and substrate specifications (Jiang et al., 2005). The dicer in *C. elegans* is a 210 kDa protein involved in exo- and endo-RNAi and in the miRNA pathways (Ketting et al., 2001). The *C. elegans dcr-1* helps in gene regulation which in turn helps the nematode to control its innate immune response against pathogens and stress from several factors (Welker et al., 2007). Unlike the *Giardia intestinalis* dicer protein crystal structure where the PAZ domain binds to the 3' end of dsRNA, the dicer itself acts as a 'ruler', starting from the PAZ domain during processing of long dsRNA into small RNAs (MacRae et al., 2006). *C. elegans* requires up to six effectors (*drh-1, drh-2, drh-3, pir-1, rde-1 and rde-4*) to process the dsRNA trigger (Duchaine et al., 2006).

MiRNAs comprise of a large gene family of noncoding RNAs found across animals, plants, and viruses. MiRNAs base-pair with the target mRNAs acting as guide molecules leading to translational repression and or mRNA cleavage. Studies have shown that miRNAs play key roles in diverse regulatory pathways, including development of timing control, cell proliferation, apoptosis, organ development and hematopoietic cell differentiation (Bartel, 2004). MiRNAs and their targets form very complex regulatory networks such that a single miRNA can bind to and regulate several mRNA targets, and conversely several different miRNAs can bind to and jointly control a single mRNA target. To understand the complex networks operated by miRNAs, it is important to understand the regulation of miRNA genes themselves.

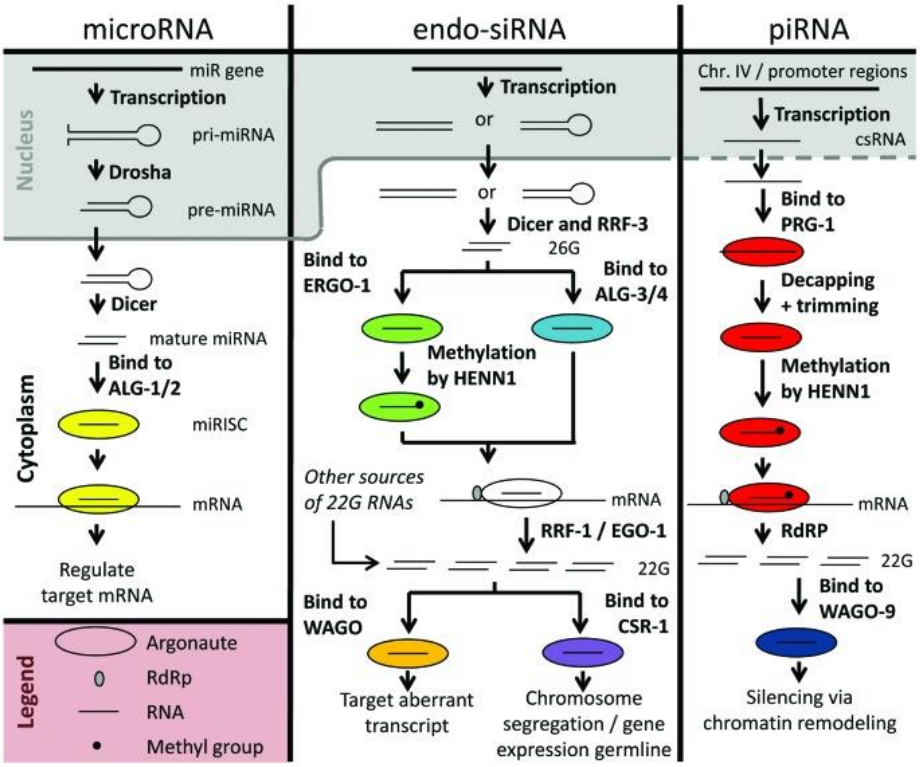


Figure 1.4. Small RNA pathways in *C. elegans*. In the piRNA pathway, the dashed line between nucleus and cytoplasm indicates that it still unknown when the piRNA enters the cytoplasm. Whilst a solid line for the miRNA and the endo-siRNAs indicate exactly when they enter the cytoplasm. The miRNA pathway is initiated by the transcription of miRNA genes, whilst siRNA pathway is initiated by dsRNA molecules and piRNAs of maternal origin initiate the piRNA pathway. Source: Hoogstrate et al. (2014).

The majority of the miRNA genes are located in the intergenic regions or in an antisense orientation to annotated genes, which indicates that they form independent transcription units (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Mourelatos et al., 2002). Many miRNA genes are found in the intronic regions and may be transcribed as a part of the annotated genes (Lee et al., 2002). The nuclear RNAse III drosha (Kim, 2004; Lee et al., 2003) and the dsRNA-binding protein (dsRBD) pasha cleave the ~70 nt long primary transcripts (pri-miRNA) into hairpin-shaped pre-miRNA (Lee et al., 2004). The intermediate, pre-miRNA is exported into the cytoplasm from the nucleus by a member of the Ran-dependent nuclear transport receptor family called exportin 5/Ran GTP after which it is cleaved by the cytoplasmic RNAse III dicer, *dcr-1* into 21-22 nt miRNA duplex (Bohnsack et al., 2004; Kim, 2004; Lund et al.,

2004; Yi et al., 2003). An unknown nuclease degrades one strand based on the internal stability of the two ends of the short-lived duplex whilst the other strand remains as a mature miRNA (Khvorova et al., 2003; Schwarz et al., 2003). ATP-independent enzyme, *dcr-1* along with its co-factor dsRBD protein called *Loquacious* (loqs) or *R3D1-L* (long) selectively cleaves near the loop of the hairpin pre-miRNA (Jiang et al., 2005; Lee et al., 2004).

In the second or the effector step of RNAi the guide strand is incorporated into the RNA inducing silencing complex (RISC). The RISC is composed of a core protein called argonaute (*ago*), this protein is a multi-member protein with several functions like cleavage of RNA. Argonautes are members of a large gene family encoding proteins characterised by a central PAZ (Piwi/Argonaute/Zwille) domain and a C-terminal piwi domain, and members of this protein family are essential components of the RISC (Rehwinkel et al., 2006). These proteins are involved in a number of processes such as small RNA pathways, chromatin modification and determination of the fate of the stem cells (Carmell et al., 2002). Five *ago* paralogs are encoded in the genome of *Drosophila melanogaster*, of which *ago-1* and *ago-2* are directly involved in the RNAi pathways *via* RISC, whilst there are 20 and eight in *C. elegans* and humans, respectively (Carmell et al., 2002; Cerutti et al., 2000; Hammond et al., 2001; Meister and Tuschl, 2004). Mature miRNA is delivered to *ago-1* containing RISC in the miRNA pathway. During the process, the miRNA is eliminated and the guide miRNA helps in the cleavage of the homologous mRNA (Jaubert-Possamai et al., 2010).

Exogenously introduced dsRNA or viral dsRNA is cleaved by a dsRNA specific RNase III family ribonuclease dicer, *dcr-1* in *C. elegans* and *dcr-2* in insects along with *loqs* to generate ~21 nt siRNA in an ATP-dependent catalytic step. In insects, the *dcr-2* and the dsRBD protein, R2D2 form a complex which delivers the duplex siRNA to *ago-2* containing RISC, where the *ago-2* converts the pre-RISC to holo-RISC by removing the passenger strand (Vodovar and Saleh, 2012). After the removal of the passenger strand, the holo-RISC contains only the guide strand whilst the pre-RISC contains siRNA duplex (Das et al., 2011). The 5'-end of the siRNA helps in target recognition during the RNAi mechanism (Matranga et al., 2005). The passenger strand is eliminated whilst the RISC recognises the 3' end 2-nt overhang of the guide strand leading to its binding and unwinding from the 5' end to form a single-stranded RNA, which in turn guides

the RISC for target RNA degradation (Bakhetia et al., 2005b; Chiu and Rana, 2002; Das et al., 2011; Hannon, 2002; Jaubert-Possamai et al., 2010; Kennedy et al., 2004).

In *C. elegans*, during the above process, the ss siRNA molecules generated from the dissociated dsRNA are released from the activated RISC and act as primers on complimentary mRNA leading to production of new dsRNA molecules which act as substrate for dicer. This process is amplified by an *ego-1* gene product called RdRp (Bakhetia et al., 2005b). During amplification, distinct secondary siRNAs showing the presence of di- or tri-phosphates at their 5' end unlike the mono-phosphate bearing primary siRNAs are generated from individual RdRp events (Pak and Fire, 2007; Sijen et al., 2007).

The phenomenon of RNAi amplification known as 'transitive RNAi' is present in plants and fungi, but found to be absent in *Drosophila* and mammals. In *C. elegans*, seven genes, *ego-1*, *rrf-1*, *smg-2*, *smg-5*, *smg-6*, *rde-10* and *rde-11* encode proteins that are involved in siRNA amplification (Sijen et al., 2007; Smardon et al., 2000; Zhang and Ruvkun, 2012). Of these genes, *ego-1* is required for efficient germline RNAi response whilst *rrf-1* is required for efficient RNAi in somatic cells suggestive of distinct roles associated with genes that are only 0.9 kb apart in tandem orientation and very closely linked in the genome of *C. elegans* (Sijen et al., 2001; Smardon et al., 2000). Similarly, the genes *smg-2*, *smg-5* and *smg-6* (Suppressor with Morphological effect on Genitalia) play a role in degrading defective mRNAs, which code for toxic protein fragments and nonsense mediated mRNA decay (Johns et al., 2007; Pulak and Anderson, 1993). Likewise, *rde-10* and *rde-11* genes are required for accumulation of secondary siRNAs within the endo and exo-RNAi pathways where they form a complex that interacts with partially degraded target mRNAs (Zhang and Ruvkun, 2012).

The synthesis of piRNAs (Piwi-interacting RNAs), amongst all the small RNAs is the least understood. It is independent of dicer enzymes and requires piwi proteins. It is processed from the single-stranded primary transcripts that are transcribed from defined genomic regions rather than the dsRNA (Vodovar and Saleh, 2012). These small noncoding RNAs have been found to regulate gene expression at both transcriptional and post-transcriptional levels and are involved in silencing of transposable elements in animal gonads (Brennecke et al., 2008; Lin, 2007). In *D. melanogaster*, proteins such as *ago-3*, *aubergine* and *piwi* are involved in the

processing of piRNA and play a role in chromatin modification within the nucleus (Aravin et al., 2007; Aravin et al., 2003; Brennecke et al., 2008; Olivieri et al., 2010). Putative nuclease Zucchini, RNA helicase Armitage, and the Tudor domain containing RNA helicase Yb are essential factors for primary piRNA biogenesis in the *in vivo* assay conducted for studying the somatic piRNA pathway in *D. melanogaster* (Olivieri et al., 2010).

1.5.4 Natural functions of RNAi

RNAi plays a crucial role in many biological processes: development, formation of heterochromatin, maintenance of genomic stability, and defence mechanism against viruses and transposable elements (Bartel, 2004; Hannon, 2002; Meister and Tuschl, 2004). In *C. elegans*, *dcr-1* and *ego-1* have shown to play a role in proper germ-line development (Knight and Bass, 2001) Similarly, the RNA deficient (rde) mutant, *rde-1* and *rde-4* play a role in silencing transposable elements in the germ-line (Sijen and Plasterk, 2003). Another mutator gene, *mut-7* has shown to play a role in the silencing of the germ-line transposon *Tc1* that is present in abundance in all the natural isolates of *C. elegans* (Ketting et al 1999).

1.5.5 Inhibitors of RNAi

RNAi inhibitors act in contrast to the process of RNAi amplification and there are about 13 known genes in *C. elegans*. The enhanced RNAi phenotype genes (*eri-1*, *eri-3*, *eri-5*, *eri-6*, *eri-7*, *eri-9*) negatively impact RNAi, for example, *eri-1* expressed in neurons of *C. elegans* specifically use siRNA as their substrate (Kennedy et al., 2004). Similarly, mRNAs of *eri-6* and *eri-7* form the RNAi inhibitor eri-6/7 that encodes for a helicase which has a function in the exogenous and endogenous RNAi pathways. Another group of RNAi inhibitors are genes (*lin15b*, *gfl-1* and *zfp-2*) that encode the RdRp family protein such as *rrf-3* which competes for templates or primers in RNAi amplification system there by inhibiting the production of additional short RNAs (Meister and Tuschl, 2004; Simmer et al., 2003). The adenosine deaminase acting on RNAi genes (*adr-1*, *adr-2*) reported in *C. elegans* function in RNAi editing by removing the complementarity between dsRNA and the target mRNA. This results in destabilisation of the edited dsRNA which yields into a poor substrate for the dicer (Knight and Bass, 2001). In *C. elegans*, XRN ribonuclease related gene *xrn-1* is involved in the degradation of mRNA in P-bodies and *xrn-2* in mature miRNA resulting in the regulation of miRNA homeostasis (Chatterjee and Großhans, 2009; Maule et al., 2011; Muhlrad et al., 1994).

1.6 RNAi in PPNs

The discovery of gene silencing *via* RNAi in *C. elegans* (Fire et al., 1998) along with the knowledge that RNAi is a widespread eukaryotic phenomenon gave the first opportunity to use reverse genetics as a tool to determine gene functions in parasitic nematodes (Kuwabara and Coulson, 2000). There are several ways of deploying *in vitro* RNAi as a tool for functional analsysis as was demonstrated in the model nematode *C. elegans* and a few economically important PPN species. There are three ways used to deliver the dsRNA for induction of RNAi in *C. elegans*: microinjection, soaking which involves oral uptake of dsRNA from solution, and feeding on transformed bacteria expressing dsRNA (Fire et al., 1998; Gouda et al., 2010; Tabara et al., 1998; Timmons and Fire, 1998). Similarly effective RNAi based control and functional assays of vital PPN genes in crop plants can be achieved by *in planta* expression of dsRNA homologues of the vital gene essential for nematode survival (Bakhetia et al., 2005a).

1.6.1 *In vitro* RNAi in PPNs

Culturing, maintaining and conducting RNAi based functional assays using *in vitro* feeding and soaking of PPNs is a challenging task due to their obligate nature. However, a significant breakthrough came to light when Urwin et al., (2002) first reported uptake of dsRNA solution conatining homologues of CN genes encoding a cysteine proteinase and C-type lectin along with a neurochemical octopamine. Urwin et al. (2002) successfully demonstrated delivery of dsRNA to J2 of CNs by using soaking solution containing a neuroactive compound called octapamine which induces pharyngeal pumping, resulting in uptake of the dsRNA. In this experiment, there was reduction in target gene transcript abundance followed by a phenotypic effects in both *G. pallida* and *H. glycines.* Subsequently, Lilley et al. (2005) and Sunko et al. (2007) also demonstrated the amenability of *in vitro* RNAi through dsRNA soaking solution in *H. glycines* (Lilley et al., 2005b; Sukno et al., 2007).

RNAi is a practical tool for gene knockout and 'loss of phenotype' studies in many organisms and this has been achieved in various ways, including microinjection, soaking, feeding bacteria expressing dsRNA and recently plants expressing vital nematode dsRNAs (Fire et al., 1998; Mitchum et al., 2013; Sharma, 2016; Timmons and Fire, 1998). Application of RNAi to certain parasitism genes has helped in understanding the role of the gene and its product such as effector proteins during plant host invasion.

In vitro RNAi targeted silencing of dual oxidase genes in *M. incognita* revealed the important role of extracellular matrix associated peroxidase and NADPH oxidase in the parasitic ability of nematodes (Bakhetia et al., 2005a). It has been observed that when nematodes form a long-term feeding relationship by formation of feeding cells, they use ligand mimicry of peptide signaling molecules which assist in altering plant cell biology. During cyst nematode infections a well-known family of CLAVATA3 (CLV3)/Endosperm Surrounding Region-related (CLE) peptide ligands appears to be primarily targeted and there are reports of the presence of CLE-like genes in several *Heterodera* and *Globodera* spp. (Lu et al., 2009; Patel, 2008; Wang et al., 2010a; Wang et al., 2010b). The nematode CLE-like genes encoded proteins possess an N-terminal secretion signal peptide and either a single or multiple conserved C-terminal CLE domains(s) separated by a domain varying in length and amino acid sequence which characteristically resemble plant CLE sequences. This variable domain in nematode CLEs exhibits a bipartite structure harbouring a unique protein trafficking function *in planta* (Wang et al., 2010a). Also, the sequences outside the CLE motif may likely contribute to CLE function and functional specificity *in vivo* (Meng et al., 2010). Cyst nematode secreted CLEs are probably delivered to the cytoplasm of feeding cells and would require protein trafficking to the apoplast in order to mimic plant CLEs which bind to extracellular receptors (Wang et al., 2010b). Mis-expression of the nematode CLEs in wild type Arabidopsis caused shoot and root meristem termination similar to mis-expressed plant CLEs and could compliment the Arabidopsis *clv3* mutant phenotype of enlarged shoot and floral meristem (Wang et al., 2010b).

All these data suggest posttranslational *in planta* modification and processing of nematode CLEs very similar to plant CLEs (Wang and Fiers, 2010). As with most plant CLE ligand-receptor pairs, nematode CLEs might interact with the receptors in the extracellular space (Hirakawa et al., 2008; Ogawa et al., 2008). A CLAVATA2 and CORYNE a heterodimeric leucine-rich repeat receptor-like kinase complex with role in maintenance of root and shoot meristem has been reported to play a role in nematode CLE signaling (Replogle et al., 2011). Most functional CLEs have been found in CNs and there are also reports of CLE-like sequence called 16D10 bearing similarity of plant CLEs in RKNs (Huang et al., 2006b). However, the 16D10 lacks a variable domain unlike the CN CLEs with mis-expression stimulating root growth in wild-type Arabidopsis (Huang et al., 2006b). The 16D10 was not able to rescue *clv3* mutant and its functional role as plant CLE ligand mimic needs further investigations. Similarly, protein

products of the *Hg-syv46* gene functionally mimic CLE family of *A. thaliana* that help in maintaining balance during meristem cell proliferation and differentiation, this gene may disrupt the normal pattern of cell differentiation at the feeding site (Bakhetia et al., 2007).

RNAi has helped to understand how SKP-1 or Ring-H2 and ubiquitin-like proteins may help the nematodes in gaining a parasitic advantage by regulating host cell protein degradation (Sindhu et al., 2009). Similarly, the role of β-1-4 endoglucanases in invasion and migration of *H. glycines* and *G. rostochiensis* by modifying plant cell walls has been demonstrated using RNAi (Bakhetia et al., 2007; Chen et al., 2005b). RNAi of *M. incognita* and *H. glycines* cysteine proteinase genes revealed their importance during host invasion by decreasing the nematodes establishment on to host roots. The essential parasitism gene 16D10, that encodes a conserved RKN secretory peptide which stimulates root growth and also functions as a ligand for the generation of a putative plant transcription factor, has been characterised using RNAi and may help in development of resistance against major RKN spp. (Huang et al., 2006a). RNAi treatment of *H. glycines* shows how macrophage mannose receptor and proteoglycan core proteins like aggrecan the two lectin domains of C-type lection, that have sequence homology with cobra venom coagulation factor, are important during parasitism (Urwin et al., 2002). Venom allergen-like proteins (VAPs) are a conserved group of secreted proteins in APNs and PPNs. VAPs in PPNs are secreted into the apoplast of host cells to selectively suppress host immunity mediated by surface-localised immune receptors (Lozano-Torres et al., 2014). Silencing of the *Gr-VAP1* in *G. rostochiensis* resulted in reduced nematode infectivity. Similarly, silencing of *Hs-VAP1* and *Hs-VAP2* in *H. schachtii* enhanced the susceptibility of *A. thaliana* to multiple unrelated plant pathogens. Ectopic VAPs in *G. rostochiensis*, *H. schachtii* and *M. incognita* modulate basal immunity of *A. thaliana* by regulating extracellular protease-based host defences and defence-related programmed cell death initiated non-photochemical quenching. It is likely that the VAPs are delivered by the migratory stages of CNs as well as cell wall-modifying enzymes, into the apoplast of host cells, thereby modulating host responses that are triggered by the release of immunogenic fragments of damaged plant cell walls (Lozano-Torres et al., 2014).

RNAi knockdown of a *G. rostochiensis* gene for amphid protein reduced the host location and invasion ability of the nematode (Chen et al., 2005b). RNAi-based functional analysis shows

that *Mi*-gsts-1 is expressed in the oesophageal secretory glands and the secretion of glutathione S- transferases (GSTs) is essential for completion of nematode life cycle within the host (Dubreuil et al., 2007).

Similarly, in the past few years several researchers have successfully used RNAi as a characterisation tool to study different phytonematode genes involved in nematode development and mRNA metabolism (Iqbal et al., 2016; Li et al., 2011a). Amenability of RNAi *via* soaking of two RLN spp., *P. thornei* and *P. zeae* has been demonstrated by silencing of *pat-10* and *unc-87* genes responsible for muscles/movement resulting in significantly reduced reproduction after dsRNA treatments (Tan et al., 2013). This data validates the hypothesis that *in vitro* and host-derived RNAi are both viable methods for functional characterisation of selected putative RLN effector genes.

1.6.2 *In planta* RNAi in PPNs

Use of plant hosts to deliver dsRNA to the parasitic nematode stages *via* their feeding site has been an valuable tool to achieve host-derived resistance agianst RKNs and CNs (Huang et al., 2006a; Yadav et al., 2006). In host-mediated RNAi, plants are first transformed to express ds mRNA sequences of target genes of the nematode, which are then delivered to the nematode in the form of dsRNA through feeding sites, leading to silencing of the homologous mRNA (Runo, 2011). The possiblity of developing a broad-range resistance mechanism against RKNs has been demonstrated through transgenic *A. thaliana* plants expressing hairpin RNA targeted at 16D10 parasitism gene resulted in upto 90% reduction in number of galls caused by *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Huang et al., 2006a). Similarly, *M. javanica* fed on transgenic tobacco plants expressing dsRNA of two house-keeping genes, splicing factor and integrase of *M. incognita* showed reduced parasitic ability (Fairbairn et al., 2007). RNAi as a tool for effective nematode control depends on two factors; first it is important to have a large amount of dsRNA at the site of delivery between the host and parasite, and second, is to have secondary amplification of the RNAi signal for efficient silencing (Fairbairn et al., 2007; Gheysen and Vanholme, 2007).

The amount of siRNA in target cells can be increased with the help of strong promoters such as 35S promoter from the cauliflower mosaic virus and the ATCT2 promoter from *A. thaliana*

(Fairbairn et al., 2007). Additionally, a strategy that employs the use of viral vectors or artificial amplicons also looks promising in the delivery of sufficient siRNAs to target cells. Virus-induced gene silencing (VIGS) may help overcome weak amplification due to insufficient siRNA within the host during *in planta* RNAi (Lipardi et al., 2001). This was demonstrated by the introduction of a nematode-specific sequence into non-deleterious virus-based vectors such as tobacco rattle virus (TRV) which allows ample production dsRNA trigers within plant cells (Valentine et al., 2004; Valentine et al., 2007). Non-target effects of dsRNA expression in transgenic plants developed to control a nematode gene *via* transgenic plants tend to be minimal. This is because dsRNA molecules used cannot produce a functional protein, their expression can be targeted towards specific nematode feeding sites, the dsRNA sequences are strictly specific to the target pathogen and not the host plant, and generally do not affect non-target speices that would feed on the transgenic plants (Bakhetia et al., 2005b; Lilley et al., 2004).

There are over 40 reports of successful RNAi silencing reported in nine species of PPNs using dsRNAs or siRNAs in both *in vitro* and host-mediated RNAi (Lilley et al., 2012; Maule et al., 2011). Several studies involving the use of RNAi to understand gene functions in PPNs, including both sedentary and migratory nematodes that account for severe economic losses globally, have also been undertaken (Lilley et al., 2012; Maule et al., 2011). In PPNs, *in planta* RNAi studies have mainly been carried out on sedentary endoparasitic cyst and root-knot nematodes such as *M. incognita* (Bakhetia et al., 2005a; Dubreuil et al., 2011; Huang et al., 2006a; Papolu et al., 2013; Yadav et al., 2006), *M. graminicola* (Nsengimana et al., 2013), *M. artiellia* (Fanelli et al., 2005) , *M. javanica* (Adam et al., 2008; Fairbairn et al., 2007; Gleason et al., 2008; Hu et al., 2013), *G. rostochiensis* (Chen et al., 2005b; Rehman et al., 2009), *G. pallida (Urwin et al., 2002)*, *H. glycines* (Urwin et al., 2002), *H. schachtii* (Sindhu et al., 2009; Tsygankova et al., 2014) and comparatively fewer studies have been done on migratory endoparasites like *Bursaphelenchus xylophilus* (Park et al., 2008) , *Radopholus similis* (Haegeman et al., 2009; Zhang et al., 2015), *P. coffeae* (Joseph et al., 2012), *P. zea (Tan et al., 2013)*, *P. thornei* (Tan et al., 2013), *P. vulnus (Fanelli et al., 2014; Walawage et al., 2013), P. penetrans* (Vieira et al., 2015).

1.6.3 RNAi in *Pratylenchus* species

Amongst the migratory PPNs such as the RLNs, several studies have been used to demonstrate the amenibility of *Pratylenchus* spp. to *in vitro* and *in planta* RNAi. Amongst the *in vitro* RNAi studies done on *Pratylenchus* spp. two are pivotal in determining the amenability of RNAi in *Pratylenchus* spp. Joseph et al., (2012) first demonstrated the potential of RNAi by investigating the function of evolutionarily conserved genes, *unc-87* and *pat-10* in controlling *P. coffeae*, a devastating PPN of banana in the tropics (Joseph et al., 2012). Similarly, Tan et al., (2013) also carried out functional analysis of *unc-87* and *pat-10* genes in *P. zeae* and *P. thornei* and found RNAi to be a useful tool for their potential control. Recently, Fanelli et al., (2014) successfully demonstrated *in vitro* RNAi using endoglucanase gene, *Pv-eng-1* to control reproduction in *P. vulnus* (Fanelli et al., 2014). Currently, there are few published studies that have tested efficacy of various target genes, especially parasitism effectors, of *P. thornei* through *in vitro* RNAi providing scope for such investigations.

There are some interesting examples of *in planta* RNAi studies on *Pratylenchus* spp. Walawage et al., (2013) demonstrated the control of *P. vulnus* by up to 32% in transgenic walnut (*Juglans regia* L.) expressing *C. elegans* orthologue Pv010 of the spliceosome subunit having RNAi phenotypes of sterility and juvenile lethality (Walawage et al., 2013). More recently, Vieira et al., (2015, 2017) demonstrated *in planta* RNAi of two locomotion genes, *Pp-pat-10* and *Pp-unc-87*, and a fatty acid and retinol-binding *Pp-far-1* gene that resulted in reduction of *P. penetrans* population (Vieira et al., 2015; Vieira et al., 2017). In *P. thornei*, so far, there are no published papers of host-mediated RNAi. However, in her Ph.D. thesis, Tan (2015) demonstrated that silencing *pat-10* and *unc-87* in *P. thornei* using *Agrobacterium*-mediated transformation to generate carrot hairy roots expressing dsRNA to these genes lead to significant reduction in nematode reproduction and target gene expression (Tan, 2015). Hence, there is much scope for research to provide information on various target genes including parasitism effectors of *P. thornei* through successful host-mediated RNAi.

Various candidate genes have been investigated for their effects of RNAi silencing across PPNs. These target genes belong to different functional classes and silencing these genes has shown to reduce transcript levels, infections, plant invasion and establishment, impaired locomotion, and development (Bakhetia et al., 2005a; Bakhetia et al., 2007; Lilley et al., 2005a; Park et al.,

2008; Urwin et al., 2002). Until now, six research articles have been published that demonstrated the application of RNAi in *Pratylenchus* spp. In these investigations, the candidate genes that were targeted played a role in: metabolism, locomotion, reproductive ability and parasitism (Fanelli et al., 2014; Joseph et al., 2012; Tan et al., 2013; Vieira et al., 2015; Vieira et al., 2017; Walawage et al., 2013). Whilst each of these functional categories seem to affect or disrupt the normal life cycle of the nematode, the parasitism effectors seem to be an interesting class of candidates. This is because, to achieve success in plant invasion, a PPN has to circumvent active and passive plant defenses and hence relies on its plethora of effectors in achieving this. These effectors have the capacity to alter host cell structure and function, such as modulating host defence responses and also amplifying processes of the host cells during parasitism. Thus, they are of paramount importance in studies related to nematode control and are discussed below (Hogenhout et al., 2009; Mitchum et al., 2013).

1.7 Effectors

PPNs, bacteria, fungi and oomycetes, secrete proteins commonly known as effectors which are required for parasitism during host tissue invasion (Alfano, 2009). In general, effectors can be collectively defined as proteins and small molecules capable of altering host-cell structure and function (Hogenhout et al., 2009). Most of the nematode effector research has focused on cloning and characterising nematode parasitism genes encoding parasitism proteins/effector proteins that are secreted *via* its stylet. Although stylet-delivered effectors are the main focus of many investigations there are effector proteins that are produced in other regions of the nematode body such as the cuticle and amphids, which are the two chemosensory organs present in the nematode head. Because PPNs are biotrophic in nature, depending on their species, it is essential for them to feed and obtain nutrition for survival from the cytoplasm of either live unmodified plant cells or from elaborately modified plants cells in the form of discrete feeding structures such as giant cells and syncytia.

PPNs penetrate the plant cell walls using their hollow protrusible feeding stylet through which they deliver oesophageal gland secretions containing effector proteins into plant cells and withdraw nutrients from their cytoplasm (Hussey, 1989; Hussey et al., 2002; Rosso et al., 2011). The effector proteins are synthesised in the two sub-ventral and one dorsal glands, and their

secretory activity have evolved with PPNs evolution, providing them with the ability to exploit more plant species as food sources (Hussey, 1989). Effector proteins are synthesised in the nuclear region of the gland cells, with N-terminal signal peptides directing them towards a secretory pathway, where they are packaged into membrane-enclosed secretory granules or dense-core vesicles which then bud from the trans-Golgi network (Hussey, 1989; Hussey et al., 1994; Hussey and Mimms, 1990). The secretory granules are transported further through a microtubule network present in the cytoplasmic extensions of the glands. The granules reach the distal elaborate valves where the effector proteins are released into the end sac of the valve by exocytosis with their subsequent secretion into the host tissue *via* the nematode stylet (Mitchum et al., 2013). In PPNs, the genes and effectors related to parasitism are developmentally regulated during the parasitic cycle. Therefore, in most sedentary PPNs the sub-ventral glands are mostly expressed during early stages of parasitism, followed by the predominant expression of dorsal gland cells after the onset of parasitism and through the remainder of the parasitic life cycle (Mitchum et al., 2013). In contrast to the sedentary endoparasites, the migratory *Pratylenchus* spp. are devoid of granules in their sub-ventral glands whilst the dorsal glands exhibit many granules. There is no detailed study yet done to illustrate this variation in a migratory species (Jones and Fosu-Nyarko, 2014).

Although advances in molecular biology have helped to identify and enable functional characterisation of many nematode effector proteins, little is known about the actual process of their secretion, and packaging into secretory granules. Also, the knowledge of regulatory mechanisms governing the production of different effectors during the parasitic life cycle, and their co-production, specific effector triggering, packaging and release *via* the stylet is not yet clear and needs investigating.

Most studies of nematode effectors have focused on the economically important sedentary endoparasitic RKNs and CNs, regarded as exhibiting the most evolutionarily advanced mode of parasitism (Rosso et al., 2011). There are various migration patterns for entry into host plant tissue, RKNs exhibit stealth during their intercellular migration in host tissue, whilst forceful intracellular migratory PPNs, trigger a path of destruction which elicits a robust plant defence response. Changes and differences in gene expression within host roots caused by PPN infection strategies have been documented (Kyndt et al., 2008). There is an assumption that

PPNs could trigger plant defence responses as found for other plant pathogenic interactions, such as damage-associated molecular patterns (DAMPs) or conserved pathogen-associated molecular patterns (PAMPs) (Win et al., 2012).

During their migratory phase, PPNs secrete effectors that degrade or modify components of the plant cell wall, and others which dampen down the plant's immune system, thereby facilitating nematode penetration and migration (Smant and Jones, 2011). Whilst in their sedentary phase, PPNs become immobile and feed near the plant vascular system, during which time there is need for sustained suppression of the plant immune system to enable successful establishment of feeding sites and continued feeding. This is done by effector proteins which help in regulating plant defences and by cellular reprogramming to induce formation of metabolically highly active feeding cells on which the PPN relies for its sustained feeding required for growth and development (Jones, 1981; Jones and Goto, 2011). If the host plant carries a gene conferring nematode resistance the above interaction may culminate in an HR-like cell death of the feeding cell leading to the death of the associated PPN (Williamson and Kumar, 2006). Despite differences in the aetiology and evolutionary history of migratory (*Pratylenchus* spp.) and sedentary PPNs, there are some similarities in their secreted enzymes and effector repertoires. Many of these characterised effectors and protein are classified into groups based on their putative or known function such as their ability to modify cell wall, suppress and manipulate plant defence, alter plant cell biology and those with unknown functions. Many of these effector genes have been acquired within the nematode parasitome *via* a horizontal gene transfer from bacteria or fungi (Bird et al., 2015; Bird et al., 2003; Dorris et al., 1999).

1.8 Role of parasitism effectors in PPNs

Various PPN effectors and the roles they play during nematode infection are categorised briefly and discussed below.

1.8.1 Cell wall modifying enzymes or effectors

Plant cell wall (PCW) degradation or modification is a crucial function that is only present in PPNs, whilst being absent in free-living or animal-parasitic nematodes. The group of genes that

produce these PCW modifying enzymes or effectors enable PPN invasion, migration and PCW modification within the plant host tissues. These enzymes are collectively annotated as Carbohydrate Active Enzymes (CAZymes) and they include: cellulases, xylanases, polygalacturonases, pectate lyases and arabinases. Most of these CAZymes are assumed to be a product of the sub-ventral gland cells produced during the nematodes migratory phase, although they are also found during stationary phases of many PPNs during feeding site formation (Caillaud et al., 2008; Hewezi et al., 2008). Several of the CAZYmes have already been identified and characterised in many species belonging to the genera *Meloidogyne*, *Globodera*, *Heterodera*, *Radopholus*, *Bursaphelenchus*, *Rotylenchus*, and *Aphelenchus* (Gao et al., 2003).

Comparative bioinformatics analysis of transcriptomic data from *P. coffeae* and *P. thornei*, and the genomic data of *P. coffeae* have revealed the presence of most of the RKN and CN CAZymes classes in *Pratylenchus* (Haegeman et al., 2011b; Jones and Fosu-Nyarko, 2014; Nicol et al., 2012) (Fosu-Nyarko and Jones, 2016; Jones and Goto, 2011). Although PPNs can have cellulases or β-1,4-glucanases from all three CAZyme families glycosyl hydrolase 5 (GH5), GH45 and GH12, most of the data available for *Pratylenchus* confirms the presence of GH5 cellulases. The presence of *M. hapla* and *R. similis* β-1,4-endoglucanase sequences in *P. coffeae* transcriptome has been confirmed using comparative bioinformatics, similarly the *P. thornei* transcriptome exhibits some sequence similarity at the contig level to *M. hapla* and *R. reniformis* (Haegeman et al., 2011b; Jones and Fosu-Nyarko, 2014; Nicol et al., 2012). These CAZymes help in modifying the principal component cellulose within the plant cell wall during nematode invasion.

The pectate lyases *via* polygalacturonase (GH28 reported in RKN)-mediated hydrolysis cleave alpha-1, 4-galacturonan polymers which form pectin backbones within PCW have also been identified amongst *P. coffeae* and *P. thornei* transcripts (Abad et al., 2008; Haegeman et al., 2009; Jaubert et al., 2002; Popeijus et al., 2000a; Vanholme et al., 2009a; Vanholme et al., 2009b). Conservation of pectate lyases amongst *Pratylenchus* spp. has been demonstrated by Nicol et al., (2011) by isolating, cloning and comparing partial pectate lyase sequences of *P. thornei* (*PtPL3*) and *P. zeae* (*PzPL3*). They reported *PtPL3* and *PzPL3* RNA probe hybridisation to the mRNA present within the sub-ventral gland cells across the two *Pratylenchus* spp. (Nicol

et al., 2012). The study also revealed the possible secretion of pectate lyases by the nematodes into the plant cells or the apoplast, despite lack of granular content within the gland cells during mRNA hybridisation (Nicol et al., 2012). As the pectin backbone may be protected against enzymatic degradation, by side branches composed of arabinans and arabinogalactans, there is an evidence that along with the PL3 pectate lyase, candidate GH53 and GH43 arabinases may be also produced by the metazoan, *M. incognita* (Abad et al., 2008). Genomes of *M. hapla* and *M. incognita* contain multi-gene families of PL3 genes, whilst the GH5 family gene expansion may be due to tandem duplication.

Apart from cell wall modifying CAZYmes that appear to facilitate nematode entry and migration in plant tissue, there are other proteins/enzymes such as cellulose binding proteins (CBPs) and expansin-like proteins that may directly or indirectly play a role in cell wall modification, or cell growth required for feeding sites in the case of sedentary PPNs. Expansins dissociate non-covalent bonds that bind PCW components together and provide access to PCW-modifying enzymes that modify the PCW structure. Expansins and expansin–like proteins with matching CBPs have been reported in *G. rostochinensis*, *M. incognita*, *X. index*, *P. thornei* and *P. zeae* (Abad et al., 2008; Cosgrove et al., 2002; Danchin et al., 2010). CBPs with or without a signal peptide are also products of oesophageal glands of some PPNs and exhibit similarity to the ones secreted by fungi (Davis et al., 2000). CBPs may promote demethylesterification of pectin backbone and facilitate access of various PCW-modifying enzymes like xylanase (*R. similis*), cellulases, expansins and expansin-like proteins which could help in softening of the cell wall by disruption of non-covalent bonds between cell wall fibrils leading to its expansion and permeability to cell wall modifying enzymes (Cosgrove et al., 2002; Haegeman et al., 2009). *P. coffeae* and *P. thornei* secrete transcripts identical to xylanase, and polygalacturonases respectively, which hydrolyse xylans and 1,4-alpha-D-galacturonic linkages of pectic polysaccharides and galacturonans.

One intriguing feature of nematode plant cell wall modifying / degrading enzymes is their total absence or rarity in other metazoans, with the exception of some GH5 and GH28 polygalacturonases which have been occasionally identified in phytophagous insects (http://www.cazy.org/). A number of CAZymes from the GH subfamilies GH4, GH12 and GH45 have been identified in *Pratylenchus* spp., and the sequence similarity exhibited to bacteria and

fungi point towards horizontal/lateral gene transfer during evolution (Chen et al., 2005b; Davis et al., 2000; Fanelli et al., 2005; Jones et al., 2005; Kikuchi et al., 2005). Detailed phylogenetic analyses of the nematode CAZyme and CBM suggests multiple lateral gene transfer events within the phylum, as the nematodes behaving as acceptors of these genes from the donors such as bacteria and fungi that share its ecological niche (Danchin et al., 2010). There is one CAZyme (beta, 1, 3- endoglucanase) identified in the *Pratylenchus* transcriptome which is not been reported in any sedentary PPNs but has similarities to many of the fungivorous nematode species (*B. mucronatus*, *B. xylophilus* and *A. avenae* (Karim et al., 2009; Kikuchi et al., 2005).

Unlike their sedentary counterparts, migratory PPNs do not induce complex differentiation of feeding cells, the most rational explanation for having the common types of cell wall modifying enzymes is that these effectors are involved in cell wall softening allowing the stylet to penetrate the cytoplasm or they directly aid nematode entry and migration by cell wall modification (Fosu-Nyarko and Jones, 2016; Jones and Fosu-Nyarko, 2014). Many PPNs also produce CAZymes such as invertases which may be involved in converting cell storage polysaccharides like starch or sucrose to glucose (Myers, 1965). In addition, since all invasive life stages of RLNs are mobile it is not surprising that unlike the sedentary PPNs, RLNs CAZYmes are required during all stages of parasitism (Jones and Fosu-Nyarko, 2014).

There are also hypotheses that in sedentary PPN (CNs) protein degradation and their influence on altering cell biology may involve mimicking host cell proteins *via* ubiquitin-mediated proteasome activities and production of C-terminal extension bearing ubiquitin peptide. Several genes encoding proteases are present in PPNs, and a few of these, namely aminopeptidases, and cysteine and serine proteases, have been detected in transcripts of *P. thornei* and *P. zeae* with the help of comparative bioinformatics, although their function in most PPNs has not been elucidated. So far, there is no data that show that proteases are secreted *vi*a the stylet of RLNs. Although some proteases may be secreted through the stylet into the host cells during feeding, others may be secreted into the digestive tract with a role in the digestion of ingested cell contents (Haegeman et al., 2012; Jones and Fosu-Nyarko, 2014).

1.8.2 Effectors manipulating plant defence

Compared to the sedentary PPNs where each host encounter offers an opportunity for the PPN to form a feeding site, migratory RLNs have additional options if the host is not amenable for continuation of their life cycles. Even though RLNs are migratory in nature they must still evade or suppress host defence responses like the sedentary PPNs. PPNs can evade or suppress host defences using characteristic molecules that are either present on their surfaces or secreted and released into the host cell apoplast. or are generated as a result of cell wall degradation during the nematode's interaction with its host. Such pathogen-associated 'molecular signatures', PAMPs are detected by the host plant cell receptors to initiate PAMP-triggered immunity (PTI) (Brown et al., 2006; Jones and Dangl, 2006; Kikuchi et al., 2006; Smant and Jones, 2011). Once the host recognises both PAMPs and DAMPs it elicits production of various enzymes and metabolites that may be toxic or anti-pathogenic. This type of plant defence reaction is observed when defence and stress-related genes are up-regulated after infection of different plant host by CNs, RKNs and RLNs (Fosu-Nyarko et al., 2009; Goto et al., 2013; Ithal et al., 2007).

Secondary metabolites such as peroxidases, polyphenol oxidase and phenylalanine ammonia-lyase are induced against migratory endoparasites such as *P. coffeae* by resistant banana cultivars as a result of defense-related expression (Devi et al., 2007; Kumar et al., 2008). Although the migratory PPNs may be able to dodge or outpace the host defence responses by moving on before the host has time to react, they still need protection against plant defence compounds like reactive oxygen species (ROS) a class of defence compounds produced by plants against invading pests. *P. thornei* and *P. coffeae* transcriptome data reveals the production of ROS metabolising antioxidants by both these RLNs (Jones and Fosu-Nyarko, 2014). Similarly, some other PPNs and animal parasitic nematodes such as *B. malayi* are also known to secrete antioxidants such as superoxide dismutase, peroxiredoxins and glutathione peroxidase, also reported in *G. rostochiensis*, which can reduce hydrogen peroxide that is present on the nematode surface and therefore protects them (Jones et al., 2004; Robertson et al., 2000).

When invading nematodes trigger a PTI response in plants they secrete some effectors to suppress the PTI response, but the plants have evolved a counter measure against pathogen

effectors called effector triggered immunity (ETI) (Jones and Dangl, 2006). The sequence of some major resistance genes encoding multidomain receptor proteins that are typically associated with ETI are used against RKN and CN in cultivated crops around the world. ETI is therefore an important factor in nematode-plant interaction. ETI-associated *Mi-1* resistance gene has been used to control *M. incognita*, *M. javanica* and *M. arenaria* infections in tomato (Castagnone-Sereno et al., 1994; Gleason et al., 2008; Triantaphyllou, 1985). Similarly, potato cyst nematode *G. palida*, has a *Gp-RBP-1* SPRYSEC gene that produces an effector that is recognised by the Gpa2 protein, a potato resistant gene product that triggers a hypersensitive type ETI in the host (Sacco et al., 2009). Some transcripts in *P. thornei* and *P. coffeae* transcriptomic data show significant similarity to *Gp-RBP-1* although any proteins encoded by the *Gp-RBP-1* in these RLNs need to be functionally characterised (Jones and Fosu-Nyarko, 2014; Nicol et al., 2012). There is limited information on nematode molecules that may trigger PTI in plant hosts. *Cg1* avirulence gene studied in *M. javanica* that gives the nematode an ability to defeat the *Mi-1* resistance gene in tomato is one of the well-known examples of PTI (Gleason et al., 2008). Thus, investigations that would provide a better idea of nematode effector interactions are needed (Smant and Jones, 2011).

Oesophageal secretions of CNs and RKNs contain variants of the enzyme chorismate mutase which is a remarkable finding as there is no shikimate or the sole substrate of chorismate mutase in animals. The shikimate pathway plays an important role during plant metabolism with chorismate being its end product. Chorismate forms the precursor of many of the secondary metabolites (auxin, salicylic acid, phytoalexins) some of which may be involved in plant defence (Doyle and Lambert, 2003; Lambert et al., 1999; Vanholme et al., 2009b). In *P. coffeae* and *P. thornei*, a form of chorismate mutase effector sequence that is slightly different (46.7%) from the one found for sedentary nematodes (*Gr-cm-1* from *G. rostochiensis*, *Hs-cm-1* from *H. schachtii*) may be secreted. However, its functional role during parasitism is not yet clear and needs validation, as it may also play a role in modulating plant defence processes as observed in the resistance-breaking ability of CN rather than feeding site formation (Abad et al., 2008; Bekal et al., 2003; Goto et al., 2013; Haegeman et al., 2011b; Jones and Fosu-Nyarko, 2014; Lambert et al., 2005; Nicol et al., 2012).

Several other parasitism genes thought to suppress plant defences, such as *14-3-3b*, secreted fatty acid retinoid binding protein, *FAR-1/SEC-2*, calreticulin, annexins. Acid phosphatase, galectin, *SKP1* (S-phase kinase associated protein), differentially expressed *R. similis* venom allergen-like proteins, and transthyretin-like proteins used by plant nematodes during host invasion have also been identified recently in *Pratylenchus* (Jacob et al., 2007; Jaouannet et al., 2013b; Jones and Fosu-Nyarko, 2014). Plants produce various toxins such as phytoalexins, isoflavonoids, or terpenoids with a broad anti-pathogen effects to defend themselves against invading pathogens; some of these toxins may have nematicidal properties (Jasmer et al., 2003; Wuyts et al., 2007; Wuyts et al., 2006).

Glutathione S-transferase (GST) plays a role during detoxification of a wide range of endogenous and xenobiotic compounds during animal-parasitic nematode infections (Campbell et al., 2001). Several GST genes have been reported in PPNs including *Pratylenchus* species such as *P. thornei*, *P. coffeae* and *P. zeae* (Nicol et al., 2012). The studies on the GST genes from *M. incognita* have revealed that at least one of its GST is expressed in the pharyngeal gland cells from where it is secreted into the host *via* the stylet (Dubreuil et al., 2007). RNAi silencing of this GST gene leads to reduced parasitic success, suggesting it does have an important role in nematode biology. Although biochemical data on their exact role during plant parasitism is lacking, it is very plausible that the secreted PPN GSTs show a conserved carboxyl group similar to *C. elegans.* It may have a common generic role during nematode development and may be involved in detoxification of host produced nematicidal compounds before their use by the nematode (Campbell et al., 2001). The lack of signal peptide in the GST proteins despite its detection in secretions and expression in the sub-ventral glands of nematode suggest a secretory pathway independent of the canonical endoplasmic reticulum-Golgi network (Haegeman et al., 2012). Other than their role in detoxification, GST may also bind auxin and flavonoids in plants and modulate their trafficking in the cells (Moons, 2005).

Typically, nematode secretions directly or indirectly aid in cell penetration, migration, suppression or evasion of host defences, induction and/or maintenance of the specialised feeding sites. Most of the non-CAZy effectors in *Pratylenchus* spp. are therefore likely be involved in evasion and suppression of host plant defences, since migratory nematodes do not

induce any specific feeding sites (Jones et al., 2013; Jones and Fosu-Nyarko, 2014). This knowledge can allow us to separate effectors involved in host cell penetration/ migration and defence suppression from those involved in endoparasitic feeding cell formation and maintenance. Amongst the different PPN effectors, apart from CAZymes, most of the effectors across plant nematode genera seem to lack conservation. This lack of conservation in effector genes even amongst same genera of PPNs is evident in studies conducted to find common resistance against different *Pratylenchus* spp. in wheat breeding programs. More work on effector identification and characterisation needs to be undertaken across both sedentary and migratory PPNs, as it can help us understand different forms of parasitism (Jones and Fosu-Nyarko, 2014). Based on the studies undertaken to understand how sedentary PPNs establish specialised permanent feeding sites, the process can be split into two stages (Jones, 1981). The first stage involves the introduction of effectors playing a regulatory role for inducing feeding cell development and modifications to the hosts metabolic pathways, the second stage is more of a source-sink arrangement facilitated by a transfer cell function. Therefore, the molecules secreted by most sedentary PPNs can modify the host morphology, responses, metabolic/signaling pathways, mimic host structure or functions. In comparison, the RLNs exhibit a less complex association with their hosts during their migratory life cycle which involves brief and intermittent periods of feeding during passage through the host root tissue (Haegeman et al., 2012).

1.8.3 Effectors manipulating plant cell biology

Auxin, cytokinin, C-terminally encoded peptide hormones (CEPs), CLE peptides, 16D10 CLE related peptides, NodL-like, 19C07, 10A06 and 7E12 are some effectors involved in manipulating plant auxin signaling pathways that help in initiation of permanent feeding sites and their maintenance (Abad and Williamson, 2010; Jones and Fosu-Nyarko, 2014; Opperman et al., 2008). Various lines of evidence from studies on CNs have shown that local changes in auxin concentrations are involved in initiation and directional expansion of induced syncytia. Auxin-insensitive tomato and Arabidopsis mutants showed various phenotypes ranging from complete inhibition of syncytium development to decreased hypertrophy and lateral formation at CN infection sites. Similarly, studies done on auxin gradients and specific activation of an auxin-responsive promoter verifies the role of auxin in developing syncytia by showing how auxin accumulate within syncytia (Goverse et al., 2000).

Various species of CN and RKN (Haegeman et al., 2012) encode proteins/peptides with a C-terminal motif similar to the plant CLAVAT3 (CLV3) peptide. The CLV signaling components are specific to the plant kingdom and include various CLV-like elements (CLE), namely CLV1, CLV2, ESR. These plant CLV proteins determine cell fate of plant parts such as flowers, shoot apical meristems, or vascular development in conjunction with cytokinin and auxin. The PPN CLE gene products mimic the plant CLV3 and play a role in cell differentiation and proliferation during feeding site formation. These CLE proteins are synthesised in the dorsal glands of PPNs and like plant CLEs are transported to the apoplast where they are processed (Wang et al., 2010a; Wang et al., 2011). In addition, to the extensively studied CLE peptides the PPNs also produce mimics of plant peptide hormones, cytokinins that have been found in *M. incognita* and *H. schachtii*, and may take part in feeding cell formation by playing a role in cell cycle activation.

Peptide hormones such as mitogenic peptides secreted by *G. rostochiensis* may contribute to re-differentiation of plant cells into feeding cells by process of cell cycle reactivation during initiation of syncytium formation. From studies conducted on *Meloidogyne* spp. by silencing of the 16D10 resulted in their reduced infectivity and development (reproduction) on the *A. thaliana* host. 16D10 thus seems to be virulence effector which disrupts plant transcription and may help in giant cell induction (Huang et al., 2006b).

RKNs may influence the host during the formation of its feeding sites by influencing the symbiont-response pathway involving a cytoskeletal response similar to the rhizobial signaling molecules [Nod factors (NF)] there by influencing their parasitic success (Weerasinghe et al., 2005). 19C07, a protein secreted by the dorsal glands of *H. schachtii* has shown to interact with LAX3 (Arabidopsis transporter protein) to regulate auxin influx during syncytium development. Similarly, the 10A06 effector secreted by the dorsal glands of *H. glycines* and *H. schachtii* have shown to disrupt the salicylic acid responses to its parasitic advantage. The 7E12 effector from the dorsal gland of *M. incognita* when constitutively expressed in tobacco plants not only increased gall numbers but also promoted faster egg-hatching, and giant cell histology revealed increase in vacuoles and cell wall invaginations with increase in number of cells surrounding the feeding cells (de Souza Junior et al., 2011).

Most of these effectors of RKNs and CNs affect root morphology, modulate host response to infection by targeting molecular pathways including host signaling pathways. None of the nine effectors named above have been reported in *Pratylenchus*, probably because they do not form elaborate permanent feeding sites (giant cells or syncytia), and have a less complex association with their plant hosts (Jones and Fosu-Nyarko, 2014). As RLNs feed briefly and irregularly they may need a smaller repertoire of effectors, and although the plant cell-manipulating effectors have not been reported their production may be restricted to certain stages during the life cycle where host response to infection needs to be regulated. Hence, their presence if any along with their role during the life cycle of RLNs needs to be investigated (Jones and Fosu-Nyarko, 2014; Mitchum et al., 2013). Parasitism genes of the sedentary PPNs that have been reported in at least one of the three transcriptomes of *Pratylenchus* spp., *P. thornei*, *P. coffeae and P. zeae* are shown in Table 1.1 (Jones and Fosu-Nyarko, 2014).

Given the importance of various known effectors and their roles in parasitism there is limited information on the effectors of migratory nematodes such as *Pratylenchus* spp. thus making them of interest in this study as RNAi targets. So far, only a few molecular studies have been carried out to investigate the role of parasitism effector genes in *Pratylenchus* spp. and there is much scope for *in vitro* and *in planta* RNAi research.

Table 1.1. List of secreted proteins and effectors of parasitism genes found in sedentary nematodes and also reported in at least one species of *Pratylenchus.* (Adapted from Jones and Fosu-Nyarko, 2014; Haegeman et al. 2011).

Gene	Putative or known function
CAZymes (cell wall modifying enzymes)	
Arabinogalactan galactosidase/arabinase	Hydrolysis of pectin
Cellulose binding proteins	Promote hydrolysis of crystalline cellulose
Endoglucanases and precursors	Hydrolysis of β 1,4-glucan
Endo-1,3-β-glucanase	Hydrolysis of β 1,3-glucan
Expansin-like proteins	Cell wall softening or extension
Pectate lyase	Hydroslysis of α 1,4-linkages in pectate
Polygalacturonase	Hydroslysis of α 1,4-D-galactosiduronic linkages
Xylanase	Hydrolysis of xylan

Protection from host defences	
Glutathione peroxidase	Detoxification of ROS
Glutathione synthetase	
Glutathione S-transferase	
Peroxiredoxin	
Superoxide dismutase	
Thioredoxin	
SPRYSEC-RBP-1	Suppression of host defences
Sec-2/FAR	Reduction in host defence response
Targeting regulation and signalling pathways	
Annexin	Protection of plant cells against stress
β-galactosidase binding lectin (Galectin)	No function data available for nematodes
Calnexin/Calreticulin	Calcium spiking
14-3-3 and 14-3-3b	No function data available for nematodes
RANBP	No function data available for nematodes
SKP-1	Involved in ubiquitination, signal transduction
Ubiquitin extension protein	Involved in ubiquitination
Feeding	
Aminopeptidase	Protein digestion/degradation
Proteases (*eg.* serine, cysteine)	
Potential effectors with unknown functions	
Transthyretin-like proteins	Unknown function
Vap-1	
SXP-RAL2	
Initiation and maintenance of feeding sites	
Chorismate mutase	Suppression of host defences

1.9 Identifying nematode effectors

Many approaches have been used to identify and isolate PPN parasitism gene products such as effector proteins. Although there are several examples of PPN secretion or gland protein discoveries, the first effector proteins discovered were cellulases identified from the CNs using monoclonal antibodies. This research was also the first to demonstrate endogenous production of cellulase by an animal (Smant et al., 1998). Before the name 'effector' was used to describe nematode proteins, several nematode proteins and peptides had already been isolated, for

example, acidic glycoproteins from *Xiphinema index* and its host tissue have been successfully detected by direct chromatographic analysis of the infected host tissue material, and whole as well as dissected nematode oesophageal bulbs secretions (Poehling et al., 1980). Other techniques like isolation of subcellular granules from homogenates of PPN juveniles (Reddigari et al., 1985) and direct collection of stylet exudates using glass microneedles or collection of stylet exudates by induced salivation using different compounds have also been used (Jaubert et al., 2002; Masler, 2007; McClure. M and Von. Mende, 1987).

As scientific techniques evolved effector discovery in nematodes moved more to the use of cDNA libraries from cytoplasm microaspirated directly from the nematode oesophageal gland cells, RNA fingerprints of nematode developmental stages or pathotypes, and proteomics on nematode secretions followed more recently by next generation sequencing (NGS). Molecular approaches such as differential screening to study spatio-temporal expression of genes expressed in glands of PPNs and cDNA-amplified fragment length polymorphism of different PPN life stages had been demonstrated previously (Haegeman et al., 2009; Qin et al., 2000; Rosso et al., 1999).

Microaspiration of gland cell cytoplasm and a selection system based on eukaryotic signal peptides in cDNAs has been used for cloning of secreted genes expressed in dorsal oesophageal glands of *H. glycines* (Wang et al., 2001). An efficient system for obtaining a complete profile of parasitism genes expressed in the PPN oesophageal glands via microaspiration of gland cell cytoplasm made use of suppression subtractive hybridisation (SSH) cDNA library (Gao et al., 2001). Sensitive methods such as tandem mass spectrometry (MS) have been deployed to directly identify 486 proteins secreted by *M. incognita* (Bellafiore et al., 2008). Techniques like *in situ* hybridisation have been used to confirm localisation of effector gene expression (Bellafiore et al., 2008). On the other hand, subcellular localisation to determine the function based on localisation of an effector once it is secreted into the host has also been used in *M. incognita,* which revealed several putative effectors present in the cytoplasm *in planta* (Rutter et al., 2014).

Novel gene discovery can be rapid and cost-effective when stage- or tissue-specific libraries are used for more targeted analysis of ESTs coupled with bioinformatic tools (Parkinson et al.,

2004a). Large-scale EST analysis can help in assessing gene expression in specific biological life stages or tissues which may help in the annotation of available genome sequence. Use of such methods has contributed to over 500,000 available ESTs from free-living, animal-parasitic and plant parasitic nematodes in the dbEST at Genebank (Parkinson et al., 2004b). Genes that are important in host-parasite interactions such as effector genes have been identified extensively from ESTs of various PPNs including some RLN species.

Some novel effector genes such as those encoding secreted proteins with an SPRY domain or the ones encoding ubiquitin-like proteins bearing an unusual C-terminal extension have been identified using RNAi and subtractive hybridisation (Jones et al., 2009). In *M. graminicola*, transcriptomic data was used to identify putative effector genes, which consisted of both novel genes and those that corresponded to already known effectors (Haegeman et al., 2013). It involved comparison of all known and annotated putatively secreted proteins from other PPNs across *M. graminicola* contigs to recognise any possible homologues. This lead to identification of putative homologues for the most common plant cell wall-modifying enzymes like β-1,4-endoglucanase, pectate lyase, xylanase, expansin and cellulose-binding protein, and also secreted proteins such as peroxiredoxin, glutathione peroxidase, glutathione S-transferase involved in detoxification of ROS. Transcripts encoding homologues of other putative of effectors, such as fatty acid- and retinol-binding protein, annexin, calreticulin, chitinase, transthyretin-like protein, annexin, calreticulin, chitinase, transthyretin-like protein, mitogen-activated protein (MAP-1), venom allergen-like protein, galectin, C-type lectin, 14-3-3 protein were also found. Some *M. incognita* proteins such as 4D03, 19F07, 35E04 (dorsal gland proteins) and 30G11 (an acid phosphatase expressed in sub-ventral glands) also showed homologues in *M. graminicola* pointing towards their possible conservation in the genus *Meloidogyne*.

The usual approach to identify putative novel effectors involves checking for the presence of a signal peptide and the absence of transmembrane domain, is not effective in the case of transcriptomic data, as the transcripts lack 5' ends which bear the region potentially encoding a signal peptide. Therefore, along with looking for proteins with signal peptide an additional BLAST search for comparing *M. graminicola* sequences with putatively secreted proteins from

the genomes of *M. hapla*, *M. incognita* and *B. xylophilus* was also conducted (Haegeman et al., 2013).

Comparative genomics allows cross-species comparisons to help understand structure and function of sequence(s) using available genomic information of multiple nematode species rather than using one species alone. At the time this research was initiated, a review of all the available effectors of PPNs identified so far using comparative genomics was undertaken to prepare a comprehensive list of reference effectors (Appendix 1).

1.10 RLN genome, ESTs and transcriptome

There are numerous challenges to improve the control of PPN infections and no single category of information or approach can overcome them all. However, the latest genomics approaches have the potential to offer an ever-growing and fundamental base of information. Together with downstream investigatory processes using functional genomics and proteomics these approaches can provide more data for the development and design of more efficient biological research tools to understand PPNs better and control them effectively (Mitreva et al., 2007). Nematode genomics can provide information on the form of genomic DNA (gDNA) and transcribed sequences (cDNA) that can help in consolidating both basic and applied biological investigations on parasitic nematodes. When compared to certain protozoan parasites, research progress on nematodes has been slow because they have larger genomes, tissue and life complexity, combined with few choices of genetic and transgenic methods for investigating or manipulating genes of interest. This lack of data limits the scope and quality of hypotheses which can assist in generating a rationale for research.

Initial genomic research on parasitic nematodes mainly involved generation of large collections of expressed sequence tags (ESTs) representing transcribed sequences. This EST database building also laid a foundation for many recent whole genome sequencing projects. ESTs offer a rapid way of gene discovery of an organism for which a cDNA library is available, large-scale EST analysis can help in estimating gene expression levels in different life stages and in tissues

of an organism. ESTs can also be used in annotation of genomic sequences (Mitreva et al., 2007; Parkinson and Blaxter, 2009; Parkinson et al., 2004b).

Nematode EST collections now span clades I-V (Blaxter et al., 1998; Parkinson et al., 2004c; Wylie et al., 2004) (http://www.nematodes.org). There are over 1.3 million ESTs from 74 species, including free-living, animal parasitic and plant parasitic nematode species combined in the dbEST (GenBank, 5 August 2017). To date, over 125,000 ESTs from more than 20 PPNs have been stored in dbEST, of which 14,645 ESTs belong to *Pratylenchus* species with a majority of them being from *P. vulnus*. Miterva et al., (2004) first described a subset of expressed genes in *Pratylenchus* species, from splice-leader 1 (SL1) driven EST library generated using mixed development stages of *P. penetrans* (Mitreva et al., 2004). These ESTs are a valuable resource for reagent development, genome analysis and functional genomics.

ESTs offer a cost-effective route for gene discovery, provide important information required for designing probes used for microarrays, and their translation can yield putative protein sequence information useful for proteomics. ESTs also provide reference data required for alternative splicing (Lee et al., 2006), verifying open reading frame and confirming exon/intron and gene boundaries. EST data assembly can help provide concrete outline of putative genes and proteins, which help create the molecular makeup of a nematode. Initial assessment of the genes expressed in tissues throughout a parasitic nematodes life cycle stages can be assessed by mRNA surveys across generation of ESTs. Quantitative EST assessments help in determining over- or under-represented transcripts in comparison to other transcripts from nematode life cycle stages or tissues. Therefore, abundance of certain gene products or transcripts could imply their importance in a biological process leading to more investigations. EST-based identification of genes may also shed light on any evolutionary interactions of the nematodes and their host. EST data combined with the use of gene ontology programs can provide insight into cellular and metabolic pathways functioning within the parasite (Geldhof et al., 2005; Jasmer et al., 2004; Maizels et al., 2000; Mitreva et al., 2007).

Transcriptomes of four *Pratylenchus* species namely, *P. coffeae* (Haegeman et al., 2011b), *P. thornei* (Nicol et al., 2012), *P. penetrans* (Vieira et al., 2015), and *P. zeae* (Fosu-Nyarko et al., 2016) have been sequenced and investigated using *de novo* analysis, providing most of the

currently available information for *Pratylenchus* species. As *P. thornei* is the main topic of this research, its transcriptome analysis is discussed briefly. Roche GS FLX sequencing was used to generate the first global transcriptome analysis of *P. thornei* where 787,275 reads were assembled into 34,312 contigs using two *de novo* assembly programs. A total of 6,989 contigs that were common to both the assemblies were annotated, resulting in 3,048 assignments. Specific transcripts that bear resemblance to cell wall degrading carbohydrate active enzymes, neuropeptides, putative plant nematode parasitism genes and transcripts that were possibly secreted by the nematode were investigated using comparative bioinformatics tools. Some transcripts showed similarity to bacterial cell wall degrading enzymes indicating a possibility of horizontal gene transfer during the PPN evolution. Similarly, there were transcripts that matched to 14 parasitism genes involved in host defence suppression and feeding site development in sedentary endoparasitic nematodes. The role of these many of the genes found in non-migratory PPNs could be modified to suite the nematodes migratory life style, however, this topic would need further investigation. Thousands of *P. thornei* contigs had matches to contigs from other sedentary (Heteroderidae and Meloidogynidae) and migratory (*R. similis*, *P. penetrans*, *P. vulnus* and *P. coffeae*) PPNs and also the free-living nematode (*C. elegans*). The transcriptomic data from *P. thornei* provides crucial new information that can increase the existing understanding of parasitic genes and their abilities of not only *P. thornei* but also other RLNs (Nicol et al., 2012).

The free-living nematode *C. elegans* was the first multicellular organism to be sequenced and since then many nematode genomes have been sequenced with many of them yet to be fully annotated. Vast amounts of genomic and proteomic information from *C. elegans* and other highly investigated parasitic nematodes serve as a guide for initial analysis of sequence data across other nematode species (McCarter et al., 2003; Rosso et al., 2009). To date, t85 nematode genome assemblies are available in the National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) genome resources which include free-living, APNs and PPNs (5 August 2017). Of these, only 12 PPN genomes have been sequenced and deposited at the NCBI genome database. They are: *M. incognita*, *M. hapla*, *M. javanica*, *M. arenaria*, *M. floridensis*, *G. pallida*, *G. ellingtonae*, *G. rostochiensis*, *H. glycines*, *D. destructor*, *B. xylophilus*, *R. reniformis*. Currently, there is no genome assembly data available for *P. thornei.*

The *C. elegans* genome is 100 Mb with approximately 20,000 genes, whilst the most sedentary PPN genomes are smaller, *M. hapla* (54 Mb), *B. xylophilus* (74 Mb), *M. incognita* (86Mb) with an exception of *G. pallida* (100 Mb) (Bird et al., 2015; Cotton et al., 2014). Currently, there is only one genome assembly amongst the *Pratylenchus* species, *P. coffeae* (Burke et al., 2015). The reported genome size for *P. coffeae* is ~20 Mb which is much smaller than other PPN genomes currently described. The smaller genome relates to factors such as fewer non-coding sequences or simpler life style with reduced requirements as no complex feeding cells or host suppression is required however, this needs further elucidation for *P. thornei* (Jones and Fosu-Nyarko, 2014). To date, there are 8,232 nucleotide sequences of Pratylenchidae curated at NCBI nucleotide database (5 August 2017) of which 8,220 sequences belong to 20 different *Pratylenchus* species with majority of the sequences belonging to *P. thornei*.

1.11 Future prospects for RNAi-based control of RLNs

To deploy RNAi technology as a strategy of crop protection against PPNs would require host-mediated delivery of dsRNA that is homologous to a target gene and would result in disruption of vital functions or mortality upon ingestion. It would be desirable to develop targets that are ultimately lethal to PPNs.

The success of *in planta* RNAi technology has been demonstrated through several examples. Michaeli et al., (2005) first illustrated near-complete resistance to *Meloidogyne* infection in tobacco expressing dsRNA homologues to a nematode-specific collagen gene (Gheysen and Vanholme, 2007; Michaeli et al., 2005). Yadav et al., (2006) demonstrated that host-mediated RNAi could be an effective strategy to control PPNs. This research used transgenic tobacco plants to express dsRNA against target genes of *M. incognita* (Yadav et al., 2006). Soon after, Huang et al. (2006) also demonstrated effective control of four major RKN species through host mediated RNAi in Arabidopsis (Huang et al., 2006a). Similarly, another research also demonstrated host-derived resistance in transgenic soybean roots against four target genes of *M. incognita* (Ibrahim et al., 2011; Lilley et al., 2012). More recently, Tan et al. (2013) successfully demonstrated *in planta* delivery of target dsRNA across to species of RLNs, *P. thornei* and *P. zeae* (Tan, 2015; Tan et al., 2013). Given the successful accounts of *in planta* RNAi, this strategy holds immense potential to control PPNs in future. *Pratylenchus* species

and other migratory nematodes lack a feeding tube which has been reported to limit the uptake of dsRNA and affect efficacy of RNAi in their sedentary counterparts; this makes *Pratylenchus* species amenable to host-mediated RNAi.

Even though RNAi-based technology has the potential, currently it is not an immediate alternative since certain questions remain to be answered and challenges overcome. For example, biosafety issues, acceptance of RNAi based crops, crop registrations, promoter activity, non-target effects, partial resistance etc. remain to be addressed. However, new research has indicated that it is possible to use comparative bioinformatics to identify high value targets and multi-gene families, carefully screen to reduce sequences that may lead to off-target effects and potentially common to non-target organisms, and finally identify genes with lethal RNAi phenotype (Danchin et al., 2013). Similarly, an approach to improving RNAi efficiency there by providing complete host resistance against PPNs would be to use RNAi construct targeting multiple genes having multiple cellular functions, not only towards one parasitic species but multiple. Using tissue-specific plant promoters which are induced via wounding or PPN infection is another way to solve the question of undesired promoter activity.

Currently, RNAi will be much safer in comparison to any of the pesticide/nematicides available as it would offer high specificity that the broad-spectrum pesticides cannot offer. This highly specific sequence-dependent mode of action that would typically target only the selected target gene it would reduce the negative impact on natural enemies of PPNs and also preserve the other natural fauna within the soil of the crop area. The efficacy of RNAi can be further improved using dsRNA delivery *via* chemically modified molecules, polymer nanoparticles, liposome, viruses or bacteria. To address the regulatory issues related to transgenic plants for controlling PPNs or other pests, non-transformative strategies offering similar efficiency can be used (Hunter et al., 2012; Scott et al., 2013). Expressing dsRNA through transplastomic plants or supplying it through irrigation water, root drench, or trunk injection would be a great strategy in controlling the PPNs (Chen et al., 2014). Additionally, the delivery *via* trunk injections or irrigation would bear minimal environmental impact or risk due to the rapid breakdown of dsRNAs in the soil and plant debris within 2 to 3 days (Dubelman et al., 2014; Petrick et al., 2013). To make such dsRNA delivery methods possible, cost-efficient methods

for mass production are being developed, including bacterial production (Joga et al., 2016; Palli, 2014).

The effectiveness of the RNAi mechanism depends on dsRNA delivery, its stability, and uptake by the target species, and this is still being investigated for efficient control of PPNs. The holistic understanding of the systemic properties of dsRNA along with improved delivery methods is still underway and eventually, it will provide innovative breakthrough applications for PPN control. Together, such studies will provide a pathway to develop environmentally-friendly and durable approaches of crop protection against RLNs.

1.12 Aims and objectives

The overall goals of this research were to investigate the effects of silencing parasitism effector genes of the root lesion nematode, *P. thornei* using RNAi-based technology as a strategy for their control. The specific aims of this research were as follows.

(i) *In silico* identification and molecular characterisation of putative parasitism effector genes in *P. thornei*.

The aim of this aspect was to identify target parasitism effector genes that are important during invasion, cell-to-cell movement and survival of RLNs in a host. Using a range of reference parasitism effectors studied so far, comparative bioinformatics was to be used to identify suitable candidate genes in the transcriptome of *P. thornei,* based on sequence homology to reference genes and RNAi data. Target sequences identified would then be amplified, validated and cloned into suitable transcription and binary vectors.

(ii). Functional characterisation of parasitism effector genes of *P. thornei via in vitro* RNAi.

The aim of this aspect was to study the effects of silencing selected target genes. The approach to be used was to generate dsRNA, followed by soaking nematodes in dsRNA solution. The immediate effects on behaviour and gene expression, and long-term effects on reproduction were then to be assessed through root infections of soybean and alfalfa.

(iii). Host-mediated RNAi of parasitism effector genes of *P. thornei.*

Based on the results obtained in part (ii) above, *in planta* RNAi testing of promising target genes would be done. The approach for this aspect included, production of axenic and non-axenic

transgenic hairy roots of soybean and alfalfa, followed by challenges with *P. thornei*, and analysis of the effects on gene expression and reproduction of *P. thornei*.

CHAPTER 02

General Materials and Methods

2.1 Carrot callus culture

Carrot (*Dacus carota*) cv. 'Nantes' was used to prepare discs for culture, reproduction and maintaining cultures of *P. thornei*. Whole carrots were first washed with tap water and then surface-sterilised by dipping in 3% sodium hypochlorite solution (sodium hypochlorite; 125 g/L chlorine) (Rowe Scientific Pty Ltd, Stock solution concentration 12% w/v) for 30 minutes on a shaker at 90 rpm in a 5 L pre-autoclaved plastic beaker sealed by sterile aluminum foil. Post-sterilisation the whole carrots were kept in autoclaved distilled water in a laminar hood for about 30 minutes before transferring into pre-autoclaved beakers (5L) to drain off excess water.

These carrots were then peeled with a pre-autoclaved steel peeler which was regularly flamed before moving to the next carrot. The steel surface of the laminar hood was also regularly cleaned with 80% ethanol. Up to 4 to 5 cm of the distal ends of the carrots were cut and discarded. Carrot discs of approximately 2 to 3 cm were then cut and flamed briefly (2 to 3 seconds) before keeping the one to three discs in pre-autoclaved polypropylene containers. These containers were left open for about one hour before sealing in the laminar hood to allow vapour and heat dissipation. Surface-sterilised carrot discs were transferred to an incubator maintained at 21 to 22°C in the dark. The carrot discs develop surface callus after 10 to 15 days and were then used for nematode multiplication.

2.2 Nematode propagation, collection, and sterilisation

P. thornei Vic33 was originally obtained from stock cultures maintained at the Department of Agriculture and Food Western Australia (DAFWA, now the Department of Primary Industries and Regional Development, DPIRD) (Perth, Western Australia, Australia). Nematode identification and verification was previously done by Dr. Jackie Knobbs (South Australian Research and Development Institute, Australia). A misting chamber was used to collect nematodes from callusing carrot discs and untransformed or transformed hairy roots. First, entire or cut pieces of nematode-infested carrot discs were put onto two sheets of coffee filters which were placed on a 5 cm plastic sieve with 0.1 cm apertures. If nematodes were to be collected from roots, then pieces or whole roots were used in the same way. This sieve was placed in a clean plastic funnel of a bigger diameter. The funnel had a 3-inch rubber tubing of

approximately 0.25 cm diameter attached to its tapering end, this tubing was bent in a U-shape and clamped using plastic clips. This apparatus was then placed inside a misting chamber that sprayed a fine mist of water for 10 seconds every 10 minutes. As the nematodes were washed off the carrot disc surface, they accumulated in the U-shaped section of the rubber tubing after which they were collected in 50 mL sterile Falcon tubes by releasing the clamp (Figure 2.1). Nematodes were collected every 24 hours for up to three days or until no more nematodes accumulated in the U-shaped bend depending on the requirement. Collected nematodes were surface-sterilised with 1% hibitane (4% chlorhexidine gluconate) for 20 minutes followed by 1% streptomycin sulphate for 5 minutes, and washed thoroughly at least five times with pre-autoclaved distilled water. The washing step included centrifuging the suspended nematodes at 1,200 g for 3 minutes. Viability of nematodes was checked under a dissecting microscope, after which approximately 1000 nematodes were used for infecting carrot calli and the cultures were placed in dark at 21°C for 6 to 8 weeks.

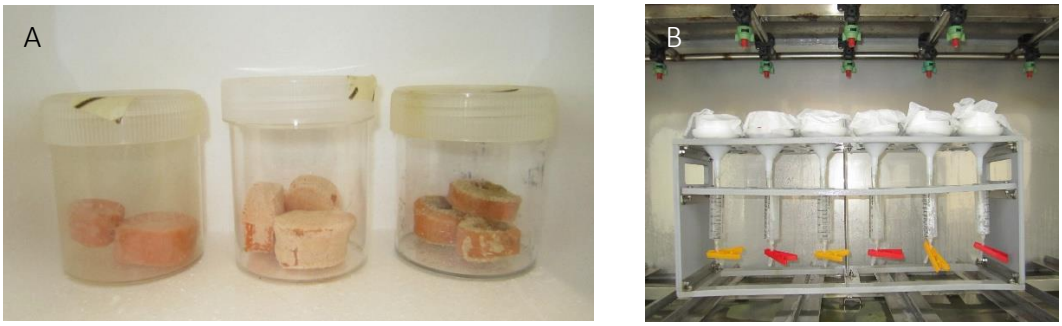


Figure 2.1. Carrot culture and nematode extraction. Stages of carrot disc culture showing pre-callusing, callusing, and infested stage (1st, 2nd and 3rd container respectively) (A). Misting chamber with apparatus for extraction of mixed stages of *P. thornei* (B).

2.3 Extraction of total RNA from *P. thornei* and transgenic hairy roots, and synthesis of cDNA

A mixed life stage population of *P.* thornei, either fresh or frozen at -80°C was used post-sterilisation for extracting RNA for synthesis of cDNA. Total RNA was extracted using the

TRIzol® reagent (Life Technologies Corporation, Australia) followed by ethanol precipitation. Tissue maceration was done using RNAse free materials treated with DEPC. Two to three steel beads were used in each 2 mL Eppendorf tube kept on ice in a laminar airflow chamber to disrupt the nematode body tissue using a tissue lyser. About 25 strokes/second for 3 minutes was used for lysis of the nematode body tissue. Tubes were immediately removed from the tissue lyser and kept on ice, then 600 µL TRIzol® and 200 µL of DEPC treated water was added in an extraction hood to maintain sterile conditions. The tubes were vortexed and incubated for 5 minutes at room temperature. Phase separation was done by addition of 200 µL chloroform ($CHCl_3$), and vigorously shaken by hand for 15 seconds. Tubes were then incubated at room temperature for 3 minutes followed by centrifugation at 4°C, 12,000 g for 15 minutes (or maximum speed). The upper phase (approximately 200-450 µL) was carefully transferred to a fresh 1.5 mL Eppendorf tube.

Ethanol precipitation was done by adding $1/10^{th}$ volume 3 M NaOAC, pH 5.2 and two volumes of ice-cold 100% ethanol. The tubes were incubated on ice and kept in a freezer for overnight precipitation at -20 °C or 2 to 4 hours at -80°C. After this, tubes were centrifuged at 4°C for 30 minutes at 12,000 g. The supernatant was discarded and the pellet was retained (may appear transparent). The pellet was washed with 500 µL 70% ice-cold ethanol, and centrifuged at 4°C for 5 minutes at 12,000 g. Again, the supernatant was discarded retaining the pellet. The pellet was washed with 500 µL 70% ice-cold ethanol twice more in the same way.

RNA was dissolved by adding 20 µL DEPC water and kept on ice or immediately store at 80°C until further use or followed with a DNAase treatment step. DNAase treatment was done by adding 20 µL RNA add 10 µL RDD Buffer, 2.5 µL DNAase, 67.5 µL DEPC-water. The mixture was incubated on bench top for 10 minutes. To verify that RNA was free from genomic DNA contamination the above RNA (1 µL) was run on a 1.5% agarose gel. RNA cleanup procedure was then done by adding $CHCl_3$ followed by the steps from phase separation (exactly as described above). Dissolved total RNA was quantified and stored at -80°C until further use.

The above protocol was also used to extract RNA from nematodes collected from transgenic hairy roots. RNA from transgenic hairy roots was also isolated using the RNeasy Plant Mini Kit (Qiagen, Australia) following the manufacturer's protocol. For this, 1-2 g fresh or -80°C frozen

root samples were macerated using the tissue lyser at 25 strokes/second and then used. DNAse treatment as described above was also applied.

Single-stranded cDNA from 100 nanogram (ng) of total RNA extracted from fresh nematodes, cleaned and frozen nematodes collected after transgenic hairy root feeding trials, or hairy root tissue, was synthesised using a MultiScribe reverse transcription (High Capacity Transcription Kit, Applied Biosystems, Australia). The 20 µL reaction mixture consisted of 1 X RT buffer, 1 X deoxynucleotide triphosphates (dNTPs), 1 X Random Primers, 2.5 U/µL MultiScribe reverse transcriptase and RNase Inhibitor. Reactions were subjected to PCR thermal cycler conditions of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes followed by 4°C. The cDNA was stored at -20°C until further use. The purity of RNA and cDNA was estimated from the 260/280 ratio quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Australia).

2.4 Extraction of genomic DNA from transgenic soybean and alfalfa hairy roots

For the analysis of transgenic soybean (*Glycine max*) and alfalfa (*Medicago sativa*) hairy roots, roots were collected and flash-frozen in liquid nitrogen. A rapid and a cost-effective method of genomic DNA extraction adapted from Henry (1997). Approximately 1-2 mg of hairy roots were placed in a 1.5 mL centrifuge tube and macerated in 200 µL extraction buffer (100 mM Tris HCL, pH 9.5, 1M KCL and 10 mM EDTA). Tubes were heated at 95°C for 10 minutes on a dry heat block followed by brief centrifugation to settle the remains of the plant tissue. One µL of supernatant was used in a 20 µL PCR.

2.5 Primer design

Gene specific primers (GSPs) were designed to amplify selected regions of the *P. thornei* transcriptome shotgun assembly (TSA) contigs after *in silico* comparison with several characterised parasitism effector genes of other PPNs, as described in Chapter 3. An *in silico* search for 18S rRNA in the *P. thornei* TSA contigs was also done to select a sequence representing the housekeeping gene, which was used as a control in semi-quantitative PCRs after RNAi. This yielded an 844 bp-long partial sequence of 18S rRNA of *P. thornei* deposited as accession number EU130826 which was used to design primers for semi-quantitative PCRs.

Of this, a 696 bp-long sequence was selected based on the choice of restriction enzyme sites that did not appear in the vector, pDoubler, and these GSPs were used in Chapters 4 and 5. The restriction enzyme (REs) sites in the regions of the all other contig sequences selected for target gene amplification were also checked for compatibility with those in cloning vectors using an online software NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/). GSPs were designed manually followed by reverse complementation using Extended Nucleic Acid Sequence Massager program (http://www.cmbn.no/tonjum/seqMassager-saf.htm). For cloning into a transcription vector, RE sites for *Kpn1* and *Xho1* were added to the GSPs. A list of primers showing the *P. thornei* TSA contig number selected, orthologous PPN genes, primer names, sequences, total length of the contig, and position of the primers are shown in Table 2.1. These primers were used for initial amplification of sequences from *P. thornei* cDNA as well as for semi-quantitative PCRs to study transcript abundance of target gene in *P. thornei*. Depending upon the purpose and the plasmid vector used, several other primers were also used. All primers were synthesised by GeneWorks Pty Ltd, Australia. These primers were used for colony PCRs and DNA sequencing: more details on there are provided in the relevant sections.

Table 2.1. List of primers used for colony, and semi-quantitative PCRs, cloning, and DNA sequencing.

No.	Primer Name	Primer Sequence (RE GSP)	Total Size; Start & Stop position	Total bp	GC %	Tm °C	Product Size (bp)
1	TSA Pt Mi Eng-1 X F	TCA CTCGAG ACGAGACATTCAATGAGC	824; 417 to 637	27	48.1	60	220
2	TSA Pt Mi Eng-1 K R	TCA GGTACC CAGCTCCTGTTTGTGGGA		27	55.6	64.7	
3	TSA Pt Mi CLP X F	TCA CTCGAG CAACTTCAGCTTCTCCTC	1149; 763 to 1107	27	51.9	61.7	344
4	TSA Pt Mi CLP K R	TCA GGTACC GCTATGTTACTCCGGTGA		27	51.9	62.2	
5	TSA Pt Mi PL X F	TCA CTCGAG CAAATCAACTGGGACCGA	593; 393 to 508	27	51.9	63.3	115
6	TSA Pt Mi PL K R	TCA GGTACC CATCATCTTCAGTTCGAC		27	48.1	59.6	
7	TSA Pt Hs UEP X F	TCA CTCGAG CCGCTGCTTATATTTGAC	2288; 124 to 288	27	48.1	60.5	164
8	TSA Pt Hs UEP K R	TCA GGTACC CCGGAGCCTTTGTAAATTA		28	46.4	61	
9	TSA Pt Mi GST X F	TCA CTCGAG GATGATGTTATTGGCTGC	642; 44 to 318	27	48.1	60.4	274
10	TSA Pt Mi GST K R	TCA GGTACC GGCGTGTCAAAATAGTAG		27	48.1	59.9	
11	TSA Pt 18S X F	TCA CTCGAG CATGCACCAAAGCTCCGA	844; 120 to 816	27	55.6	65.4	696

12	TSA Pt 18S K R	TCA GGTACC GTTAACCATTATCTCGGC		27	48.1	59.8	
13	S Intron	TCATCATCATCATAGACACAGGA					Insert + 311
14	S 35 S	GATTGATGTGACATCTCCACTGA					
15	AS Nos T	ATTGAGACGCAATCCACACGCT					Insert + 159
16	AS Intron	TCGTGTGTCTATGATGATGATGA					
17	35 S ART	GTCTTGATGAGACCTGCTGCGTA					760 (For orientation check)
18	SP 6	ATTTAGGTGACACTATAGAAT					
19	sNpt II-F	AATATCACGGGTAGCCAACG					356
20	sNpt II-R	AGCACGTACTCGGATGGAAG					
21	M13-F	TAA AAC GAC GGC CAG T					361
22	M13-R	CAG GAA ACA GCT ATG AC					

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Routine PCRs

PCRs to amplify target genes from *P. thornei* cDNA were performed using My*Taq* (Bioline, Australia). The total volume in a PCR was 20 µL with concentrations of the components as follows; 1 X buffer, 0.2 mM dNTPs, 1 U polymerases, 2 mM $MgSO_4$, 10 pm/µL primers, 300 ng of cDNA or 100 ng of plasmid DNA. PCRs were carried out in a thermocycler (Applied Biosystems 2720 or Veriti 96 wells) machine. Settings for the thermal cycler for most PCRs were 94°C or 96°C for 3 minutes for the initial denaturation stage, 25 to 45 cycles of denaturation at 94°C or 96°C for 15 to 30 seconds, primer annealing at 55°C or 60°C for 15 to 30 seconds, extension at 72°C for 30 seconds to 1 minute and a final extension at 72°C for 7 to 10 minutes. Touch-down PCRs were also used for amplifying several genes using GSPs with restriction enzyme sites; in these PCRs the annealing temperature was decreased in steps of 1°C beginning at 65°C. Specificity of the initial primer-template duplex formation was enhanced by this process.

PCRs on genomic DNA of transgenic hairy roots to amplify short fragment (356 bp) in the sequence of the selectable marker gene, neomycin phosphotransferase II (*npt II*) were accomplished using My*Taq* Red Polymerase (Bioline, Australia) and *sNpt II* primer pair as described in section 2.6. Similarly, to confirm the presence of inserts and/or check orientation of the hairpin cassette in bacterial clones, and expression of the hairpin cassette in cDNA of selected transgenic hairy roots, PCRs were carried out using the GSPs listed in Table 2.1. The total volume in a PCR reaction was 20 µL and the concentrations of the components were as follows; 1 X buffer containing 0.2 mM dNTPs and 2 mM $MgCl_2$, 1 U DNA polymerase, 10 pm/µL primers. These PCRs were carried out using the conditions described above.

2.6.2 Semi-quantitative PCRs

Semi-quantitative PCRs were carried out to determine expression of target genes in *P. thornei* extracted from infested transgenic hairy roots. A total of 100 ng of RNA was used in reverse transcription reactions and the first-strand synthesis of cDNA was done as described in section 2.3. The total volume of PCR was 20 µL and the concentrations of the components were as follows; 1 X reaction buffer comprised of 0.2 mM dNTPs and 2 mM $MgCl_2$, 1 U DNA Polymerases, 1:10 dilution of cDNA and clean water to make up the volume; the same master

mix used for each experiment. Semi-quantitative PCRs were carried out in triplicate for each biological replicate. PCRs were carried out in an Applied Biosystems 2720 thermocycler and the conditions used were as follows: 96°C for 3 minutes for the initial denaturation stage, 25, 30 and 35 cycles of denaturation at 96°C for 30 seconds, primer annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 7 to 10 minutes.

2.7 Agarose gel electrophoresis and DNA markers

Gel electrophoresis were carried out in submerged horizontal electrophoresis system (Bio-Rad, Australia). Generally, 1.2 to 2 % agarose gels were prepared in 1 X TAE buffer (40 mM Tris acetate, 1 mM EDTA) (Sambrook *et al.*, 1989) with SYBR Safe DNA stain (Life Technologies, Australia) for visualisation of DNA, depending on the expected amplicon sizes. Electrophoresis was performed at 70 to 80 V. The expected sizes of amplicons were compared to 100 bp DNA ladders (Axygen Biosciences, Fisher Biotech, Australia or New England Biolabs, Genesearch, Australia) or 1 kb DNA ladders from Promega, Australia. Electrophoresis carried out to visualise differences in gene expression after semi-quantitative PCRs included consistent conditions such as: electrophoresis system, 1.5% agarose gel, well combs, SYBR Safe DNA stain, and buffer. Gels were observed with a gel documentation system attached to a camera system (Fisher Biotech, Australia) and analysed with BioVision software (www.vilber.com). Inverse image capture of gels for visualising semi-quantitative PCRs were done at the same default settings and images were saved in .tiff format to ensure no loss of image quality.

2.8 Gene expression analysis using ImageJ

Densitometric analyses was carried out to study the changes in gene expression in nematodes fed on dsRNA and transgenic hairy roots of soybean and alfalfa. This made use of the gel electrophoresis images resulting from semi-quantitative PCRs being analysed in an open-source image analysis software, ImageJ developed at the National Institute of Health, Maryland, USA. ImageJ 1.51j8 bundled with 64-bit Java 1.8.0_112 for Windows was downloaded from https://imagej.nih.gov/ij/download.html. ImageJ manual was used to analyse DNA as bands on gel. Inverse images of gels in tiff format with consistent exposure parameters were used for all analyses. Background subtraction was done in ImageJ followed

by measurements for percent density for each band. Gel images were analysed for three replicates of semi-quantitative PCRs. These data were then exported in Microsoft Excel for further analysis. Relative density of controls and samples, and reduction in transcript accumulation (gene expression) relative to the house-keeping gene control, 18S RNA was calculated. This was done for each sample and was a ratio of average density of sample to the control, 18S ribosomal RNA (rRNA). The difference in relative densities between control and sample was then calculated and expressed as a percentage of reduction.

2.9 DNA purification from agarose gel

DNA purification from agarose gels was achieved using the Wizard SV Gel and PCR Clean-up System (Promega, Australia). Desired DNA bands were excised using a sterile surgical blade, placed in pre-weighed 1.5 mL or 2 mL centrifuge tubes and membrane binding solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate pH 5.0) at a ratio of 10 µL per 10 mg agarose gel was added. Gel slices were melted at 50 to 65°C in a heat block for 10-12 minutes and tubes were vortexed intermittently. The dissolved gel mixture was transferred to an SV minicolumn assembly and centrifuged for 1 minute at 16,000 g. DNA bound to the column was washed twice; first with 700 µL followed by 500 µL membrane wash solution (after addition of ethanol: 10 mM potassium acetate pH 5.0, 80% ethanol, 16.7 µM EDTA pH 8.0) previously diluted with 95% ethanol. Spin column assembly was centrifuged at 16,000 g first for 1 minute and then for 5 minutes. All centrifugation steps were performed in a benchtop centrifuge at room temperature. Finally, DNA was eluted in 20 to 50 µL pre-warmed clean RNase-free water quantified and stored at -20°C until further use.

2.10 Vectors used for cloning

The plasmid vectors pDoubler and pCleaver-NosT used in this research were developed and kindly provided by Dr. John Fosu-Nyarko (formerly Nemgenix Pty. Ltd.) and were used for *in vitro* transcription and cloning of a hairpin cassette respectively. The binary vector, pART27 (Gleave, 1992) was used for plant transformations. Target sequences of *P. thornei* were cloned into pDoubler which was then used for dsRNA synthesis (Figure 2.2). To clone fragments, purified PCR products and the plasmid vector, pDoubler, were digested with restriction

enzymes, *XhoI* and *KpnI* to yield complementary ends used for ligation. For generating sense (S) and antisense (AS) fragments, restriction enzyme pairs, *XhoI/KpnI* and *BamHI/XbaI* respectively were used to digest the fragments from pDoubler. Both fragments were subsequently sub-cloned into the plasmid vector, pCleaver-NosT which was also cleaved with the same restriction enzyme pairs prior to ligation. Plasmid pCleaver-NosT was designed with a CaMV35S promoter, bean catalase intron, and Nos terminator (Figure 2.3). Finally, the RNAi hairpin cassette was cleaved from pCleaver-NosT using *NotI* and ligated to the binary vector, pART27 which was also cleaved with *NotI* prior to ligation. Figure 2.4 is a schematic representation of the steps in cloning of the RNAi hairpin cassette.

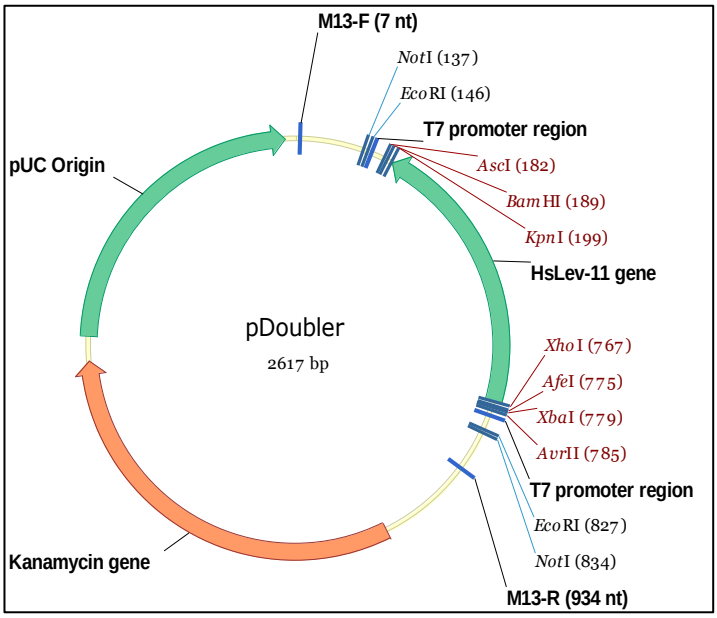


Figure 2.2. *In vitro* transcription vector pDoubler used for cloning of *P. thornei* sequences and dsRNA synthesis.

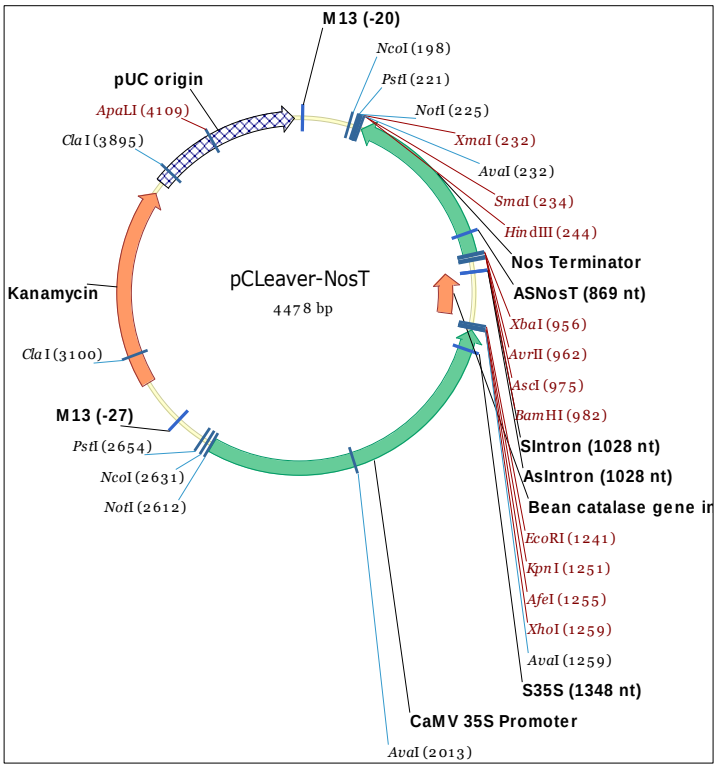


Figure 2.3. Vector pCleaver-NosT used for cloning sense and antisense fragments of target *P. thornei* sequences.

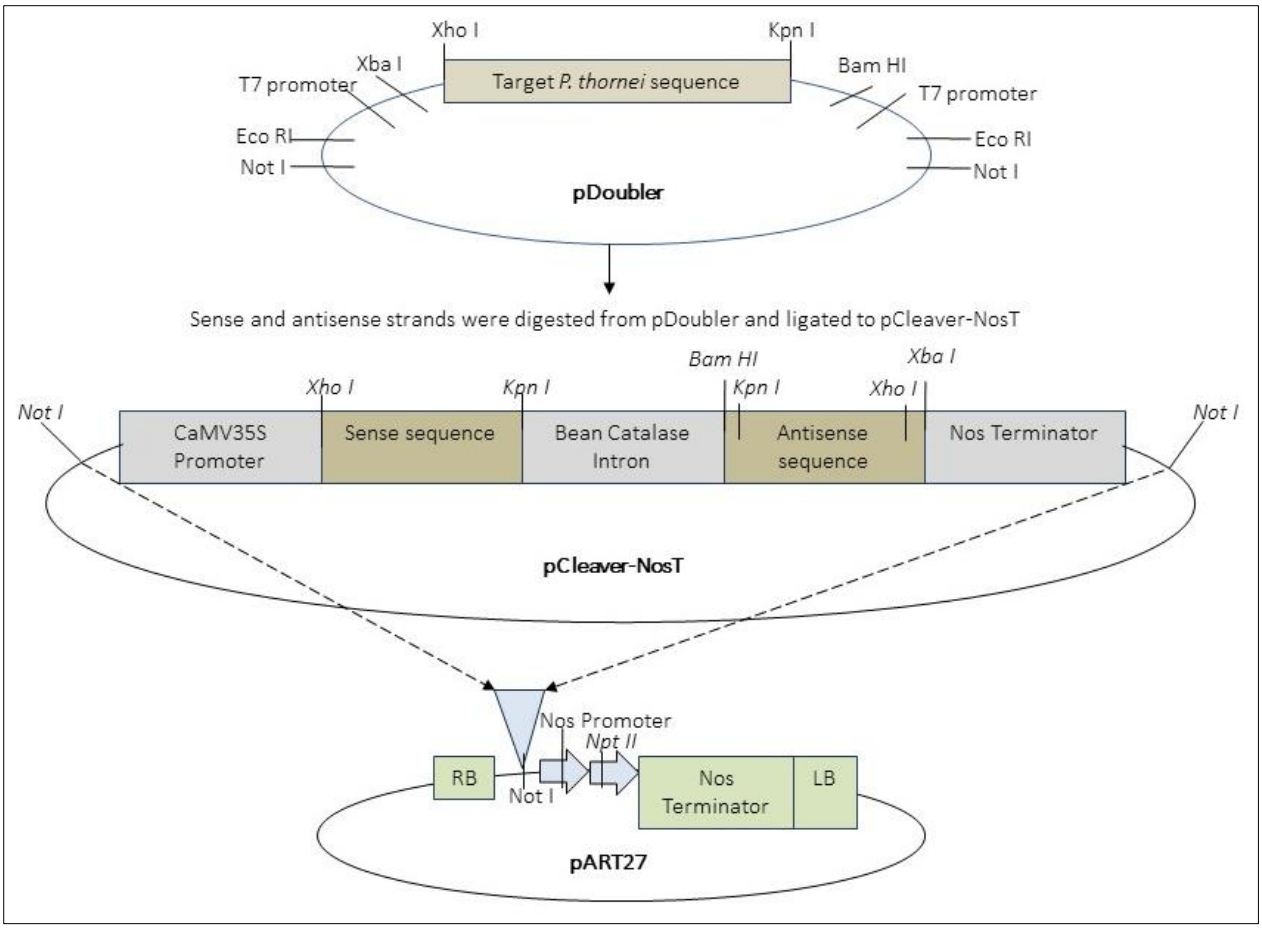


Figure 2.4. A schematic representation of cloning of a RNAi hairpin cassette in pART27 (*not to scale*).

2.11 Dephosphorylation of the binary vector, pART27

The hairpin RNAi cassettes that were cleaved from pCleaver-NosT using *NotI* were ligated to dephosphorylated pART27. For this, Antarctic Phosphatase (AP) (NEB, Genesearch, Australia) was used. AP catalyses the removal of 5' phosphate from DNA thereby preventing self-ligation of the vector. A 20 µL dephosphorylation reaction containing 1 X AP Reaction Buffer, 0.5 U AP, 1 µg *NotI* digested pART27 and RNase-free water was incubated at 37°C for 30 minutes followed by heat inactivation at 65°C for 5 minutes.

2.12 Ligation

Amplified PCR products purified from gels were used to set up ligation reactions. Purified amplicons were ligated to pDoubler, sense and antisense fragments were ligated to pCleaver-NosT, whilst the final hairpin RNAi expression cassettes were ligated to a dephosphorylated pART27 in a 3:1 insert:vector molar ratio following the formula:ng of insert=(concentration of vector in ng) X (size of insert in kilobase (kb))/size of vector in kb X 3/1. All ligation reactions were carried out in a total reaction volume of 10 or 20 µL overnight at 16°C. Reaction components were 1 X T4 DNA Ligation Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT), 1 U T4 DNA Ligase (NEB, Genesearch, Australia). Heat inactivation of ligase at 65°C was carried out in a thermal cycler, and reactions were stored at -20°C until further use.

2.13 Bacterial transformation and DNA purification

Chemically competent *Escherichia coli* JM109 cells were used for transformations. Recombinant plasmid DNA was isolated and purified from cell culture. The recombinant pART27 vector containing the hairpin cassettes was transformed into chemically competent *Agrobacterium rhizogenes* K599 cells and used for plant transformations.

2.13.1 Preparation and transformation of competent *E. coli* JM109 cells

All plasmids in this study were propagated in *E. coli* JM109 cells. For this, the heat-shock transformation method was used to transform chemically competent *E. coli* JM109 cells. Aliquots of a commercial stock of chemically competent *E. coli* JM109 (Promega, Australia) stored at -80°C were first thawed, and 5 to 10 µL of the ligation reaction was added to 25 to 50 µL cells in 1.5 µL centrifuge tubes. The tubes were gently flicked two to four times to mix the cell-ligation mixture before incubating on ice for 20 minutes. This was followed by a heat-shock treatment at 42°C in a water bath for 45 to 60 seconds after which the tubes were immediately placed on ice for 5 to 10 minutes. About 500 to 800 µL LB medium without any antibiotics was added and this culture was incubated at 37°C on a shaker at 225 rpm. After 2 to 3 hours, when bacterial growth seen as swirls or ribbons in the culture was evident about 200 to 400 µL of culture was streaked on LB agar plates containing appropriate antibiotics depending on the plasmid vector used. For pDoubler and pCleaver-NosT, 25 mg/L kanamycin

was used whilst for pART27 100 mg/L spectinomycin was used. Plates were incubated at 37°C overnight in dark.

2.13.2 Preparation and transformation of competent *A. rhizogenes* K599 GV3101 cells

Glycerol stock of *A. rhizogenes* K599 was used to prepare competent cells using calcium chloride ($CaCl_2$) treatment. Glycerol stock was first thawed on ice and culture was streaked on LB medium plates with 50 mg/L gentamicin and 25 mg/L rifampicin. Plates were incubated at in the dark 28°C overnight. A single colony was picked and 10 mL of LB broth with antibiotics in McCartney bottles was inoculated. Cultures were placed at 28°C on a shaker (225 strokes per minute) overnight. One tenth volume of the overnight culture was used to inoculate 100 mL of LB with antibiotics in 2 L conical flasks and incubated at 28°C on a shaker (225 strokes per minute) until the OD_{600} reached 0.4 to 0.5. Using the stated volumes, this step required 6 to 7 hours. The cell culture was divided into two sterile 50 mL Falcon tubes and centrifuged at 1000 to 1,500 g for 30 minutes at 4°C in a pre-cooled Beckman Coulter Allegra® X-15R benchtop centrifuge. The supernatant was discarded carefully retaining the pellet which was then resuspended in 10 mL of 20 mM $CaCl_2$ on ice. The tubes were centrifuged at 1100 g for 10 minutes at 4°C. Again, the supernatant was discarded carefully retaining the pellet which was then resuspended in 20 mL of 20 mM $CaCl_2$ on ice. Finally, 100 µL cell suspension was aliquoted in 0.5 mL centrifuge tubes, flash-frozen in liquid nitrogen immediately, and stored at -80°C until further use.

A freeze-thaw transformation procedure was used to transform competent *A. rhizogenes* K599 cells with recombinant plasmid vectors (pART27 containing the hairpin cassettes). Aliquots of cells were thawed on ice and 500 ng plasmid vector was added to 100 µL cell suspension. Tubes were gently flicked to mix the cells, frozen immediately by immersing into liquid nitrogen for 5 minutes followed by thawing for 5 minutes at room temperature. After this, tubes were incubated at 37°C for 5 minutes in a water bath. A total of 500 to 800 µL LB medium without antibiotics was added and this culture was incubated at 28°C for 4 to 5 hours on a shaker at 225 rpm. Cell cultures were centrifuged at 12,000 g for 2 minutes at room temperature in a benchtop centrifuge. The supernatant was discarded and the pellet was resuspended in 100 µL of LB broth containing 100 mg/L spectinomycin and 25 mg/L rifampicin. About 250 µL of

this suspension was plated on LB agar plates with antibiotics. Inverted plates were carefully wrapped in aluminium foil and incubated at 28°C for 2 to 3 days in dark.

2.13.3 Plasmid DNA isolation

Plasmid DNA was isolated using Wizard *Plus* SV Minipreps DNA Purification System (Promega, Australia) kit. Cell cultures were divided into 2 mL centrifuge tubes and centrifuged for 5 minutes at 10,000 g on a benchtop centrifuge at room temperature. The pellet was resuspended in 250 µL of cell resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg /mL RNase A) by inverting the centrifuge tubes. To this, 250 µL of cell lysis solution (0.2 M NaOH, 1% SDS) was added, tubes inverted four times, and incubated for 5 minutes until the suspension appeared clear. A total of 10 µL alkaline protease solution was added, mixed by inverting four times, and incubated for 5 minutes. Then, 350 µL of neutralisation solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, final pH 4.2) was immediately added, tubes were inverted four times, and resulting bacterial lysate was centrifuged at maximum speed for 10 minutes. A spin column assembly was prepared simultaneously on to which the cleared bacterial lysate was poured without transferring the white fluffy precipitate followed by centrifugation at maximum speed for 1 minute. DNA bound to the column was washed twice, first with 750 µL and then 250 µL column wash solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl pH 7.5, 0.109 mM EDTA pH 8.0) previously diluted with 95% ethanol. The assembly was centrifuged for 1 minute at maximum speed both times. Finally, plasmid DNA was eluted in 50 to 75 µL clean pre-warmed RNase-free water and stored at -20°C until further use.

2.14 Analyses of transformants

Depending on the vectors used for cloning, transformants were selected on appropriate antibiotic media as explained above, followed by PCRs and restriction digestion as described below.

2.14.1 PCR analyses

For these PCRs, single colonies were picked with sterile 10 µL pipette tips and resuspended in 20 to 25 µL of clean injection water of which 5 µL of the culture was used for PCRs. Routine

PCRs were performed using 1 X buffer containing 0.2 mM dNTPs and 3 mM $MgCl_2$, 0.5 to 1U *Taq* polymerases (My*Taq*, Bioline, Australia) and 10 pm/µL primers. PCR conditions set in the thermal cycler were: initial denaturation at 96°C for 3 minutes followed by 25 cycles of denaturing at 96°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72 for 30 seconds, and a final extension at 72°C for 7-10 minutes. PCR products were electrophoresed and visualised on agarose gel. To identify positive pDoubler clones, either M13-F and M13-R or gene specific primer pairs were used. If M13-F and M13-R primers were used, the amplicon size included insert plus 361 bp of the vector. To confirm the presence of sense fragments in pCleaver-NosT, primer pair SIntron and S35S was used whereas, antisense fragment was confirmed with the primer pair ASNosT and ASIntron. To identify positive pCleaver-NosT clones with sense and antisense fragments the amplicon size included insert plus 311 bp and 159 bp respectively. The presence and orientation of hairpin cassettes in pART27 were identified by a PCR product of 760 bp as a result of the primer pair SP6 (designed on the SP6 primer binding site on pART27) and 35SART (designed on CaMV35S promoter on pCleaver-NosT).

2.14.2 Restriction digestion

After antibiotic selection and PCR analyses, positive clones were selected for restriction digestion of plasmid preps. To do this, 5 to 10 µL of culture water used for PCRs above, was used to inoculate 10 mL of LB broth with appropriate antibiotics in McCartney bottles and was incubated at 37°C on a shaker at 225 rpm overnight. Plasmid DNA from bacterial cell cultures was isolated as described in section 2.12.3. Restriction digestion with appropriate restriction enzyme(s) was done to confirm the presence, size, direction of inserts in a vector. For confirming presence and size of inserts in clones of pDoubler, *XhoI*/*KpnI*, for confirming the presence of sense fragment in clones of pCleaver-NosT *XhoI*/*KpnI* whereas, *XbaI*/*BamHI* were used to confirm and antisense fragments in pCleaver-NosT. To confirm the presence and direction of a hairpin cassette in pART27 *XhoI*/*XbaI* were used. All restriction enzymes were obtained from NEB, Genesearch, Australia, and reactions were set up as per the manufacturer's protocol. Depending on the enzyme(s) used in either a single or double digest(s), recommended concentrations of DNA and enzymes, buffers, incubation temperatures for digestion with or without BSA were incubated at 37°C for varying number of hours. Sometimes higher concentrations of enzymes or longer incubations were needed for

complete digestion. When recommended, reactions were heat inactivated at 65°C in a thermal cycler.

2.15 DNA sequencing and analyses

DNA sequencing of all fragments or recombinant vector clones was done at the WA State Agricultural Biotechnology Centre (SABC) Sanger sequencing facility using the dye terminator (version 3.1) chemistry-based ABI 3730 Sanger Sequencer (Applied Biosystems, Life Technologies, Australia). To sequence pDoubler clones, either M13 forward or gene specific forward primer was used whereas, for sense and antisense fragments cloned in pART27, either primer SIntron or ASIntron was used.

Instructions given in the "Sequencing guide for beginners" developed by Ms Frances Brigg was used to set up the reaction. A modified version of the Big Dye terminator v3.1 Cycle Sequencing protocol from Applied Biosystems was used. Template for sequencing was prepared with 1/16th dilution of the recommendation reaction mixture. For this, 3.2 pm/µL M13 forward primer, 1 X reaction buffer, Big Dye terminator (dye T), and 150-300 ng plasmid DNA in a total volume of 10 µL was prepared. Reactions were placed in a thermal cycle set at a hold temperature of 96°C for 2 minutes followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C, and an extension at 60°C for 4 minutes. After this, ethanol precipitation was done to remove any salts or unincorporated dye terminator from the reactions. To do this, 1 µL of 125 mM EDTA, 1 µL of 3M sodium acetate pH 5.2, and 30 µL of 100% ethanol in the same order was added without making a master mix for samples. This mixture was pipetted thoroughly, incubated at room temperature for 20 minutes, after which it was centrifuged at maximum speed for 30 minutes at room temperature. The supernatant was discarded and 125 µL of 70% ethanol was added followed by centrifugation at 12,000 g for 5 minutes at room temperature. Finally, the tubes were air-dried in the dark for 20 minutes and submitted to the SABC Sequencing facility. Sequencing was carried out by Frances Brigg. Raw sequencing results were viewed and edited with FinchTV (www.geospiza.com/products/finchtv.html) and annotated using the BLAST suite at NCBI (https://www.ncbi.nlm.nih.gov/).

2.16 Staining of *P. thornei* in soybean and alfalfa roots

A simple and effective method of staining roots was adapted from Thies et al. (2002) with slight modifications, to check for the presence of *P. thornei* in untransformed and transformed hairy roots (Thies et al., 2002). Generally, the staining was undertaken two weeks after inoculations with *P. thornei* to check that the nematodes had entered the roots. The procedure involved placing the roots in a small, steel fine-mesh tea infuser (4.5 cm), followed by washing and blotting the roots dry. If the roots were very long and weighed over 10 g then they were cut into 1-2 cm long pieces, otherwise they were left intact. The root pieces were submerged in 50 mL of 1.5% sodium hypochlorite in a 200 mL conical flask placed on a shaker at 60 rpm for 5 minutes. Roots were transferred into the infuser and rinsed under running tap water for 30 seconds after which they were submerged in fresh water for 15 minutes. The roots were removed from the infuser, placed in a 200 mL conical flask containing a 12.5% solution of red food dye (Queen Fine Foods, Australia), brought to boil for 30 seconds on an oxidising flame of a Bunsen burner, and allowed to cool down at room temperature. The roots were placed in the infuser and rinsed thoroughly under running tap water followed by gentle blot drying. After this, the roots were transferred to 10 mL of acidified glycerin preheated to 40°C for 15 seconds. Acidified glycerin was prepared by adding 1 to 2 drops of 5 N HCl to 10 mL glycerin. These roots were allowed to cool at room temperature in a sterile Petri dish. After this, they were placed on a slide with a drop of glycerin, sealed with a coverslip, and observed under bright-field of an Olympus BX-51 microscope having a camera attachment.

CHAPTER 03

Identification and Molecular Characterisation of Target Putative Parasitism Effectors of *Pratylenchus thornei.*

3.0 Introduction

The discovery of pest and pathogen effectors has provided new targets for their control (Vleeshouwers and Oliver, 2014). In order to study gene function and evaluate the efficacy of such target genes for potential use in breeding programs, comparative bioinformatics *in silico* has been used to identify and characterise some of them in *P. thornei*. This has been followed by functional characterisation using RNAi. It is thus possible to mine for target parasitism effectors that manipulate host cell structure and function to facilitate infection, and those that modulate host defence responses. Several effectors are important early in the infection as they help enable PPNs to enter and migrate in root tissues. These effectors are potential targets for RNAi-based management of PPNs since they can discourage nematode entry, feeding and reduce their impact. Functional characterisation of these genes *via* RNAi can help in understanding how crucial these genes are for nematode invasion and life processes.

Unlike the sedentary PPNs that must keep the host root cells alive for longer term feeding, development and reproduction, RLNs, such as the *Pratylenchus* spp., cause more cell damage during migration and feeding (Bird et al., 2009; Lin et al., 2012; Zunke, 1990b). Root damage is caused both by mechanical forces exerted by the nematode stylet and by the effects of stylet-delivered effector proteins from the oesophageal gland cells (Jones and Fosu-Nyarko, 2014; Vieira et al., 2015; Zunke, 1990a, b). These effectors are thought to play a key role in parasitism, not only during penetration and migration but also during brief periods of feeding during which they may help in attenuation of plant defences (Fanelli et al., 2014; Jones and Fosu-Nyarko, 2014; Nicol et al., 2012; Yang et al., 2017). Hence, it is important to target suitable effector genes that are vital to PPNs during plant invasion or feeding processes.

Some effectors which have been identified and their function validated have been idenitified, with most emphasis on the sedentary PPNs. PPN effectors are generally include the cell wall-degrading enzymes (CWDEs) β-1,4-endoglucanase (Ledger et al., 2006; Rosso et al., 1999), pectate lyase (De Boer et al., 2002; Doyle and Lambert, 2002), polygalacturonase (Jaubert et al., 2002), β-1,4-endoxylanase (Dautova et al., 2001), cellulose-binding protein (Ding et al., 1998), and other effectors like chorismate mutase (Bekal et al., 2003; Lambert et al., 2005), venom allergen-like protein (Ding et al., 2000), glutathione S-transferase (GST) (Dubreuil et al., 2007), acid phosphatase, and sodium/calcium/potassium exchanger (Huang et al., 2003).

Novel and known effectors have been identified in different PPN species using techniques such as microaspiration of oesophageal gland cells, direct effector protein analysis using two-dimensional electrophoresis followed by microsequencing based identification, mass spectrometry, genome and transcriptome-based comparative bioinformatic identification (Abad et al., 2008; Bellafiore et al., 2008; Caillaud et al., 2008; Fanelli et al., 2014; Fosu-Nyarko et al., 2016; Haegeman et al., 2011b; Huang et al., 2003; Jaubert et al., 2002; Nicol et al., 2012; Opperman et al., 2008).

Despite the agricultural importance of *Pratylenchus* as pests of economically important crops such as wheat, barley and chickpea, most data generated by NGS technologies (454 and Illumina) has focused on gaining insights for molecular mechanisms of the sedentary PPN - RKNs and CNs (Castillo et al., 1998; Doyle et al., 1987; Gokte-Narkhedkar, 2015; Nicol et al., 1999; Thompson et al., 1999; Van Gundy et al., 1974). Nevertheless. transcriptome analyses have now been published for *P. coffeae*, *P. thornei*, *P. zeae* and *P. penetrans* (Fosu-Nyarko et al., 2016; Haegeman et al., 2011b; Maier et al., 2013; Vieira et al., 2015). In general, the transcriptomes of *P. thornei*, *P. coffeae*, *P. zeae* and *P. penetrans* exhibit common features, including transcripts encoding a similar range of CWDEs, with a slight reduction in number of such enzymes compared to RKNs, CNs and false root-knot nematodes (Vieira et al., 2015).

Comparative bioinformatics of the *P. thornei* transcriptome data show some *P. thornei* effector protein orthologues to known parasitism genes including those from other PPNs (Jones and Fosu-Nyarko, 2014; Nicol et al., 2012). Hence, in the absence of *P. thornei* genomic data, the currently available transcriptomic data provides crucial new information that can increase the existing understanding of parasitic genes and their functions in not only *P. thornei* but also other RLNs. To date, there are 8,232 nucleotide sequences of Pratylenchidae curated at NCBI nucleotide database (5 August 2017) of which 8,220 sequences belong to 20 different *Pratylenchus* species with majority of the sequences (6,667 mRNA nucleotide sequences) belonging to *P. thornei* transcriptome assembly, PRJNA67871 (Nicol et al., 2012).

3.1 Aims and objectives of Chapter 3

Therefore, the overall aims of this chapter were to identify potential parasitism effectors of *P. thornei in silico* that can be deployed as RNAi targets. The objectives were to perform *in silico* comparisons of all available *P. thornei* TSA contigs with the available mRNA nucleotide sequences or complete coding sequences (complete cds) of known and published PPN effectors that may have a direct role in pathogenicity, and to identify sequence homologies. Selected putative parasitism effector sequences identified in *P. thornei* were used for further characterisation by routine and Rapid Amplification of cDNA Ends (RACE) PCRs to identify partial or full-length sequences followed by Sanger sequencing and annotation using publicly available sequences.

3.2 Materials and methods

3.2.1 Bioinformatic analyses of *P. thornei* TSA for effector identification

All available 6,667 *P. thornei* TSA mRNA nucleotide sequences as contigs deposited as BioProject PRJNA67871 at the NCBI were downloaded on November 9, 2014. A sequence homology search using, TBLASTX 2.2.26+ (Altschul et al., 1997) was performed using CLC Genomics Workbench 7.0.3 (www.clcbio.com). To find sequence similarities between selected effector cds and *P. thornei* TSA sequences for putative parasitism effectors, a BLAST search was conducted with an expected value (e-value) cut-off of 1.0E-05 against the local database created from all the available *P. thornei* TSA contigs. The resulting alignments were retrieved with e-values, accession details, bit scores, etc. for further analysis useful in molecular characterisation.

3.2.2 Selection of target effector genes

Complete cds of effectors identified in four publications were downloaded from NCBI and a list of 88 PPN effectors without duplicates was generated after visual comparison between the published lists or tables (Abad et al., 2008; Haegeman et al., 2013; Haegeman et al., 2011b; Nicol et al., 2012). Although most of these effector genes played some role during nematode parasitism, their role in the life of the migratory PPNs, such as *P. thornei* was not clear, for example, 10A06, 14-3-3b, 16D10, 19C07, 7E12, annexin, calreticulin, CLE peptides, ERp99, and galectin. Hence, only 21 of the total 88 effectors were selected based on their possible direct

role during parasitism of host roots and are indicated by an asterisk in Appendix 1. The selected effector genes may play a direct role in host invasion, migration and feeding in host tissue, plant cell wall degradation, intracellular digestion, plant defence suppression, detoxification of reactive oxygen species (ROS), protein degradation and avirulence. Additionally, the selected effector genes have also been identified *in silico* in either or all of the three transcriptomes of *Pratylenchus* species, namely, *P. thornei*, *P. coffeae* and *P. zeae,* (see Table 1.1 (Jones and Fosu-Nyarko, 2014).

3.2.3 Molecular characterisation of putative effector sequences of *P. thornei*

Selected putative parasitism effector sequences identified in *P. thornei* further characterised by PCRs, Sanger sequencing and annotation using publicly available sequences. Broadly, two types of PCRs were done; routine PCRs to amplify the selected fragment subsequently used for cloning into *in vitro* transcription and binary vectors, and RACE PCRs to obtain partial or full-length sequences of the selected putative parasitism effectors.

3.2.3.1 Characterisation and validation of putative effector sequences of *P. thornei* using routine PCRs

RNA extraction from mixed stages of *P. thornei* and cDNA synthesis was done as per section 2.3. GSPs with suitable restriction enzyme sites designed manually to amplify five target putative parasitism effector sequences as per Table 2.5 were used to carry out routine PCRs as described in section 2.6.1. Agarose gels electrophoresis and clean-up of excised fragments was done as described in sections 2.7 and 2.9, respectively. Cleaned fragments were then cloned into pDoubler and clones sequenced as described in sections 2.10 and 2.15, respectively. Sequence chromatograms generated from Sanger sequencing were used to retrieve the nucleotide information in FASTA format, and this was then used to confirm the identities of cloned sequences by carrying out an alignment of the cloned sequences and the originally selected *P. thornei* contigs in BLASTN using the default algorithm parameters. Wherever necessary, sequences were checked for the presence or absence of restriction enzyme sites using NEB cutter (http://nc2.neb.com/NEBcutter2/).

3.2.3.2 Characterisation and validation of putative effector sequences of *P. thornei* using RACE PCRs

A mixed-stage *P. thornei* population was collected and cleaned as described in section 2.2 and used for kit-based RNA extraction.

3.2.3.2.1 RNA extraction for RACE PCRs

RNA was extracted using a NucleoSpin® RNA XS kit (Macherey-Nagel, Germany), following the manufacturer's protocols. Fresh samples were prepared as a *P. thornei* pellet, approximately 300 mg, and stored at -80°C in a 1.5 mL sterile microfuge tube until the procedure was started. First, 200 µL buffer RA1 and 4 µL TCEP was added to the whole nematode sample and vortexed vigorously twice for 5 seconds. Nematode tissue was disrupted using a rotor-stator homogeniser or with a shaker and sterile steel beads. Working solution of carrier RNA at the volume of 5 µL was added to every 20 ng of lysate, followed by vortexing twice for 5 seconds after which the tubes were centrifuged at 1000 g for less than 5 seconds to clear the lid. The lysate was filtered through the NucleoSpin Filter to make it clear by reducing the viscosity. This was done by placing the filter in a collection 2 mL tube then applying the lysate mixture and centrifugation at 11,000 g for 30 seconds. The NucleoSpin Filter was discarded and 200 µL of 70% ethanol was added to the filtered homogenised lysate and mixed by pipetting five times. A stringy precipitate was visible after addition of ethanol; the precipitate was mixed well prior to loading the ethanolic lysate onto the column in order to prevent pelleting of the precipitate. For each preparation, a single NucleoSpin RNA XS Column was placed in a collection tube and not more than 600 µL lysate was loaded onto the column. The column was centrifuged for 30 seconds at 11,000 g. Then, 100 µL membrane desalting buffer was added to the column and centrifuged at 11,000 g for 30 seconds to help dry the membrane.

DNA digestion was done first by preparing rDNase reaction mixture in a sterile microfuge tube; 3 µL of this reconstituted rDNase was added to 27 µL reaction buffer for rDNase. The tube was mixed well by flicking, 25 µL of this rDNase reaction mixture was applied directly onto the center of the silica membrane of the column followed by closing the lid and incubation at room temperature for 15 minutes. The silica membrane was then washed and dried. This process involved three washes; in the first wash, 100 µL buffer RA2 was added to the NucleoSpin RNA

XS column followed by incubation for 2 minutes at room temperature and centrifugation for 30 seconds at 11,000 g. Before the second wash, the column was placed in a new sterile 2 mL collection tube. Then, 400 μL of buffer RA3 was added to the column and centrifuged for 30 seconds at 11,000 g after which the flow-through was discarded and the column placed back into the collection tube. For the third wash, 200 μL of buffer RA3 was added followed by centrifugation for 2 min at 11,000 g. Finally, the column was placed in a nuclease-free 1.5 mL collection tube before proceeding to the final elution step.

RNA elution was done using 5 to 30 μL RNase-free water and centrifugation for 30 seconds at 11,000 g. For higher concentrations of RNA, elution through the same column was repeated using 15 μL RNase-free water. The final RNA eluate was stored on ice and no clean-up and concentration step was done. Total RNA was quantified using a Nanodrop and Bioanalyser after which it was stored at -80°C until further use.

3.2.3.2.2 RACE PCR Primers

The first set of primers were designed from the TBLASTX results and alignments of five target putative parasitism effector sequences of *P. thornei* with the known effectors. SMARter™ RACE cDNA Amplification Kit recommended multiple parameters for designing GSPs, to which 15 bp overlapping sequence homologous to the vector, pRACE, are added for infusion cloning. However, the 15 bp homology sequence was not mandatory if the pRACE vector was not used downstream. The parameters for primer design were: specificity to the sequence of interest, 23-28 nt in length, 50-70% GC content, Tm ≥ 65°C, and the primer sequence should not complement the 3'-end of the universal primer mix. Where ever necessary, nested gene specific primers (NGSPs) were designed based on the parameters recommended by the manufacturer. These were: primers should be designed only when universal primer mix and GSP did not generate a clear RACE product, primers should not overlap with the outer GSP, and meet all previous parameters listed above.

Table 3.1. List of primers used for RACE PCRs, *P. thornei* TSA contigs used for primer design and the total size of the primers.

No.	Primer Name	PCR type	Primer Sequence	TSA Contig used for primer design	Total size of primer
1	PtEng1-GSP1	5' RACE	GCCTTGTTCCGCAGCTCCTGTTTG	JN052046	24
2	PtEng1-GSP2	3' RACE	CTACGAGACATTCAATGAGCCACTGC	JN052046	26
3	PtEng1-NGSP2-A	3' Nested RACE	GGTCAGGAGTGAAGTCCTACCACCAGGCAG	JN052046	30
4	PtCLP-GSP2	3' RACE	CTCTTGCTGGGCCTTCAGTTCTAC	JO845319	24
5	PtPL-GSP1	5' RACE	CGA CAG AAG TTC ACA ACA GCA CAG C	JO845337	25
6	PtUEP-GSP2	3' RACE	GACTCTCACCGGAAAGACCATTACC	JO845330	25
7	PtGST-GSP2	3' RACE	CCAATGCGCCTGATGTTCCATTAC	JL8649731	24

3.2.3.2.3 RACE PCRs

A SMARter™ RACE cDNA Amplification Kit (Clontech, now, Takara Bio, USA) was used to amplify target gene sequences. For generating RACE-ready first-strand cDNA between 10 ng to 1 μg of total or poly A+ RNA could be obtained. A positive control using 1 μg of the supplied mouse heart total RNA was also done. First, buffer mix containing 4 μL of 5X first-strand buffer, 0.5 μL of dithiothreitol (100 mM) and 1 μL of random primer mix (20 mM) using 5.5 μL was prepared and incubated at room temperature. To appropriately labelled tubes of 5' and 3' reactions, about 1 μg *P. thornei* RNA was added, followed by 1 μL 5' or 3' RACE CDS Primer A

(12 μM) and appropriate volumes of sterile RNase-free water to a final volume of 11 μL and 12 μL for 5' and 3' reactions, respectively. The contents of the tubes were mixed by pipetting, quickly centrifuged and incubated at 72°C for 3 minutes followed by 42°C for 2 minutes in a thermocycler. Tubes were centrifuges at 14,000 g for 10 seconds, kept at room temperature after which 1 μL of SMARTer II A Oligonucleotide (24 μM) was added to 5' reactions. To the previously prepared buffer mix tubes, 0.5 μL of RNase inhibitor (40 U/μL), 2 μL of SMARTScribe Reverse Transcriptase (100 U/μL) was added and 8 μL of this mixture was added to the 5' and 3' reactions. The 20 μL reaction was then mixed gently, briefly centrifuged, incubated at 42°C for 90 minutes followed by 70°C for 10 minutes and temporarily held at 4°C. The first-strand cDNA synthesis reactions were diluted by adding 90 μL tricine-EDTA buffer to all the reaction tubes. These stock tubes of 3' and 5'-RACE-ready cDNA were stored at -20°C for up to three months or longer at -80 °C until proceeding to RACE PCR reactions.

RACE PCRs were done using 5' and 3' RACE-ready cDNA, with appropriate negative controls included. First, master mix consisting of 25 μL of SeqAmp PCR buffer, 1 μL of SeqAmp DNA polymerase, with appropriate volumes of sterile RNase-free water was prepared to make a reaction volume of 41.5 μL on ice. RACE reaction tubes were then labelled to which 2.5 μL of 5' or 3' RACE-ready cDNA, 5 μL of 10X universal primer A mix, 5' or 3' GSPs, and 41.5 μL of master mix was added. PCRs were done using the following settings: 94°C for 1 minute, 30 cycles of 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 3 minutes, and final extension at 72°C for 7 minutes.

RACE PCR products were visualised on 1.2% agarose gel and compared to 1 kb DNA ladder. For this, a 5 μL aliquot of the samples were used. If a clear signal was observed the DNA band was carefully excised and stored in a pre-weighed Eppendorf at 4°C. If a weak signal was observed, then the sample was subjected to five more PCR cycles and then visualised once again on a gel. If the signal was found to be weak or smeared then, nested primers were designed and nested RACE PCRs were done using the universal primer short (10 μM). To do this, 5 μL of the primary PCR product was added to 245 μL of Tricine-EDTA buffer and PCRs were setup using 5 μL of the diluted primary PCR product instead of RACE-ready cDNAs, 1 μL of universal primer short

(10 μM) and 1 μL of 10 pm/μL appropriate nested GSPs. RACE products were cleaned as described in section 2.9 and stored at -20 °C until further use.

3.2.3.2.4 Sequencing and analysis of RACE products

RACE PCR products were sequenced by Sanger sequencing using appropriate GSPs depending on the product, either 3' or 5' RACE. Due to high GC content and previous unsuccessful attempts using 1/16th reaction volumes, template for sequencing was prepared with 1/2 reaction volumes of the recommendation reaction mixture. For this, 3.2 pm/μL of the appropriate primer, 1 X reaction buffer, 4 μL each of Big Dye terminator (dye T) and dGTP mix, and 5-20 ng or 10-40 ng DNA for 500 bp-1 μg or1 kb-2kb, respectively, in a total volume of 10 μL was prepared. Reactions were placed in a thermal cycle set at a hold temperature of 96°C for 2 minutes followed by 25 cycles of denaturation at 98°C for 10 seconds and annealing/extension at 60°C for 4 minutes with a final hold at 4°C. Clean up of the sequencing product was done as in section 2.15.

To validate the identity of the RACE products, sequence chromatogram files generated from sequencing were used for *in silico* comparison of nucleotide sequences retrieved in FASTA format. For a quick assessment, the nucleotide sequences were used as queries in BLASTX with an e-value cut-off of 1e-05 to identify the sequences producing significant alignments or top matches. However, this information was not enough to identify multiple sequence alignments and conserved domains within those alignments, and hence, other approaches were also used. First, these nucleotide sequences were converted into virtual amino acid sequences in different reading frames using the ExPasy Translate tool (http://web.expasy.org/translate/) with the default settings: output format (Verbose -'Met', 'Stop', spaces between residues) and standard genetic code (Artimo et al., 2012; Gasteiger et al., 2003). The open reading frame yielding maximum information or length of amino acid sequence was selected to carry out an InterPro scan to identify protein families and conserved domains within the selected sequence (http://www.ebi.ac.uk/interpro/search/sequence-search) (Finn et al., 2016; Marchler-Bauer et al., 2006; Marchler-Bauer et al., 2010). The virtual amino acid sequences of each of the RACE PCR products representing the target genes used for analysis is given in Appendix 2.

Additionally, BLASTP to obtain detailed information on the sequences producing most significant alignments (top matches based on e-values) and putative conserved domains was done. BLASTP with an e-value cut-off of 1e-05 against the non-redundant protein (nr) sequence databases was done using the same query. BLASTP provided results that were also supported by other useful reports such as taxonomy reports, distance tree of results, related structures and multiple alignments. The resulting matches were downloaded and then assessed using CLUSTAL OMEGA multiple alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) and constraint-based multiple alignment (COBALT, https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) where protein sequences resulting from BLASTP were aligned to the protein sequences of the RACE product using default parameters. In this way, conserved domains within the alignment were checked thus validating and annotating the identity of the RACE products (Sievers et al., 2011).

3.3 Results

3.3.1 *In silico* comparison and selection of putative parasitism effector sequences in *P. thornei*

The TBLASTX program using an e-value cut-off of 1.0E-05 to identify any sequence similarities between the nucleotide sequences of 21 selected known effectors was used as query and the 6,667 available *P. thornei* TSA contigs yielded seven effector matches to different contigs. The remaining 14 selected effectors did not match any TSA contigs. The TBLASTX results for the seven effectors that showed sequence homologies to TSA contigs is given in Table 3.2. Of these, five effectors, indicated by asterisks in Table 3.2, were selected for molecular characterisation, whilst the remaining two effector genes were not advanced. The latter were *P. thornei* matches to ubiquitin extension protein mRNA of *H. glycines* and the *Tpx* encoding peroxiredoxin of *G. rostochiensis*. Between the *P. thornei* sequences matching to the two ubiquitin extension proteins used as queries, the *Ubi1* of *H. schachtii* was chosen. This was because, it had a lower e-value, greater number of high-scoring segment pairs (HSPs) and HSP length compared to the *P. thornei* sequence that matched the ubiquitin extension protein mRNA of *H. glycines.* The *P. thornei* sequence that matched the *Tpx* gene encoding peroxiredoxin of *G. rostochiensis* was selected initially, however, as addressed in the discussion of this chapter, this gene was not advanced for molecular characterisation.

In silico comparison of selected effectors against the *P. thornei* TSA contigs in TBLASTX resulted in a collection of matching contigs each having variable numbers of HSPs, thus generating a summary of HSPs to select from as viewed in CLC Workbench. Since this summary had overwhelmingly large worksheets, it has been condensed in text here.

The selection of contigs as the top hit was based on three main criteria: the corresponding contig sequence matched the query sequence within its cds, the sequence had the lowest e-value, and highest alignment and bit scores. An overview of the resulting contig selection based on these criteria is given in Table 3.2. For example, one contig, JO845337, consisted of four HSPs matching to the *M. incognita* pectate lyase mRNA query, however, the sequence with the best e-value, alignment and bit scores from the contig JO845337 was selected. On the other hand, four contigs, JN052046, JN052045, KM593897 and KM593896 consisting of eight, six, three and three HSPs, respectively, that matched the *M. incognita Eng-1* query. Again, based on the best e-value, alignment and bit scores, the sequence from the contig JN052046 as the top hit was selected. Similarly, 10 contigs, JO845319, JL86713, JO845320, JO845321, JO845322, JO845323, JO845324, JO845325, JO845326 and JL861771 consisting of fifteen, nine, nine, six, three, four, three, three, one and four HSPs, respectively, matched the *M. incognita Cpl-1* query, however, the sequence of the best matching JO845319 was chosen. Likewise, nine contigs, JO845330, JO845331, JL861073, JO845332, JO845333, JO845334, JL865744, JO845335 and JO845336 consisting of eight, eleven, six, four, seven, three, four, one and one HSP(s), respectively, matched the *H. schachtii Ubi* query of which, the sequence from the contig JO845330 was selected. Finally, two contigs, JL864973 and JL863495 consisting of five and two HSPs, respectively, matched the *M. incognita Gsts-1* query, of which the sequence from JL864973 was chosen.

The sequence of each effector gene queried that best matched to *P. thornei* TSA contigs is shown as a yellow line in Figure 3.1A to 3.1E. Then, using an online tool, NEB cutter, the corresponding contig sequences were checked for the presence of restrictions enzymes that appeared in the *in vitro* and binary vectors designed for subsequent cloning purposes. If a restriction enzyme site was found within the corresponding contig sequence then this area was not chosen thus making the available sequence length smaller. This resulted in nine restriction enzyme sites for *AscI*, *BamHI*, *KpnI*, *XhoI*, *AfeI*, *XbaI*, *AvrII*, *NotI* and *EcoRI* that were

not within the sequence of interest on the contig. Subsequently, the sequence marked in red on the selected contigs was used for cloning and dsRNA synthesis (Figure 3.1A to 3.1E).

A 146 bp-long sequence of the *P. thornei* contig, JO845337, best matched the *M. incognita* pectate lyase mRNA query from 367 nt to 513 nt, however, due to the presence of the desired restriction enzyme sites within the sequence itself, a 115-bp long sequence between 393 nt and 508 nt of JO845337 was selected (Figure 3.1A). Similarly, a 164 bp-long sequence of the contig JO845330 was selected that best matched the *H. schachtii Ubi* query between 70 nt to 294 nt (Figure 3.1B).

Likewise, the sequence between 10 nt to 333 nt of *M. incognita Gsts-1* query best matched the contig JL864973 of which 274 bp sequence and was chosen (Figure 3.1C). A 344 bp-long sequence of the contig JO845319 that best matched the *M. incognita Cpl-1* query within the area between 760 nt to 1152 nt was chosen (Figure 3.1D). Finally, a 220 bp-long sequence of the contig JN052046 was chosen which best matched the *M. incognita Eng-1* query between 406 nt to 711 nt (Figure 3.1E). Thus, five sequences were chosen as target putative parasitism effector sequences for further RNAi studies in Chapters 4 and 5.

Based on the TBLASTX results and alignments of five target putative parasitism effector sequences of *P. thornei* with the known effectors (Table 3.2 and Figure 3.1), primers for RACE PCRs were designed. The nucleotide region of the TSA contigs corresponding to the sequence of the known effectors (yellow horizontal line in Figure 3.1) was used for RACE PCR primer design. In summary, the aligning nucleotide regions on the *P. thornei* TSA contigs were 146 bp, 224 bp, 323 bp, 393 bp and 305 bp-long for pectate lyase (JO845337), ubiquitin extension protein (JO845330), glutathione S-transferase (JL8649731), cathepsin L-protease (JO845319) and endoglucanase (JN052046), respectively.

Table 3.2. TBLASTX results for the subset of seven selected effectors showing matches to *P. thornei* TSA contigs.

No.	Effector gene	Organism	Accession used as query	Total length of sequence (bp); total length of cds (bp);start and stop position of cds (nt)	TBLASTX against the TSA of *P. thornei*						
					Accession with top hit	Total length of accession (bp)	No. of HSPs	E-value	Greatest identity %	Greatest HSP length	Greatest bit score
1*	Pectate lyase mRNA	*M. incognita*	AF527788	1124; 816; 32-847	JO845337	593	4	5.64E-25	57.14	147	67.97
2	Ubiquitin extension protein mRNA	*H. glycines*	AF473831	318; 318; 1-318	JO845331	572	40	1.67E-37	100	219	149.53
3*	*Ubi1* (Ubiquitin extension protein)	*H. schachtii*	AY286305	369; 369; 1-369	JO845330	2288	45	1.78E-43	100	225	169.69
4*	*Gsts-1* (Gluthione-S-transferase)	*M. incognita*	EF429119	747; 612; 40-651	JL864973	642	7	6.89E-44	62.96	324	171.97
5†	*Tpx* (Peroxiredoxin)	*G. rostochiensis*	AJ243736	846; 600; 48-647	JL866118	401	3	1.27E-47	82.42	273	184.35

6*	*Cpl-1* (Putative cathepsin-L-protease)	*M. incognita*	AJ557572	1152; 1152; 1-1152	JO845319	1149	57	6.18E-88	90	393	221.55
7*	*Eng-1* (β-1,4-endoglucanase)	*M. incognita*	AF100549	1168; 1521; 26-1546	JN052046	824	20	8.49E-107	100	315	210.95

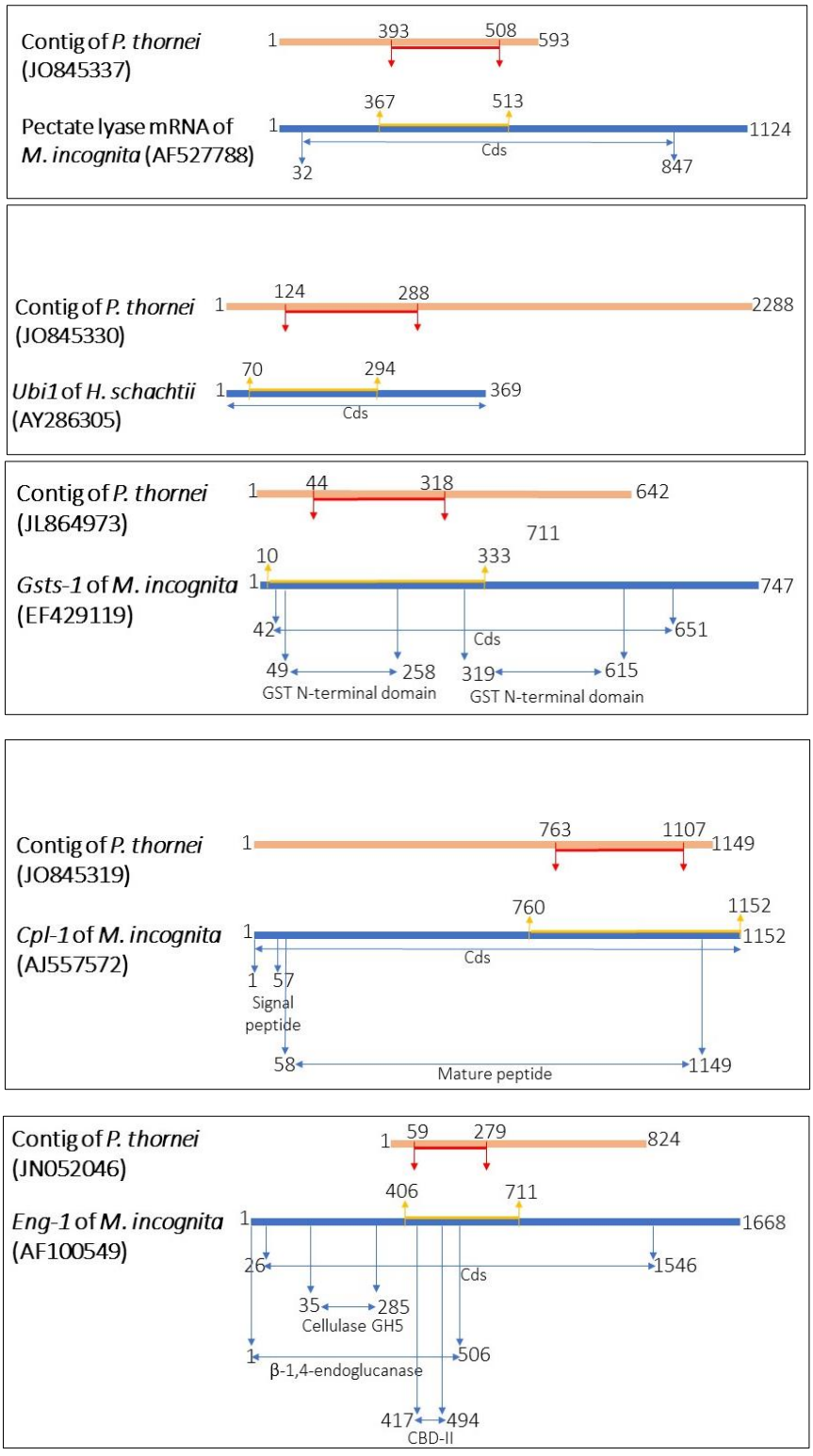


Figure 3.1. Diagrammatic representation of the alignment of five target putative parasitism effector sequences in *P. thornei* with the known effectors used as query for *in silico* searches. Alignments of *P. thornei* contigs matching nucleotide query sequences of pectate lyase (A), ubiquitin extension protein (B), glutathione S-transferase (C), cathepsin L-protease (D) and endoglucanase (E). Yellow horizontal lines on the query show the region of sequence having

the best matches to *P. thornei* contigs and the nucleotide position number is given by yellow arrows. Red horizontal lines on the contigs show the corresponding matching area used for cloning and characterisation and is marked by red arrows to indicate the nucleotide position. All information marked on the known effector sequences are adapted from NCBI. (Images not to scale).

3.3.2 Characterisation and validation of putative parasitism effector sequences of *P. thornei* used for RNAi studies

The cloned sequences were referred to as *Pt-Eng-1*, *Pt-CLP*, *Pt-PL*, *Pt-UEP* and *Pt-GST* representing β-1, 4-endoglucanase, cathepsin L-protease, pectate lyase, ubiquitin extension protein and glutathione S-transferase, respectively, and their clones were referred by the same label except with the prefix 'pDo' that stands for the vector, pDoubler. The identities of cloned sequences were validated by carrying out an alignment with the originally selected *P. thornei* contigs in BLASTN using the default algorithm parameters. These results gave positive matches to original sequences and showed high percent identities and low e-values thus validating the identities of the cloned sequences (Table 3.3). Subsequently, these sequences were targeted through *in vitro* and host-mediated RNAi in Chapters 4 and 5.

Table 3.3. BLASTN results showing alignment of cloned sequences to the selected *P. thornei* TSA contigs.

pDoubler clone	Maximum score	Query cover (%)	E-value	Percent (%) identity	*P. thornei* contig accession number
pDo*Pt-Eng-1*	172	33	5e-47	90	JN052046
pDo*Pt-CLP*	423	23	5e-122	92	JO845319
pDo*Pt-PL*	99	5	1e-24	92	JO845337
pDo*Pt-UEP*	86	5	9e-21	95	JO845330
pDo*Pt-GST*	250	17	4e-70	89	JL864973

3.3.3 Determination of quality and quantitation of RNA for RACE PCRs

The quality and integrity of RNA samples used for RACE-ready first-strand cDNA synthesis was checked by Nanodrop spectrometry and also on an Agilent 2100 BioAnalyzer (Agilent Technologies, CA, USA). All procedures that used the bioanalyser were kindly done by Frances Brigg. The SMARter™ RACE cDNA Amplification Kit (Clontech, now, Takara Bio, USA) manual recommended RNA concentrations greater than 20 ng/µL as acceptable after purification and useable for checking RNA integrity number (RIN), which is suggested to be 7 or higher for RACE protocols.

From the Nanodrop, the concentration of RNA was found to be 85 ng/µL with acceptable ratios of 2.0 for absorbance at 260/280 and 260/230. However, it did not give information on the ratio of 28S:18S rRNA. The results of assessing total RNA integrity using a bioanalyser provided information on concentration and integrity (Figure 3.2). The first peak represents the marker followed, by very small peaks that represent small RNAs, the second and third peaks indicate the intactness of 18S and 28S rRNA, respectively. The fluorescence emitted by 28S rRNA indicates its proportion with respect to 18S rRNA. These can be visualised as clear bands in the lane on the right of Figure 3.2; the green line indicates that the marker peaks were aligned to the ladder. The RIN value of 9.6 was of good quality and this RNA sample was used for RACE-ready first-strand cDNA synthesis.

3.3.4 Characterisation and validation of putative parasitism effector sequences of *P. thornei* using RACE PCRs

RACE PCRs were done to try to obtain full-length or partial sequences of the five target putative parasitism genes. However, only four of them were successfully amplified and characterised. These final RACE PCR products were referred to as *Pt-Eng-1*, *Pt-CLP*, *Pt-PL*, and *Pt-GST,* representing β-1, 4-endoglucanase, cathepsin L-protease, pectate lyase, ubiquitin extension protein and glutathione S-transferase, respectively. These were sequenced by Sanger sequencing. The identities of the nucleotide sequences were validated by carrying out alignments and BLAST functions to compare with nr protein databases on the NCBI as described below for each gene.

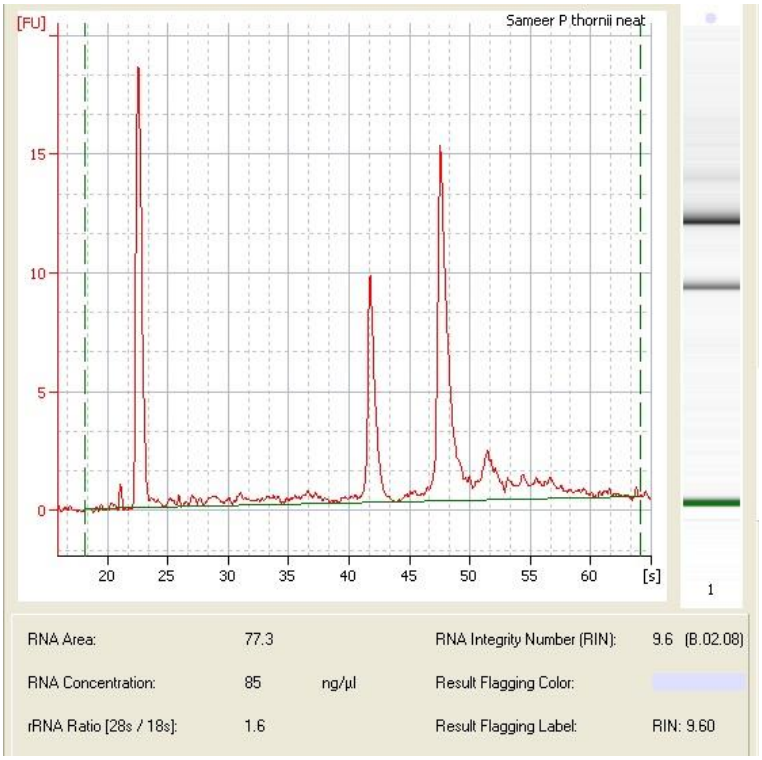


Figure 3.2. Assessment of integrity of total RNA extracted from *P. thornei* using an Agilent 2100 BioAnalyzer.

3.3.4.1 Characterisation and validation of *Pt-Eng-1*

For *Pt-Eng-1*, a 5'RACE PCR and 3' nested RACE PCR were done which resulted in the amplification of DNA bands at approximately 800 bp and 1.1 kb, respectively, on 1.2% agarose gel. The identities of these nucleotide sequences were first subjected to BLASTX for a quick assessment of the matching proteins. For 5'*Pt-Eng-1*, the results based on lowest e-values and highest Hsp bit scores revealed matches to cellulase glycoside hydrolase family 5 (GHF5) from eukaryotic and bacterial genera. The eukaryotic matches comprised protein sequences belonging to PPN genera *Meloidogyne*, *Radopholus*, *Aphelenchoides*, *Ditylenchus*, *Hirschmanniella*, *Heterodera* and *Globodera,* with majority belonging to *Pratylenchus* spp. In the *Pratylenchus* genera the protein sequences matched to *P. vulnus*, *P. thornei*, *P. neglectus*, *P. penetrans*, *P. coffeae*, *P. pratensis*, *P. convallariae*, *P. goodeyi* species. The top five protein matches belonged to endoglucanase-2 precursor of *P. vulnus* (CDM79919), β,1-4 endoglucanase-1 precursor of *P. vulnus* (CDM79917), and partial β,1-4 endoglucanase-1

sequences of *P. thornei* (AER27782), *P. vulnus* (AER27792) and *P. neglectus* (AER27769). The BLASTX result also indicated the presence of conserved domains belonging to cellulase GHF5 at 17 to 358 nt.

The virtual protein or amino acid sequence of 274 aa derived from ExPasy used as a query in BLASTP resulted in similar protein matches as described for BLASTX with four of the top five hits (CDM79919, CDM79917, AER27782 and AER27769) being the same in addition to partial β,1-4 endoglucanase-1 sequence of *P. neglectus* (AER27770). The taxonomy BLAST report indicated most matching hits to *Pratylenchus* spp., a total of 27 hits, with *P. vulnus* and *P. penetrans* having 11 and five, respectively. The BLASTP results showed conserved domains of cellulase GHF5 and aryl-phospho-beta-D-glucosidase BglC GHF1 (carbohydrate transport and metabolism) at amino acid intervals 87-266 and 154-244, respectively. The constraint-based multiple alignment of the amino acid sequence of 5'*Pt-Eng-1* query against the top five hits using COBALT resulted in similarity of conserved domains across the matches (Figure 3.3). Additionally, when the virtual protein of 5'*Pt-Eng-1* was analysed for protein classification using InterPro, detailed signature matches yielded glycoside hydrolase superfamily (IPR017853) between 75 to 266 aa of which 90 to 266 aa represented the conserved cellulase domain (Finn et al., 2016).

A phylogenetic tree was produced using BLASTP pairwise alignments of 100 closest homologous sequences that matched to 5'*Pt-Eng-1* query as a result of BLASTP and is shown in Figure 3.4. The 5'*Pt-Eng-1* query is shown in yellow highlight and was most closely related to partial β,1-4 endoglucanase-1 sequences of *P. thornei* (AER27781 and AER27782) followed by several other *Pratylenchus* spp. and one partial β,1-4 endoglucanase-1 sequences of *M. artiellia* (AER27761) (Figure 3.4).

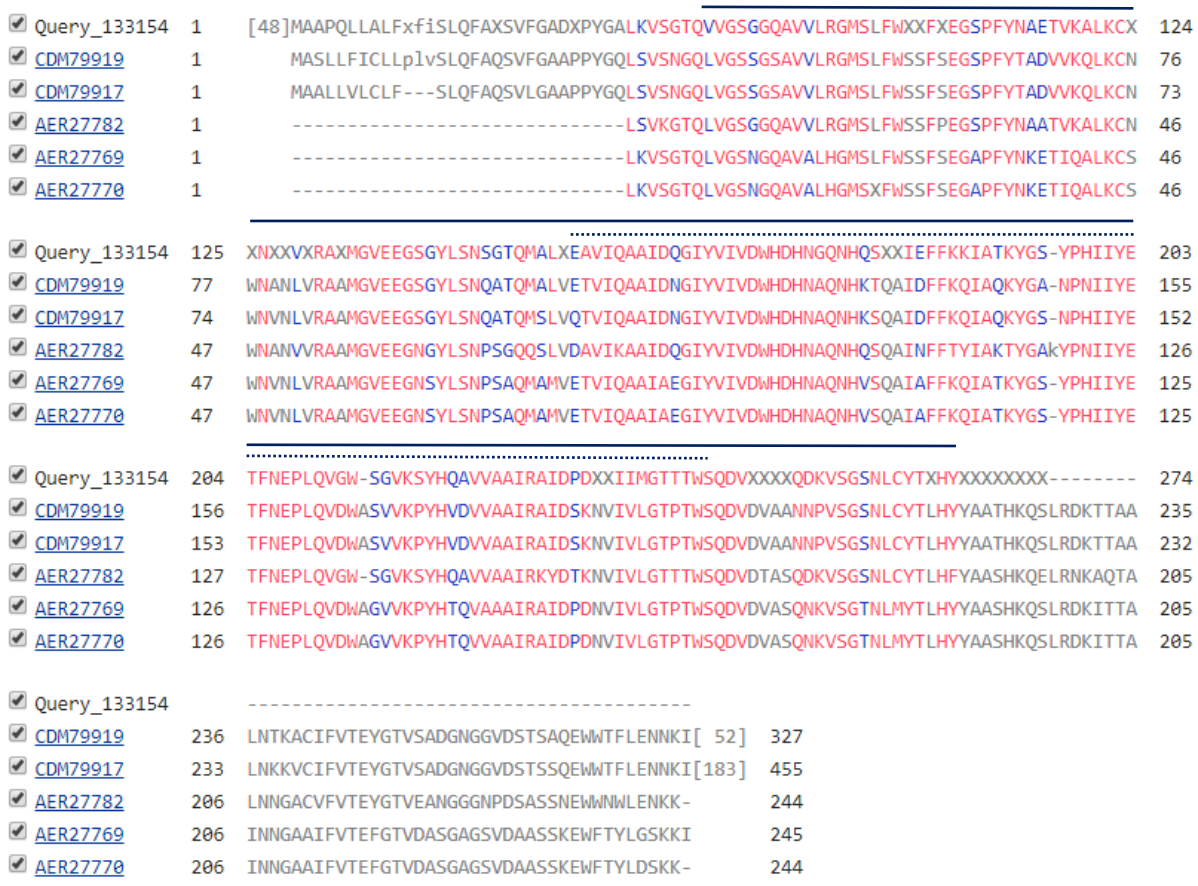
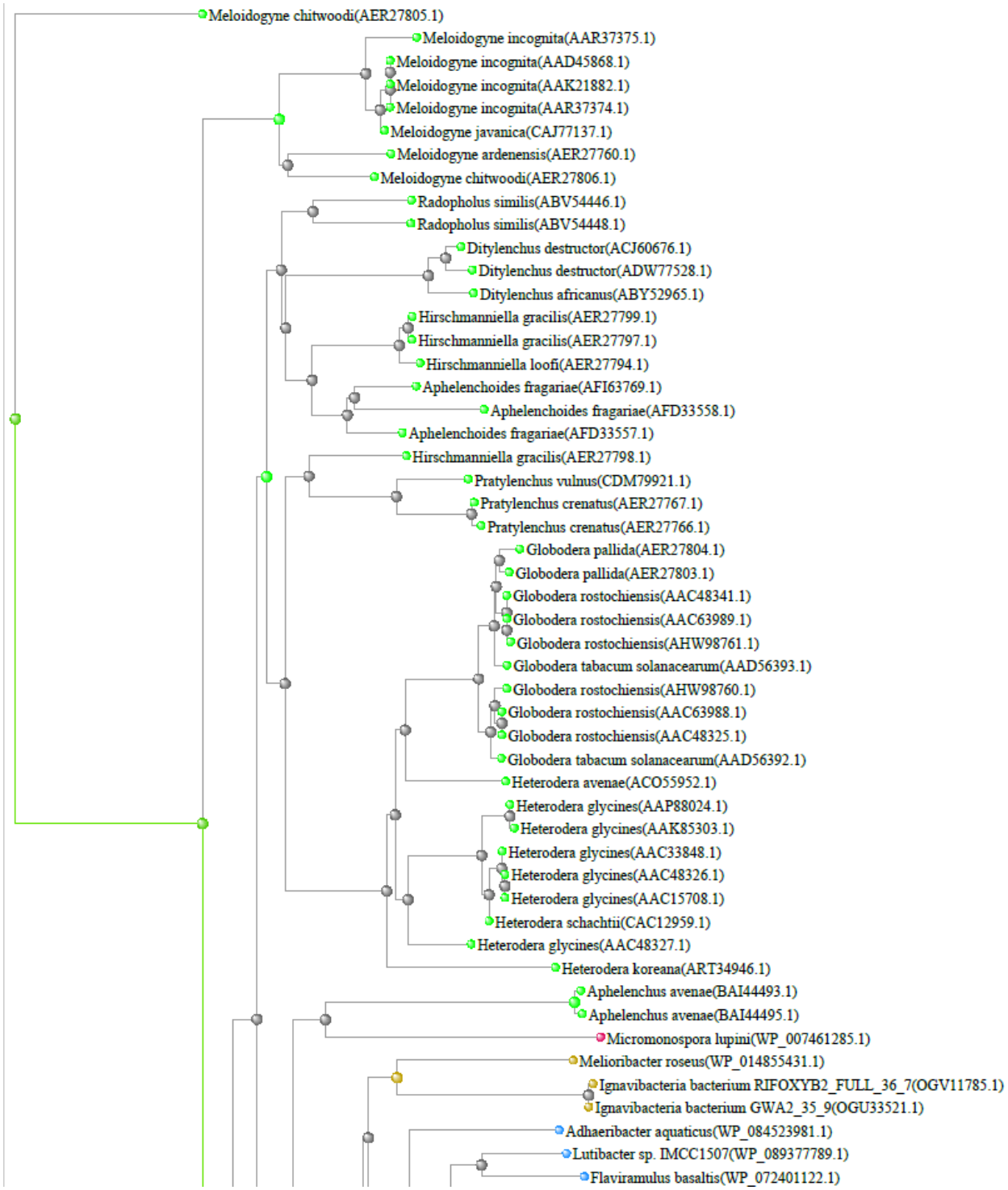


Figure 3.3. Multiple sequence alignment of the amino acid sequence of 5'*Pt-Eng-1* query against the top five hits as a result of BLASTP. The red and blue coloured letters indicate highly conserved and less conserved residues, respectively. The solid and dotted horizontal lines indicate the presence of a conserved cellulase domain and aryl-phospho-beta-D-glucosidase BglC GHF1.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Meloidogyne incognita(AAR37374.1)
Meloidogyne javanica(CAJ77137.1)
Meloidogyne ardenensis(AER27760.1)
Meloidogyne chitwoodi(AER27806.1)
Radopholus similis(ABV54446.1)
Radopholus similis(ABV54448.1)
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Ditylenchus destructor(ADW77528.1)
Ditylenchus africanus(ABY52965.1)
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Hirschmanniella loofi(AER27794.1)
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Aphelenchoides fragariae(AFD33558.1)
Aphelenchoides fragariae(AFD33557.1)
Hirschmanniella gracilis(AER27798.1)
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Pratylenchus crenatus(AER27767.1)
Pratylenchus crenatus(AER27766.1)
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Globodera rostochiensis(AHW98761.1)
Globodera tabacum solanacearum(AAD56393.1)
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Globodera rostochiensis(AAC63988.1)
Globodera rostochiensis(AAC48325.1)
Globodera tabacum solanacearum(AAD56392.1)
Heterodera avenae(ACO55952.1)
Heterodera glycines(AAP88024.1)
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Heterodera glycines(AAC33848.1)
Heterodera glycines(AAC48326.1)
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Lutibacter sp. IMCC1507(WP_089377789.1)
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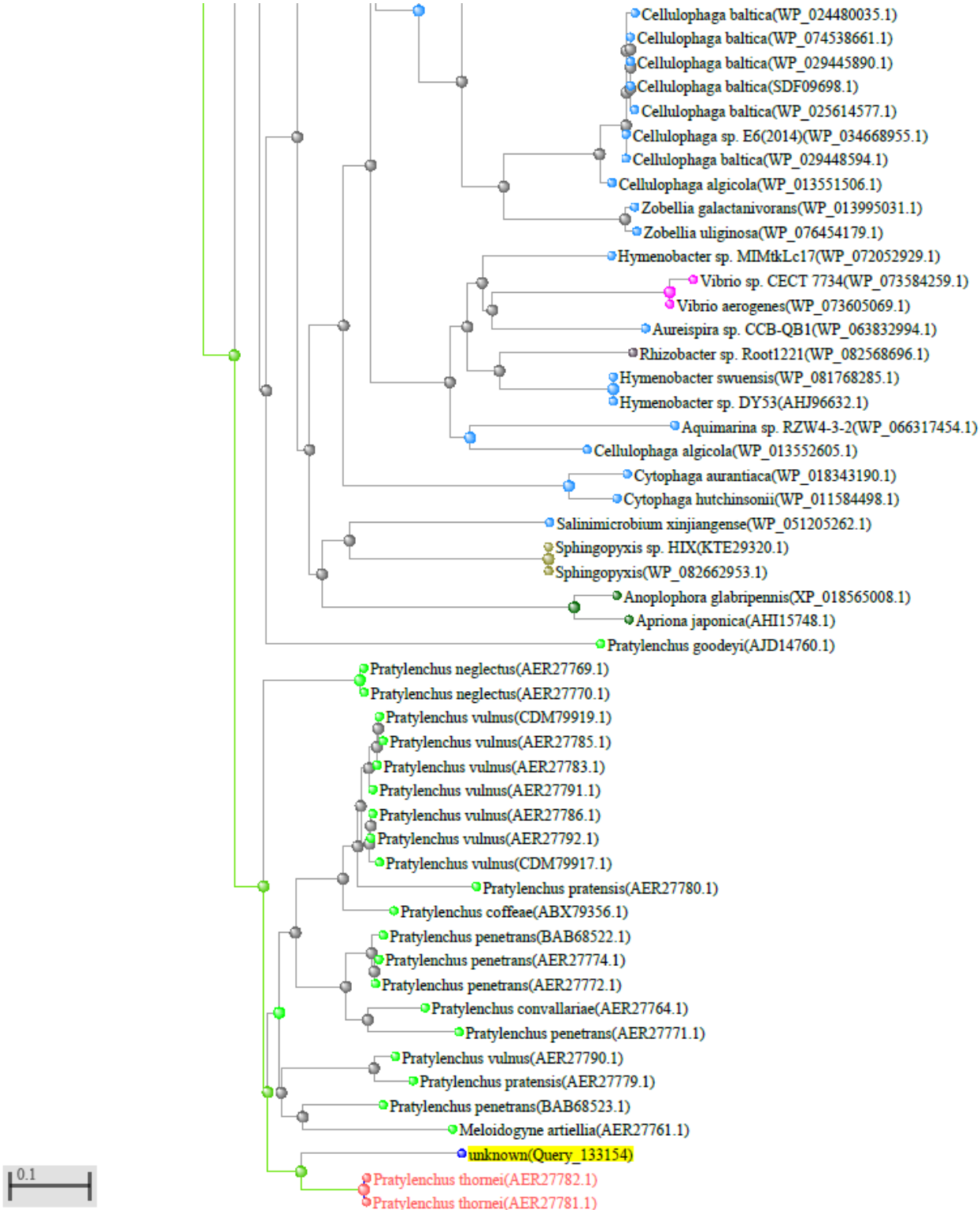
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Pratylenchus neglectus(AER27770.1)
Pratylenchus vulnus(CDM79919.1)
Pratylenchus vulnus(AER27785.1)
Pratylenchus vulnus(AER27783.1)
Pratylenchus vulnus(AER27791.1)
Pratylenchus vulnus(AER27786.1)
Pratylenchus vulnus(AER27792.1)
Pratylenchus vulnus(CDM79917.1)
Pratylenchus pratensis(AER27780.1)
Pratylenchus coffeae(ABX79356.1)
Pratylenchus penetrans(BAB68522.1)
Pratylenchus penetrans(AER27774.1)
Pratylenchus penetrans(AER27772.1)
Pratylenchus convallariae(AER27764.1)
Pratylenchus penetrans(AER27771.1)
Pratylenchus vulnus(AER27790.1)
Pratylenchus pratensis(AER27779.1)
Pratylenchus penetrans(BAB68523.1)
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Pratylenchus thornei(AER27781.1)
0.1

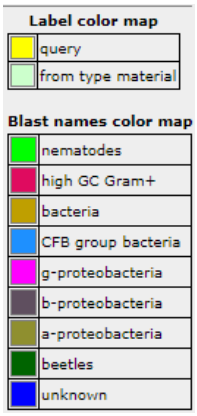


Figure 3.4. Phylogenetic tree representing the position of 5'*Pt-Eng-1* query as a result of BLAST pairwise alignment with respect to its 100 closest homologous amino acid sequences. The horizontal branches represent evolutionary lineages changing over time whilst the horizontal bar of 0.1 at the bottom of the figure provides a scale for the genetic change.

For 3'*Pt-Eng-1*, the absence of a solid band accompanied by smearing required the use of nested RACE PCR which resulted in a 1.1 kb band on gel. Sequencing results were subjected to BLASTX and they indicated that based on lowest e-values and highest Hsp bit scores matches for cellulase GHF5 from eukaryotic and bacterial genera. The eukaryotic matches were comprised of protein sequences belonging to the PPN genera *Meloidogyne*, *Radopholus*, *Aphelenchoides*, *Ditylenchus*, *Hirschmanniella*, *Heterodera*, *Globodera* with majority belonging to *Pratylenchus* spp. In the *Pratylenchus* genera the protein sequences matched to *P. vulnus*, *P. thornei*, *P. neglectus*, *P. penetrans*, *P. coffeae*, *P. pratensis*, *P. convallariae*, *P. goodeyi* species. The top five protein matches belonged to GHF5 endoglucanase precursor of *P. coffeae* (ABX79356), endoglucanase-2 precursor of *P. vulnus* (CDM79919), β,1-4 endoglucanase-1 sequences of *P. penetrans* (BAB68523 and BAB68522) and β,1-4 endoglucanase-1 precursor sequence of *P. vulnus* (CDM79917). The BLASTX results also indicated the presence of conserved domains belonging to cellulase GHF5 between 18 to 338 nt.

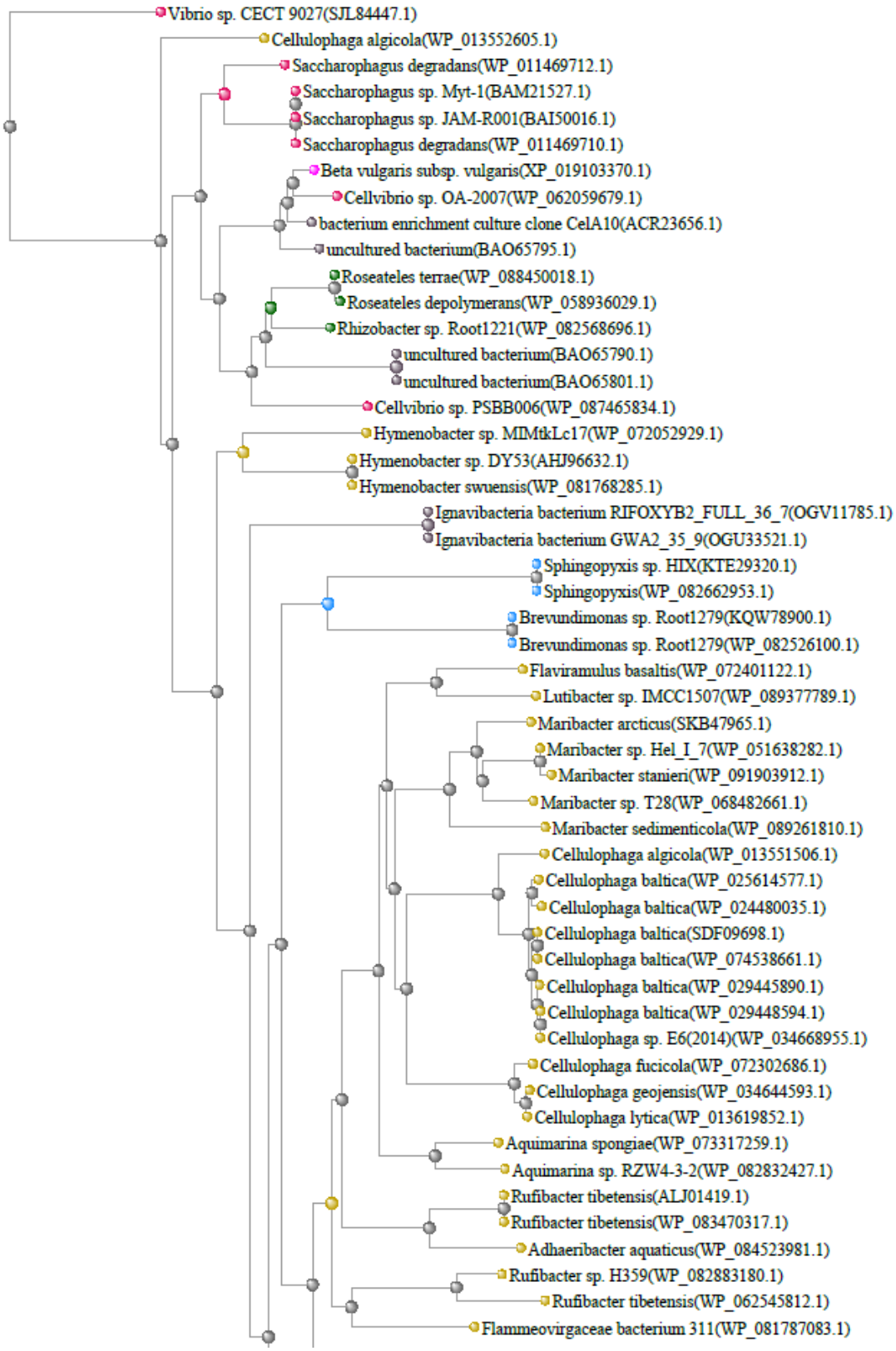
The virtual protein or amino acid sequence of 296 aa derived from ExPasy used as a query in BLASTP resulted in similar protein matches as described for BLASTX with three of the top five hits (ABX79356, BAB68522 and CDM79917) being the same in addition to β,1-4 endoglucanase-1 sequences of *D. destructor* (ADW77528) and GHF5 endoglucanase of *D. africanus* (ABY52965). The taxonomy BLAST report showed the highest number of matching

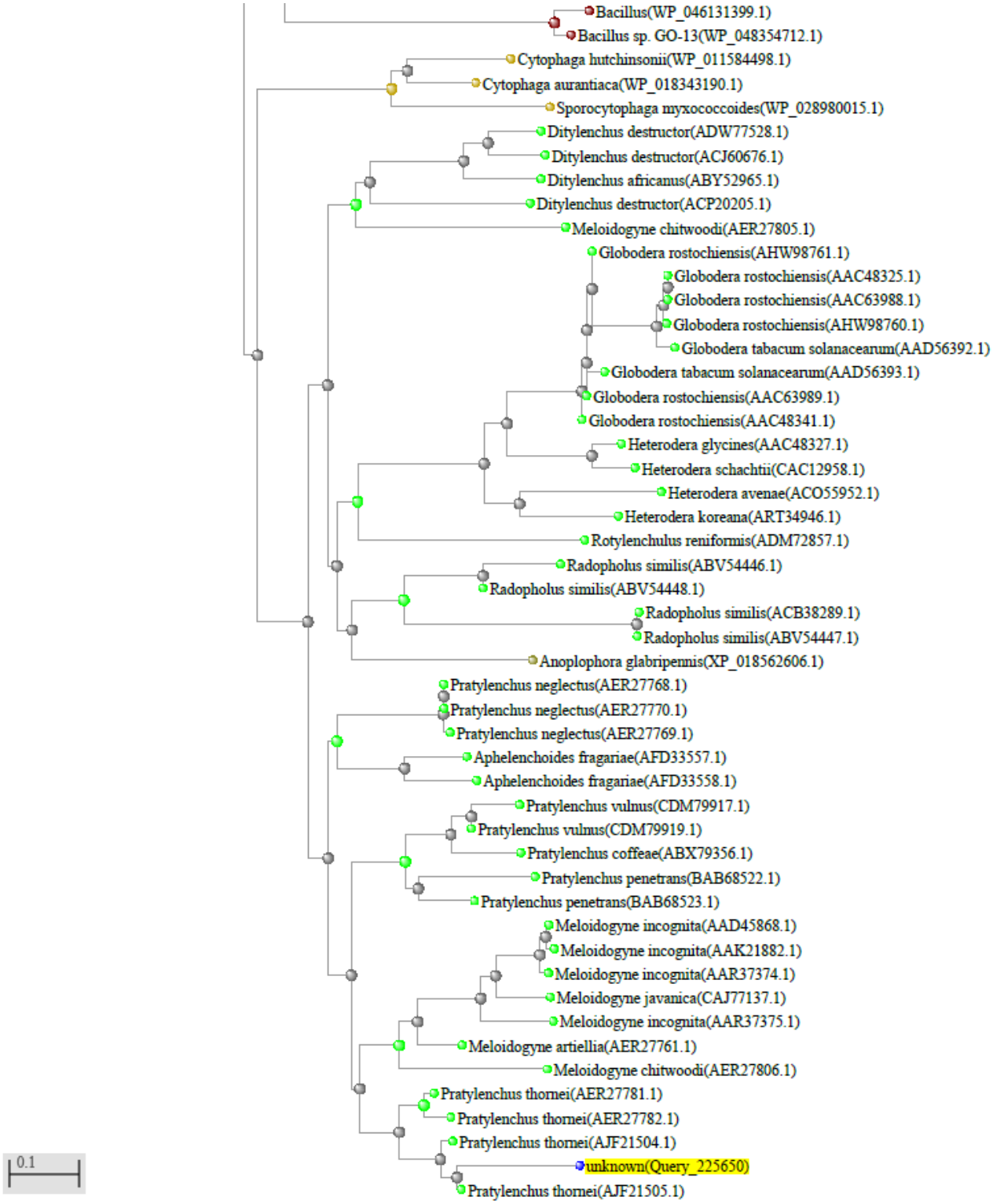
hits to *Pratylenchus* spp., a total of 14 hits, of which four hits each were for *P. vulnus* and *P. thornei.* The BLASTP results showed conserved domains of cellulase GHF5 and cellulose binding domain at 10 to 116 aa and 207 to 296 aa, respectively. Alignment using COBALT for the amino acid sequence of 3'*Pt-Eng-1* query against the top five hits resulted in similarity of conserved domains across the matches (Figure 3.5). The two tryptophan (W) residues involved in cellulose binding were found in the 3'*Pt-Eng-1* virtual amino acid at 212 and 266 aa represent. Also found was a conserved cysteine (C) at 157 aa preceded by the highly conserved residues, glycine (G), valine (V) and serine (S) at 154 to 156 aa across all the top five hits. Moreover, when the virtual protein of 3'*Pt-Eng-1* was analysed for protein classification using InterPro detailed signature matches yielded glycoside hydrolase superfamily (IPR017853) between 10 to 145 aa of which 10 to 116 aa represented the conserved cellulase domain, and carbohydrate binding domain between 201 to 296 aa.

A phylogenetic tree was produced using BLAST pairwise alignments of 100 closest homologous sequences that matched to 3'*Pt-Eng-1* query as a result of BLASTP and is shown in Figure 3.6. The 3'*Pt-Eng-1* query is shown in yellow highlight and was most closely related to partial β,1-4 endoglucanase-1 sequences of *P. thornei* (AJF21505) followed by several other *Pratylenchus* and *Meloidogyne* spp. (Figure 3.6).

Figure 3.5. Multiple sequence alignment of the amino acid sequence of 3'*Pt-Eng-1* query against the top five hits as a result of BLASTP. The red and blue coloured letters indicate highly conserved and less conserved residues, respectively. The solid and dotted horizontal lines

indicate the presence of conserved cellulase and cellulose binding domains, respectively. The two red and one black stars indicate two tryptophan and cysteine residues, respectively.

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Heterodera schachtii(CAC12958.1)
Heterodera avenae(ACO55952.1)
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Meloidogyne incognita(AAR37374.1)
Meloidogyne javanica(CAJ77137.1)
Meloidogyne incognita(AAR37375.1)
Meloidogyne artiellia(AER27761.1)
Meloidogyne chitwoodi(AER27806.1)
Pratylenchus thornei(AER27781.1)
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0.1

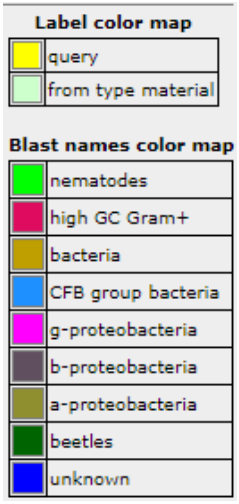


Figure 3.6. Phylogenetic tree representing the position of 3'*Pt-Eng-1* query as a result of BLAST pairwise alignment with respect to its 100 closest homologous amino acid sequences. The horizontal branches represent evolutionary lineages changing over time whilst the horizontal bar of 0.1 at the bottom of the figure provides a scale for the genetic change.

The 5'*Pt-Eng-1* and 3'*Pt-Eng-1* virtual amino acid sequences were analysed for overlapping regions and a combined sequence of 486 aa referred to as *Pt-Eng-1* amino acid sequence with a methionine residue at 22nd position was deduced based on the common regions and is given in Appendix 2. The *Pt-Eng-1* amino acid sequence used to generate the nucleotide sequence referred to as *Pt-Eng-1* nucleotide sequence using EMBOSS Backtranseq tool using the only nematode codon usage table available for *C. elegans* in the selection parameter (McWilliam et al., 2013). Appendix 2 shows the nucleotide sequence with the start codon (ATG) in red letters. The last cysteine residue however, could not be identified in the nucleotide sequence due to the limited data generated by the tool itself.

A BLASTP using the *Pt-Eng-1* virtual amino acid as query showed the presence of cellulase GHF5, BglC GHF1, CBD-II and cellulose binding domain (CBM-2) at regions spanning 60 to 306 aa, 127 to 300 aa, 400 to 485 aa, and 397 to 486 aa, respectively (Figure 3.7). The multiple sequence alignment using COBALT as a result of BLASTP showed these conserved domains that were shared amongst majority of the hits. The top five hits matched to GHF5 endoglucanase precursor of *P. coffeae* (ABX79356), β,1-4 endoglucanase-1 precursors of *P. vulnus* (CDM79917 and CDM79919), β,1-4 endoglucanase of *P. penetrans* (BAB68522) and β,1-4 endoglucanase

of *M. incognita* (AAD45868). The methionine residue encoded by the start codon (ATG) was also found in the beginning of the 486 aa-long *Pt-Eng-1* and was also found to be conserved in the top five hits that matched the query (Figure 3.7). Additionally, when the virtual amino acid sequence of *Pt-Eng-1* was queried at Interpro, the results indicated that the glycoside hydrolase superfamily (IPR017853) and CBD (IPR008965) were present between 47 to 346 aa and 391 to 486 aa, respectively. A conserved GHF5 site spanning 173 to 182 aa was also found and is shown as a dotted orange line in Figure 3.7.

The *Pt-Eng-1* sequence beginning with the methionine residue resulting in a 465 aa sequence was checked in the SignalP 4.1 server for the presence and location of a predicted signal peptide cleavage site. Interestingly, the output predicted a signal peptide in the query and is indicated by the S-score that showed the highest peak at 14th residue (Figure 3.8). The cleavage site was predicted at 24th residue from the N-terminal is shown as a C-score with significant elevation; generally, a SignalP value greater than 0.45 is considered significant (Figure 3.8) (Melhem et al., 2013; Petersen et al., 2011). However, there was another cleavage site before the 24th residue which could have been as a result of unknown residues found in the query. The 465 aa *Pt-Eng-1* sequence beginning with the methionine residue was also checked in TMHMM server v. 2.0 for prediction of transmembrane helices in proteins. This resulted in an output that indicated no predicted transmembrane helices (Figure 3.9) (Krogh et al., 2001). However, there was a small sequence of amino acids that did indicate a transmembrane segment with a low probability score. The 465 aa *Pt-Eng-1* sequence beginning with the methionine residue was then checked in SecretomeP 2.0a server (Bendtsen et al., 2004). This resulted in an NN-score/SecP score of 0.95 which was above the threshold of 0.5 for bacterial sequences indicating a non-classically secreted protein. When the same sequence was checked in TargetP 1.1 server for prediction of subcellular location of a eukaryotic protein based on predicted presence of any of the N-terminal presequences: chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (SP) (Emanuelsson et al., 2000; Nielsen et al., 1997). The highest NN-scores of 0.965 indicated that the sequence was predicted to be localised in the secretory pathway and contained a signal peptide. Additionally, the TPlen value of 23 indicates the pre-sequence length of the cleavage site.

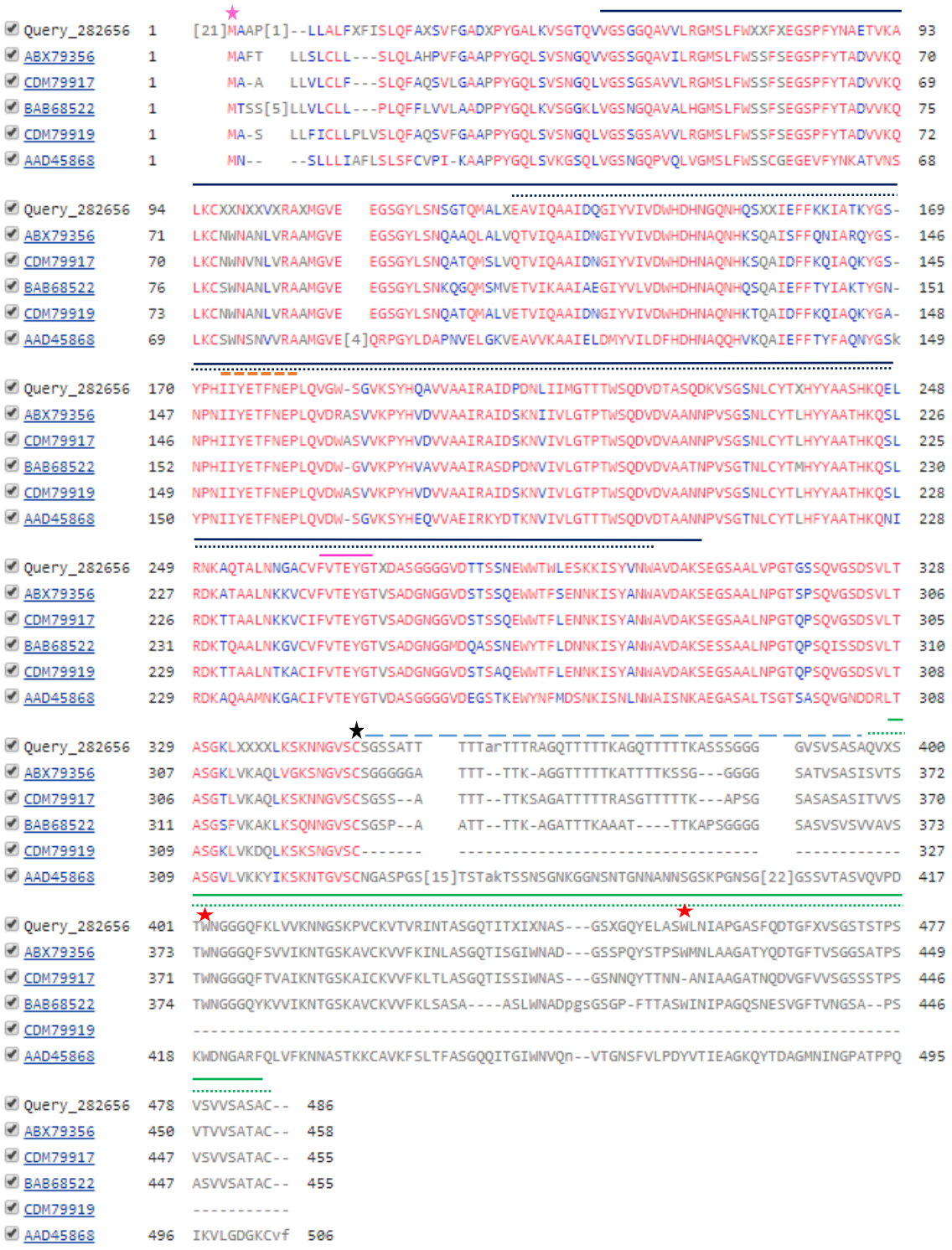
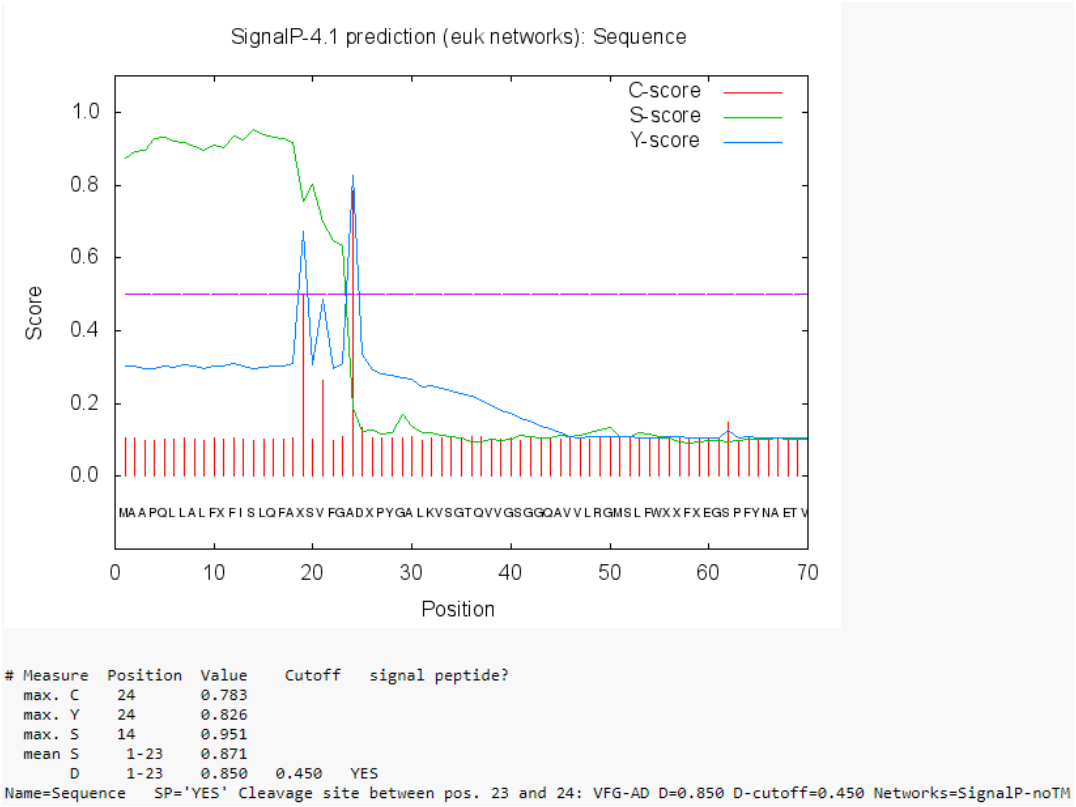


Figure 3.7. Multiple sequence alignment of the 486 aa sequence of *Pt-Eng-1* query against the top five hits as a result of BLASTP. The red and blue coloured letters indicate highly conserved and less conserved residues, respectively. The solid and dotted horizontal blue lines indicate the presence of conserved cellulase GHF5 and BglC GHF1 domains, respectively. The solid and dotted horizontal green lines indicate the presence of conserved CBD-II and CBM-2 domains,

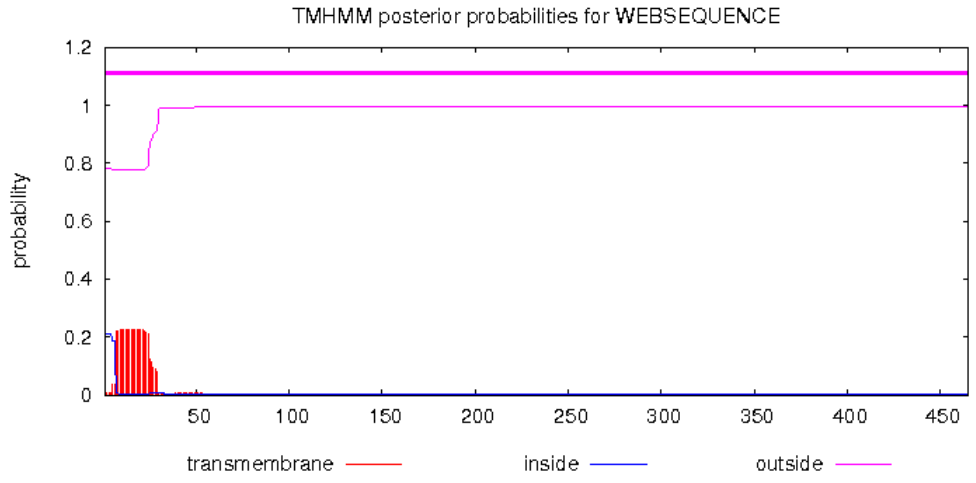
respectively. The orange and the blue dotted line indicates the conserved site within GHF5 and the linker region between the two domains, respectively, whilst the pink line is a conserved site within the GHF5 domain. The two red, one black and one pink star(s) indicates two tryptophan, cysteine and methionine residues, respectively.




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    24       0.783
  max. Y    24       0.826
  max. S    14       0.951
  mean S     1-23    0.871
       D     1-23    0.850   0.450   YES
Name=Sequence   SP='YES' Cleavage site between pos. 23 and 24: VFG-AD D=0.850 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure 3.8. SignalP 4.1 analysis of the *Pt-Eng-1* amino acid. Red, green and blue lines indicate C-score (raw cleavage site score), S-score (signal peptide score) and Y-score (combined cleavage site score).

```
# WEBSEQUENCE Length: 465
# WEBSEQUENCE Number of predicted TMHs:  0
# WEBSEQUENCE Exp number of AAs in TMHs: 4.75155
# WEBSEQUENCE Exp number, first 60 AAs:  4.74018
# WEBSEQUENCE Total prob of N-in:        0.21823
WEBSEQUENCE     TMHMM2.0        outside      1   465
```



plot in postscript, script for making the plot in gnuplot, data for plot

Figure 3.9. TMHMM analysis for prediction of transmembrane helices in *Pt-Eng-1* amino acid sequence.

A multiple sequence alignment using Clustal-Omega was done to identify conserved domains and amino acid residues, probable residues for unknowns that were found, and or stop codons, amongst *Pt-Eng-1* amino acid sequence and full-length coding sequences for cellulase and β,1-4 endoglucanase-1 of various PPNs. The choice of PPN protein sequences used for comparisons satisfied at least one of the following criteria: it was amongst the closest homologous hits that appeared in the BLASTP results when virtual amino acid sequences of 5'*Pt-Eng-1,* 3'*Pt-Eng-1* and *Pt-Eng-1* were used as queries, it was the original parasitism effector that was initially selected as reference, and it was a partial known sequence in *P. thornei*. Some of the chosen protein sequences were common for the three BLASTP results and have been indicated by an asterisk. A total of 11 proteins, nine with full-length coding sequences and two with partial sequences, belonging to five generas of PPNs were used. These were: *P. penetrans* (BAB68523, BAB68522*), *M. incognita* (AAR37375*, AAD45868), *M. javanica* (CAJ77137), *M. chitwoodi* (AER27806), *H. avenae* (ACO55952), *A. fragariae* (AFD33558*), *D. destructor* (ACP20205), *P. thornei* (AER27781 and AER27782).

The multiple sequence alignment of the *Pt-Eng-1* amino acid sequence with the 11 PPN parasitism effector proteins showed several contiguous common sequences and conserved domains: cellulase GHF5, BglC GHF1 domain, CBD-II and CBM-2 (Figure 3.10). Whilst nine protein sequences encoded by full-length genes had the methionine residue representing the start codon, its alignment was exact/common between *Pt-Eng-1* and β,1-4 endoglucanase-1 of *P. penetrans* (BAB68523). The highly conserved residues, glycine(G), valine (V), serine (S) and cysteine (C), found in the linker region between cellulase and CBD-II domains was common in seven of the 11 proteins with only two PPNs effectors, β,1-4 endoglucanase-1 of *D. destructor* (ACP20205) and cellulase of *M. incognita* (AAR37375) that did not share similarity. The two tryptophan (W) residues essential in the cellulose binding function were also found to be common amongst some sequences aligned. The first of the tryptophan residues that appeared in *Pt-Eng-1* aligned exactly with those of β,1-4 endoglucanase-1 of *P. penetrans* (BAB68522), *H. avenae* (ACO55952), *M. incognita* (AAD45868) and cellulase of *M. javanica* (CAJ77137), whilst the second tryptophan residue in *Pt-Eng-1* aligned exactly with those of β,1-4 endoglucanase-1 of *P. penetrans* (BAB68522) and *H. avenae* (ACO55952).

```
ACO55952.1      ------------------MCRLRSMFIHLCLLLALCIALANSLTAVPPPYGQLSVSGTKL    42
ACP20205.1      --------------------------MLI-LFTLLFIVYGSMAVDPPYGQLSVEGPNL    31
AFD33558.1      ------------------------MIQV----AAVLFVFIGFANAVAPPYGQLSVKGNKI    32
AER27806.1      ---------------------------MSFIILFSIFFYSFCEAAPPYGQLSVKDSKL    31
AAR37375.1      ------------------------MFSLSLVAFL--SLTFCIQINAAPPYGQLSVKGSQL    34
AAD45868.1      ------------------------MNSLLLIAFL--SLSFCVPIKAAPPYGQLSVKGSQL    34
CAJ77137.1      ------------------------MNSLLLIAFL--SLSFCIPIKAAPPYGQLSVKGSQL    34
BAB68522.1      ------------------MTSSSSSMALLVLCLLPLQ-FFLVVLAADPPYGQLKVSGGKL    41
BAB68523.1      --------------------MV---LQLLFCLLMVSTTLHLANSAAPPYGQLSVKGKNV    36
query           SSSSSHPLPFTQFNFNDTSTTMAAPQLLALFXFISLQ-FAXSVFGADXPYGALKVSGTQV    59
AER27781.1      ------------------------------------------------------------    0
AER27782.1      ----------------------------------------------------LSVKGTQL    8

ACO55952.1      VGSNGQQVQLIGNSLFWHQWYG--NYWNAETVKALKCQWNANVVRGAMGADS-----GGY    95
ACP20205.1      KGEKGDTVALHGMSLFWSQWMG--KYWNADTIKALKCPWNSNVVRAAMGVDQ-----GGY    84
AFD33558.1      LGADGKPVALHGMSLFWSQWGEGAPFYNKDTVQALKCSWNANLVRAAMGVEE-----GGY    87
AER27806.1      LGSNGQPVQLVGMSLFWSNCGEGGQVFYNYDTLKILKCNWNSNVVRAAMGVESDGCQRPGY    91
AAR37375.1      VGSNGQPVQLVGMSLFWSSCGEGEGFYNRETVNSLKCSWNSNVVRAAMGVEYSGCQRPGY    94
AAD45868.1      VGSNGQPVQLVGMSLFWSSCGEGEVFYNKATVNSLKCSWNSNVVRAAMGVEYSGCQRPGY    94
CAJ77137.1      VGSNGQPVQLVGMSLFWSSCGEGEVFYNKATVNSLKCSWNSNVVRAAMGVEYSGCQRPGY    94
BAB68522.1      VGSNGQAVALHGMSLFWSSFSEGSPFYTADVVKQLKCSWNANLVRAAMGVEE----GSGY    97
BAB68523.1      VGSNGQAVALHGMSLFWSSFSEGSPFYTADVVKALKCQWNANVVRAAMGVEE----GSGY    92
query           VGSGGQAVVLRGMSLFWXXFXEGSPFYNAETVKALKCXXNXXVXRAXMGVEE----GSGY    115
AER27781.1      ----------------------------------------NVVRAAMGVEE----GNGY    15
AER27782.1      VGSGGQAVVLRGMSLFWSSFPEGSPFYNAATVKALKCNWNANVVRAAMGVEE----GNGY    64
                                                         : *. **.:       **

ACO55952.1      VQDPGTAYKLMSAVIEAAISNGIYVIVDWHAHD--PFQDKAIEFFTKIAKTYG-SYPHIL    152
ACP20205.1      LTDQATNEKMVETVVDAAIAGGIYVIIDWHAD--EKHQAEAVAFFDKMSKKYA-GVKNVL    141
AFD33558.1      LTNQATEQGKLEAVVKAAIDLGIYVIIDWHDHHAYQHPDKAVEFFSAMSKKYA-GVPNII    146
AER27806.1      LEAPITEREKVEAVVKAAIELDMYVIIDFHTNNAPAHLDKAIEFFTYFATNYGSKYPNIL    151
AAR37375.1      LDAPNVELAKVEAVVKAAIELDMYVILDFHDHNAQGHVKQAKQFFAYFAQNYGSKYPNII    154
AAD45868.1      LDAPNVELGKVEAVVKAAIELDMYVILDFHDHNAQQHVKQAIEFFTYFAQNYGSKYPNII    154
CAJ77137.1      LDAPNVELGKVEAVVKAAIELDMYVILDFHDHNAQSHVKQAIEFFTYFAQNYGSKYPNII    154
BAB68522.1      LSNKQGQMSMVETVIKAAIAEGIYVLVDWHDHNAQNHQSQAIEFFTYIAKTYG-NNPHII    156
BAB68523.1      LSNKQNQRNMVDTVIKAAIAQGIYVIVDWHDHNAQNHLSQANEFFTYIAQTYGSKNPNII    152
query           LSNSGTQMALXEAVIQAAIDQGIYVIVDWHDHNGQNHQSXXIEFFKKIATKYG-SYPHII    174
AER27781.1      LSNPSGQQSLVDAVIKAAIDQGIYVIVDWHDHNAQNHQSQAINFFTYIAKTYGSKYPNII    75
AER27782.1      LSNPSGQQSLVDAVIKAAIDQGIYVIVDWHDHNAQNHQSQAINFFTYIAKTYGAKYPNII    124
                :          .:*:.***  .:**::*:* .    .      **  :: .*.    :::

ACO55952.1      YETYNEPVGVSWNDVLVSYHTKVVGAIRAIDQKNVIILGTPMYSQDVDIASQNPIKGYKN    212
ACP20205.1      YEDINEPKDVSWSGELVPYHKAVIDAIRKNDKDNIIILGTPTYSQKVDEASQAPIKDEKN    201
AFD33558.1      YEIFNEPLSVSWTSDLVPYHNKVISAIRANDPLNLIILGTPNWSQDVDIAAQNPVAG-KN    205
AER27806.1      YETFNEPLQVDWSG-VKAYHEKVVATIRKYDQKNIIILGTTTWSQDVDIASRNPVAG-SN    209
AAR37375.1      YETFNEPLQVDWNG-VKSYHEQVVAEIRKYDNKNVIVLGSTTWSQDVDTAANNPVRG-SN    212
AAD45868.1      YETFNEPLQVDWSG-VKSYHEQVVAEIRKYDTKNVIVLGTTTWSQDVDTAANNPVSG-TN    212
CAJ77137.1      YETFNEPLQVDWNG-VKSYHEQVVAEIRKYDTKNVIVLGTTTWSQDVDTAANNPVSG-TN    212
BAB68522.1      YETFNEPLQVDWGV-VKPYHVAVVAAIRASDPDNVIVLGTPTWSQDVDVAATNPVSG-TN    214
BAB68523.1      YEVFNEPLQVDWNSVIKPYHQSVVATIRKYDTKNIIVLGTRTWSQEVDTAANSPVSG-SN    211
query           YETFNEPLQVGWSG-VKSYHQAVVAAIRAIDPDNLIIMGTTTWSQDVDTASQDKVSG-SN    232
AER27781.1      YETFNEPLQVGWSG-VKSYHQAVVAAIRKYDTKNVIVLGTTTWSQDVDTASQDKVSG-SN    133
AER27782.1      YETFNEPLQVGWSG-VKSYHQAVVAAIRKYDTKNVIVLGTTTWSQDVDTASQDKVSG-SN    182
                **  ***  *.*   :  **  *:  **  *  *:*::*:  :**.** *:   : . .*
```

continued…


ACO55952.1 LMYTFHFYASSHFVDGLGAKVRTAINNGLPIFVTEYGTCEASGAGSLNSGSMNSWWKMLD 272
ACP20205.1 IMYTLHYYAGTHK-EELRAKAETAIKAGLPLFVTEYGTVNADGGGAVDEASSKAWWDFLD 260
AFD33558.1 LAYTLHYYAASHK-QALRDKTQAALDKRLAIFVTEYGTVDASGGGSVDVQSSNEWWAFLD 264
AER27806.1 LCYTLHFYAATHK-QNIRDKAQTALNNNKCVFVTEYGTVDNNGGGNPDNQSTNDWWTFMD 268
AAR37375.1 LCYSLHYYAATHK-QNLRDKAQAAINKGACIFVTEYGTVDASGGGGVDEGSTKEWYNFLD 271
AAD45868.1 LCYTLHFYAATHK-QNIRDKAQAAMNKGACIFVTEYGTVDASGGGGVDEGSTKEWYNFMD 271
CAJ77137.1 LCYSLHFYAATHK-QNLRDKAQAAINKGACIFVTEYGTVDASGGGGVDEGSTKEWYNFLD 271
BAB68522.1 LCYTMHYYAATHK-QSLRDKTQAALNKGVCVFVTEYGTVSADGNGGMDQASSNEWYTFLD 273
BAB68523.1 LCYTLHYYAASHK-QDLRNKAQAALNKNVCIFVTEYGTVNADGNGGMDQASSQEWWTFLD 270
query LCYTXHYYAASHK-QELRNKAQTALNNGACVFVTEYGTXDASGGGGVDTTSSNEWWTWLE 291
AER27781.1 LCYTLHFYAASHK-QELRNKAQTALNNGACVFVTEYGTVSADGGGGVDTASSNEWWNWLE 192
AER27782.1 LCYTLHFYAASHK-QELRNKAQTALNNGACVFVTEYGTVEANGGGNPDSASSNEWWNWLE 241
ACO55952.1 ELKISYVNWSICDKGEACSALKPGSSSA--NVASPSSWTESGNMVASHHKTKPTGVSCNG 330
ACP20205.1 KNKISYVNWSVADKDEGASALKPGTPATTAGVGDDANLTASGKLVKEKYKTQDKWSEVRK 320
AFD33558.1 ANKISYANWAIGNKAEAAAALTPGTTAQ--QVGDDSRLTASGKLVKQKLKSQNNGVSC-- 320
AER27806.1 KNRMSYLNWALDNKGEGAAALFPNTVAS--LAGNEDRLTQSGRLVKNKLRKMNTGVSCSG 326
AAR37375.1 SKKISNLNWAISNKAEGAAALTPGTTSS--QVGNDDRLTASGRLVKSYIKSKNTGVRCNG 329
AAD45868.1 SNKISNLNWAISNKAEGASALTSGTSAS--QVGNDDRLTASGVLVKKYIKSKNTGVSCNG 329
CAJ77137.1 SNKISNLNWAISNKAEGASALTSGTSAS--QVGNDDRLTASGLIVKKYIKSKNTGVSCSG 329
BAB68522.1 NNKISYANWAVDAKSESSAALNPGTQPS--QISSDSVLTASGSFVKAKLKSQNNGVSCSG 331
BAB68523.1 NNKISYVNWALDAKNEKSAALNPGTQPS--QVGSDSVLSESGKLVKAQLKKQNNGVSCSG 328
query SKKISYVNWAVDAKSEGSAALVPGTGSS--QVGSDSVLTASGKLXXXXLKSKNNGVSCSG 349
AER27781.1 NKK 195
AER27782.1 NKK 244
ACO55952.1 APAPSAGGAPSPAQPSPPSA----------Q 351
ACP20205.1 VKKTDQTQATK-----CEY----NIFF 338
AFD33558.1 320
AER27806.1 GKSNNALPSLPV------NKDVVAIK-NSTSGNK---NNNGDSSDNKGYPQGNPV----L 372
AAR37375.1 GGAAKKGSSSSNTGSKKDKQKFKEQKFKEKI 360
AAD45868.1 ASPGSGSG-SNPSGNKPSNSQTSTAKTSSNSGNKGGNSNTGNNANNSGSKPGNSGSNTGN 388
CAJ77137.1 ASSSSGSG-SKPSGNKPSNSESSTAKTSGNSGNKGGNSNTGSNANNSGSKSGNSGSNTGN 388
BAB68522.1 SPAATTTTKA----------GATTT----------K-------A 348
BAB68523.1 328
query SSATTTTTARTTTRA----------GQTTTTT-------KAG-------Q 375
AER27781.1 195
AER27782.1 244
ACO55952.1 P-----APPPAQPVPGPSGSASASINVLSVSSWNGGGQLNIEVKNTGSAPLCGVQFRLNL 406
ACP20205.1 338
AFD33558.1 320
AER27806.1 TQTTKKQASNDNKNGYPQGNPVLTQTT----------KKXSLK----- 405
AAR37375.1 360
AAD45868.1 TGSNAG-ASSGN-TGTSTSGSSVTASVQVPDKWDNGARFQLVFKNNASTKKCAVKFSLTF 446
CAJ77137.1 TGSNAG-ANSGN-TGTSTGSSSVTASVQVPDKWDNGARFQLVFKNNASTKKCGVKFSLTF 446
BAB68522.1 AATTTK-APS-----GGGGSASVSVSVVAVSTWNGGGQYKVVIKNTGSKAVCKVVFKLSA 402
BAB68523.1 328
query TTTTTK-ASS-----SGGGGVSVSASAQVXSTWNGGGQFKLVVKNNGSKPVCKVTVRINT 429
AER27781.1 195
AER27782.1 244
ACO55952.1 PQGTTIGNAWNADPAGQGQYKLPSWIRIEPGQDSKQAGMTYNGNGKP-TAQIVSTKPC-- 463
ACP20205.1 338
AFD33558.1 320
AER27806.1 405
AAR37375.1 360
AAD45868.1 ASGQQITGIWNVQNVTGNSFVLPDYVTIEAGKQYTDAGMNINGPATPPQIKVLGDGKCVF 506
CAJ77137.1 ASGQQITGIWNVQNVTGNNFVLPDYVTIEAGKQYTDAGMNINGPATPPQIKVLGDGKCVH 506
BAB68522.1 SAASLW--NADPGSG-SGPFTTASWINIPAGQSNESVGFTVNGSA--PSASVVSATAC-- 455
BAB68523.1 328
query ASGQTITXIXNASGS-XGQYELASWLNIAPGASFQDTGFXVSGSTSTPSVSVVSASAC-- 486
AER27781.1 195
AER27782.1 244

Figure 3.10. Multiple sequence alignment of the 486 aa sequence of *Pt-Eng-1* query against 11 proteins encoded by full-length or partial parasitism effector genes of PPNs as a result of Clustal-O. An asterisk indicates positions having a single, fully conserved residue, colon indicates conservation between groups of strongly similar properties, and full-stop indicates conservation between groups of weakly similar properties. Residue colours red, blue, pink, green and grey represent physicochemical properties of small hydrophobic, acidic, basic, hydroxyl+sulphahydryl+amine+G, and unusual amino/imino acids, respectively.

Interestingly, AER27782 was a partial β,1-4 endoglucanase-1 encoded by the *P. thornei* TSA contig JN052046 that was selected in this study for RNAi studies and RACE PCRs. However, AER27782 was only 244 aa-long and lacked the methionine residue, and the CBD-II and CBM-2 domains after the linker region. The *Pt-Eng-1* sequenced and annotated in this study was 486 aa in length, had the methionine residue (22nd position) along with the all the conserved domains separated by the linker sequence thus validating its identity as β,1-4 endoglucanase-1.

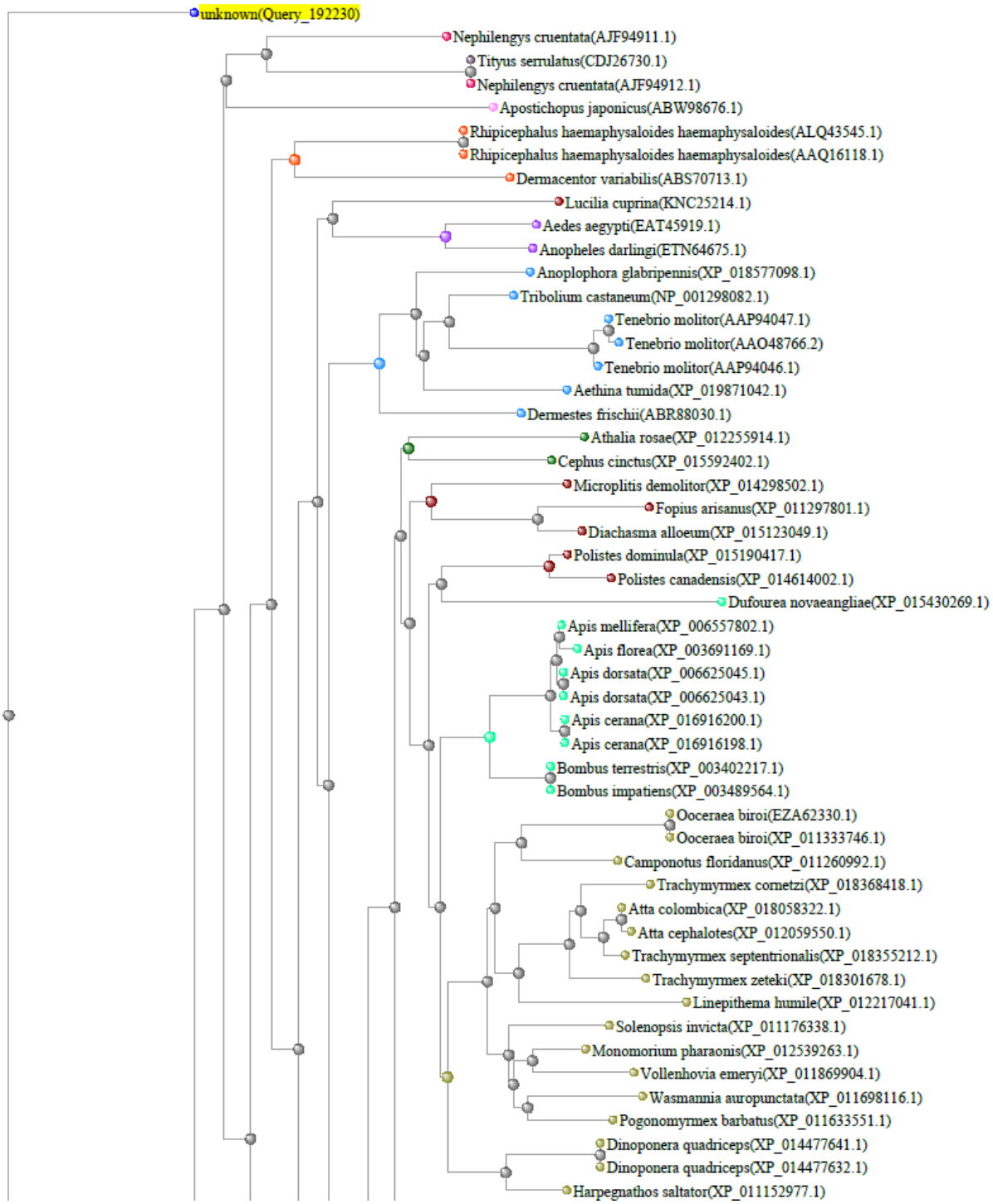
3.3.4.2 Characterisation and validation of *Pt-CLP*

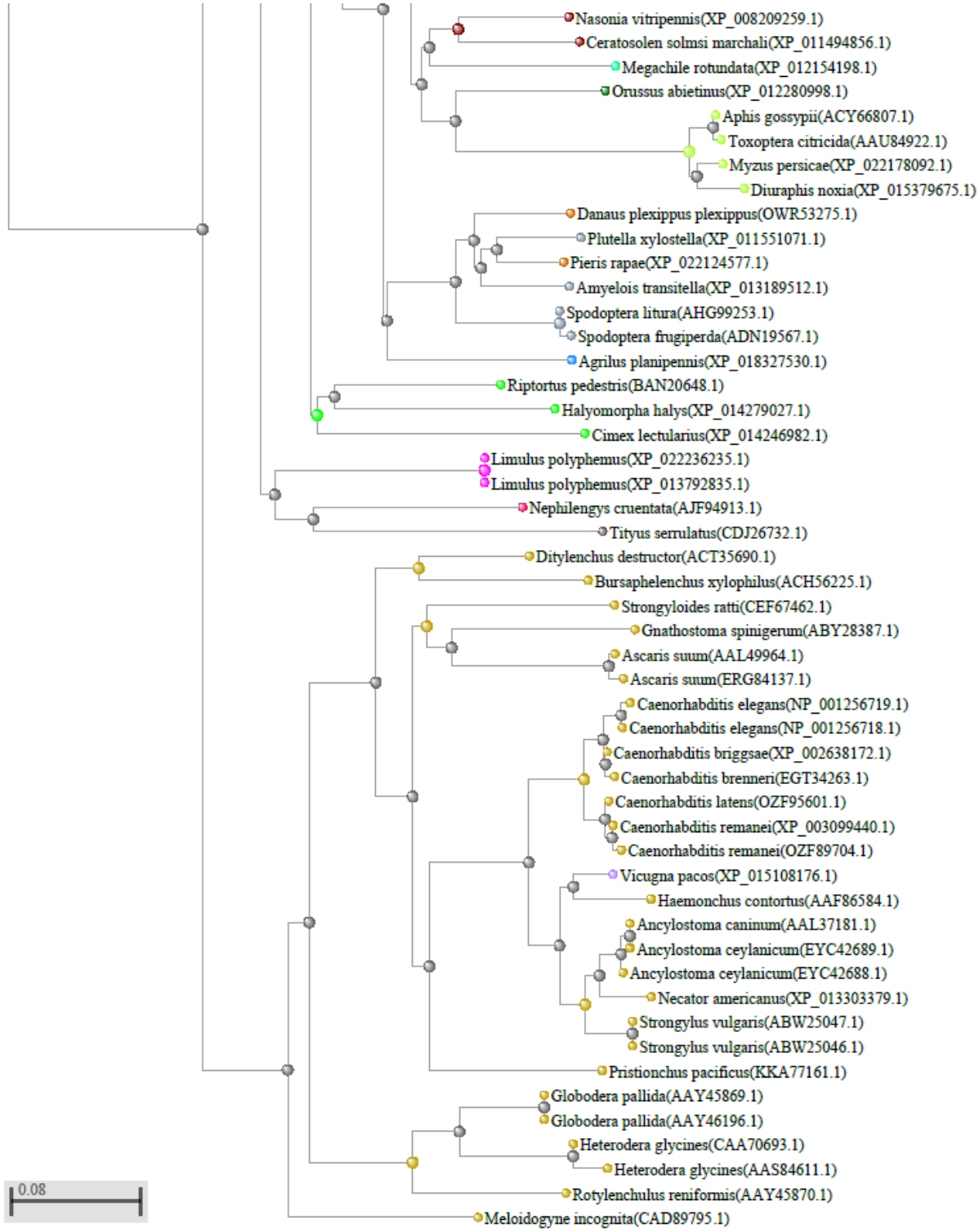
For *Pt-CLP*, both 5' and 3' RACE PCRs were done, however, only 3' RACE PCR resulted in a clear band at approximately 800 bp band on the gel thus giving only a partial sequence. Sequencing results were subjected to BLASTX and they indicated that based on lowest e-values and highest Hsp, bit scores matches cathepsin L- and cathepsin-like proteases from eukaryotic genera of free-living nematodes, PPNs, APNs and insects. The PPN genera were: *Meloidogyne*, *Ditylenchus*, *Globodera*, *Heterodera*, *Bursaphelenchus* and *Rotylenchulus*, however, there were none from *Pratylenchus* spp. The top five protein matches belonged to cathepsin L of *Riptortus pedestris* (BAN20648), putative cathepsin L protease of *M. incognita* (CAD89795), cathepsin L-like cysteine proteinase of *D. destructor* (ACT35690), cathepsin L precursor of *Tribolium casteneum* (NP001298082), and cathepsin L1-like isoform X2 of *Limulus polyphemus* (XP022236235). Interestingly, amongst the top five hits, only two hits were of PPNs, whilst the other three hits belonged to arthropods and insects. The BLASTX result also indicated the presence of conserved domains belonging to peptidase C1 superfamily spanning 88 to 558 nt.

The virtual protein or amino acid sequence of 189 aa derived from ExPasy used as a query in BLASTP resulted in the same five protein matches as described for BLASTX. The taxonomy BLAST report indicated matches to primarily to insects, free-living nematodes, APNs and PPNs. The BLASTP results showed conserved domain belonging to cathepsin L protease from papain family cysteine protease between 32 to 189 aa. Since the top five hits as a result of BLASTP were from insects in addition to PPNs, the output was manually curated to identify those most related PPNs, resulting in seven hits which were then used to carry out multiple alignment using COBALT. These hits were: putative cathepsin L-protease of *M. incognita* (CAD89795), cathepsin L-like cysteine proteinases of *D. destructor* (ACT35690), *B. xylophilus* (ACH56225), *G. pallida* (AAY45869 and AAY46196), *H. glycines* (CAA70693) and *R. reniformis* (AAY45870). The amino acid sequence of *Pt-CLP* query against the seven PPN effector proteins resulted in similarity of conserved domains across the matches (Figure 3.11). However, the pre-peptide cleavage site (R), conserved N-glycosylation site (R) and cysteine (C) active site found in all seven proteins, marked by blue, green and red stars in Figure 3.11, were not found in the *Pt-CLP* sequence, as the sequence was incomplete or partial. The conserved GCNGG motif common in the seven PPN proteins was also found in the *Pt-CLP* sequence but the central N residue (aspargine) was found to be E (glutamic acid) and is shown in Figure 3.11 as a dotted line. Additionally, when the virtual protein of *Pt-CLP* was analysed for protein classification using InterPro, detailed signature matches yielded peptidase C1 family (IPR013128) between 10 to 188 aa which included the peptidase C1-A papain C-terminal domain and a histidine (H) active site between 133 to 143 aa (shown as a green dotted line in Figure 3.11). Of the three residues, C (from the pre-peptidic cleavage site), H and N which form the catalytic triad in the active site of the mature enzyme only H and N were found at the C-terminal of the *Pt-CLP* (Figure 3.11). A phylogenetic tree was produced using BLAST pairwise alignments of the 100 closest homologous sequences that matched to *Pt-CLP* query as a result of BLASTP and is shown in Figure 3.12. The *Pt-CLP* shown in yellow highlight seemed to be related to various cathepsin L-protease and cathepsin L-like protease of insects, free-living nematodes, APNs and PPNs.

Figure 3. 11. Multiple sequence alignment of the 189 aa sequence of *Pt-CLP* query against the seven PPN effector proteins as a result of BLASTP. The red and blue coloured letters indicate highly conserved and less conserved residues, respectively. The solid and dotted horizontal blue line indicate the presence of conserved cathepsin L protease domain of the peptidase C1 superfamily and GCNGG motif, respectively. Blue, green and red stars are pre-peptide cleavage site, conserved N-glycosylation site and cysteine active site, respectively, found in all

seven proteins whilst the yellow star represents the aspargine residue of the catalytic triad. Green dotted line represents the conserved histidine (H) active site between 133 to 143 aa.

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Ceratosolen solmsi marchali(XP_011494856.1)
Megachile rotundata(XP_012154198.1)
Orussus abietinus(XP_012280998.1)
Aphis gossypii(ACY66807.1)
Toxoptera citricida(AAU84922.1)
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Halyomorpha halys(XP_014279027.1)
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Nephilengys cruentata(AJF94913.1)
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0.08

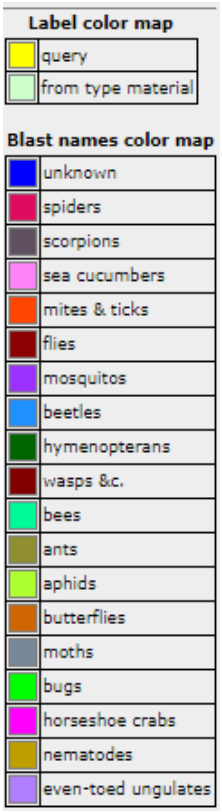

Figure 3.12. Phylogenetic tree representing the position of 3'*Pt-CLP* query as a result of BLAST pairwise alignment with respect to its 100 closest homologous amino acid sequences. The horizontal branches represent evolutionary lineages changing over time whilst the horizontal bar of 0.08 at the bottom of the figure provides a scale for the genetic change.

3.3.4.3 Characterisation and validation of *Pt-PL*

For *Pt-PL*, both 5' and 3' RACE PCRs were done, however, only the 5' RACE PCR resulted in a clear band at approximately 900 bp band on gel, hence only a partial sequence was available. Sequencing results used as query in BLASTX showed hits from eukaryotic and bacterial genera. The eukaryotic matches included protein sequences from *M. incognita*, *M. javanica*, *M. graminicola* and *M. enterolobii.* Based on lowest e-values and highest Hsp bit scores the matches, the top five hits belonged to pectate lyases of *M. graminicola* (AID59201), *M. javanica* (AAL66022), *M. incognita* (AAQ09004), *M. enterolobii* (ADN87334), and pectate lyase-1 of *M. incognita* (AAS88579). BLASTX results also showed the presence of the pectate lyase conserved domain between 122 to 730 nt.

The 282 aa virtual protein derived from ExPasy used as a query in BLASTP resulted in the same five top hits as the BLASTX results. The taxonomy BLAST report showed that the maximum number of hits that matched the query belonged to *Meloidogyne* species with the remaining hits being bacterial species. The BLASTP results also showed a conserved domain of pectate lyase between 37 to 239 aa (Figure 3.13). Multiple alignment using COBALT for the amino acid sequence of *Pt-PL* query against the top five hits resulted in similarity of several contiguous sequences and conserved domains across the matches (Figure 3.13). because of the absence of 3'*Pt-PL* RACE product, the sequence obtained was partial and did not yield data beyond the conserved domain. Interestingly, for all five homologous matches of the *Pt-PL* query, the methionine residues aligned exactly, however, due to the alignment algorithm the first 10 aa of the query were not visible, but which in fact did contain a methionine residue. The first 10 aa were TFKLKMLHSN. The alignment also showed the well conserved seven charged amino acid residues glutamic acid (E), aspartic acid (D), E, D, lysine (K), K and arginine (R) in the pectate lyase domain, and are indicated by a yellow star in Figure 3.13 with the exception of the second lysine residue to be an arginine in the query, thus making EDEDKKR to read as EDEDKRR. Similarly, within the catalytic domain was also found two closely spaced conserved cysteine (C) residues separated by glycine (G) and aspargine (N) residues towards the N-terminal whereas, two more conserved cysteine residues were also found in the other PPNs, however, only one of them was present in the query sequence whilst the other was unknown; these are indicated by blue stars in the figure below. The four conserved regions, designated I to IV, hallmark of the BF domain which a characteristic of pectate lyase class III or family 3 was also found in the query (Figure 3.13). Also indicated in a green star is an N-linked glycosylation site which was conserved amongst all the sequences (Figure 3.13). Protein sequence analysis and classification using Interpro also indicated the presence of a pectate lyase domain (IPR011050) between 36 to 249 aa.

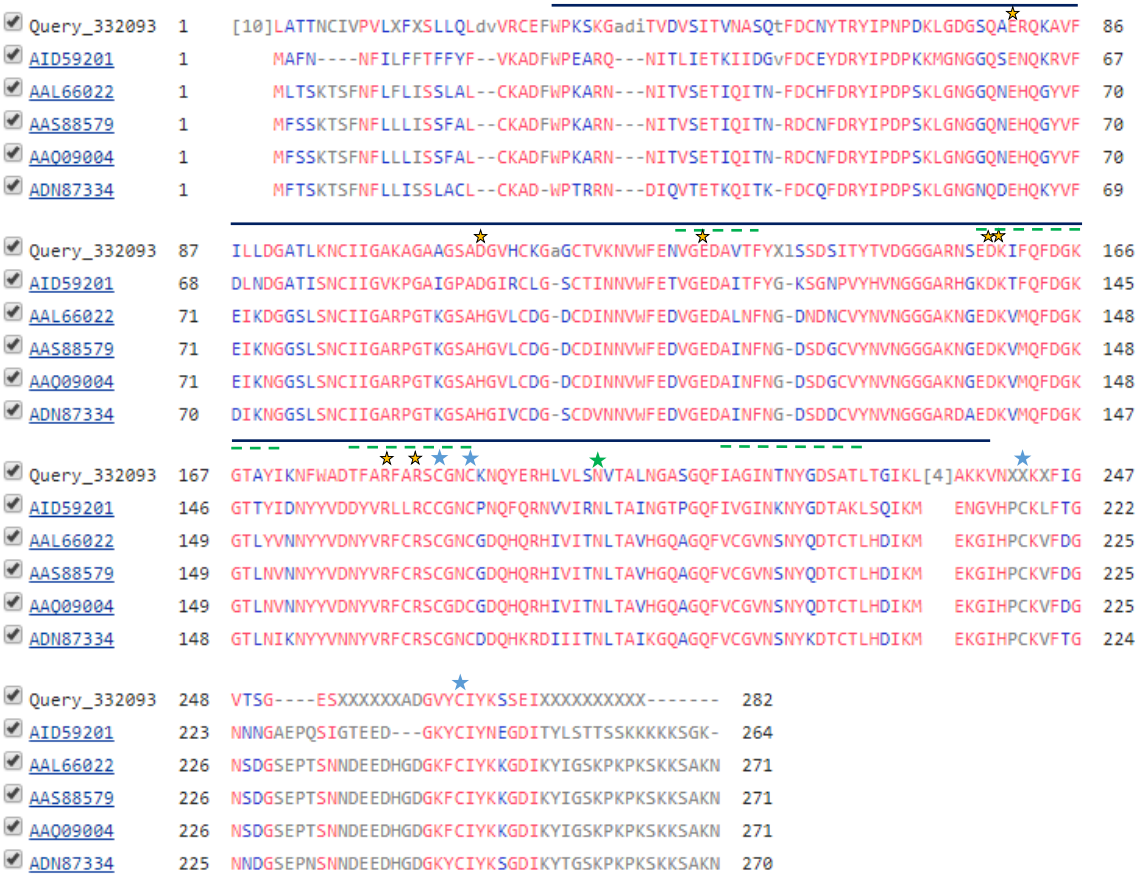

Figure 3. 13. Multiple sequence alignment of the 282 aa sequence of *Pt-PL* query against the five PPN effector proteins as a result of BLASTP. The red and blue letters indicate highly conserved and less conserved residues, respectively. The solid horizontal blue and four dotted green lines indicate the presence of pectate lyase domain and four (I to IV in the order of their appearance from the N-terminal) conserved regions of the BF domain pectate lyase family 3, respectively. The seven yellow stars indicate the well conserved charged amino acid residues glutamic acid (E), aspartic acid (D), E, D, lysine (K), K and arginine (R). The four blue stars indicate two closely spaced conserved cysteine residues towards the N-terminal two cysteines towards the carboxyl end; one of which was not unknown in the query sequence. The green star indicates the conserved N-linked glycosylation site.

The virtual 277 aa sequence of *Pt-PL* beginning with methionine was checked using the SignalP 4.1 server for the presence and location of predicted signal peptide cleavage site. Whilst the result showed presence of a signal peptide, the cleavage site predicted at the 29th residue had a C-score of 0.459 and was less than the acceptable threshold score of 0.5, and hence, the

sequence was manually modified at the 17th and 19th residue to leucine (L) and isoleucine (I), respectively, based on the consensus of the top five BLASTP and BLASTX hits and then checked. It is of interest that the output predicted a signal peptide in the query and was indicated by the S-score that showed the highest peak at 16th residue. The cleavage site was predicted at 29th residue from the N-terminal and had a significant C-score of 0.725 whilst another cleavage site following this site was also predicted but with a lower C-scores. (Figure 3.14). The TMHMM search resulted in no transmembrane helices and a very short sequence at the first 30 residues indicated a transmembrane segment but had a very low probability score (Figure 3.15). The results from SecretomeP resulted in an NN-score/SecP score of 0.91 and was above the threshold of 0.5 for bacterial sequences indicating a non-classically secreted protein. The results of TargetP indicated highest NN-scores of 0.965 indicated that the sequence was predicted to be localised in the secretory pathway and contained a signal peptide. Additionally, the TPlen value of 29 indicates pre-sequence length of the cleavage site. The phylogenetic tree produced using BLASTP pairwise alignments of 100 closest homologous sequences that matched to 5'*Pt-PL* query (shown in yellow) as a result of BLASTP showed its proximity to pectate lyases of *Meloidogyne* spp. and various bacterial species (Figure 3.16).

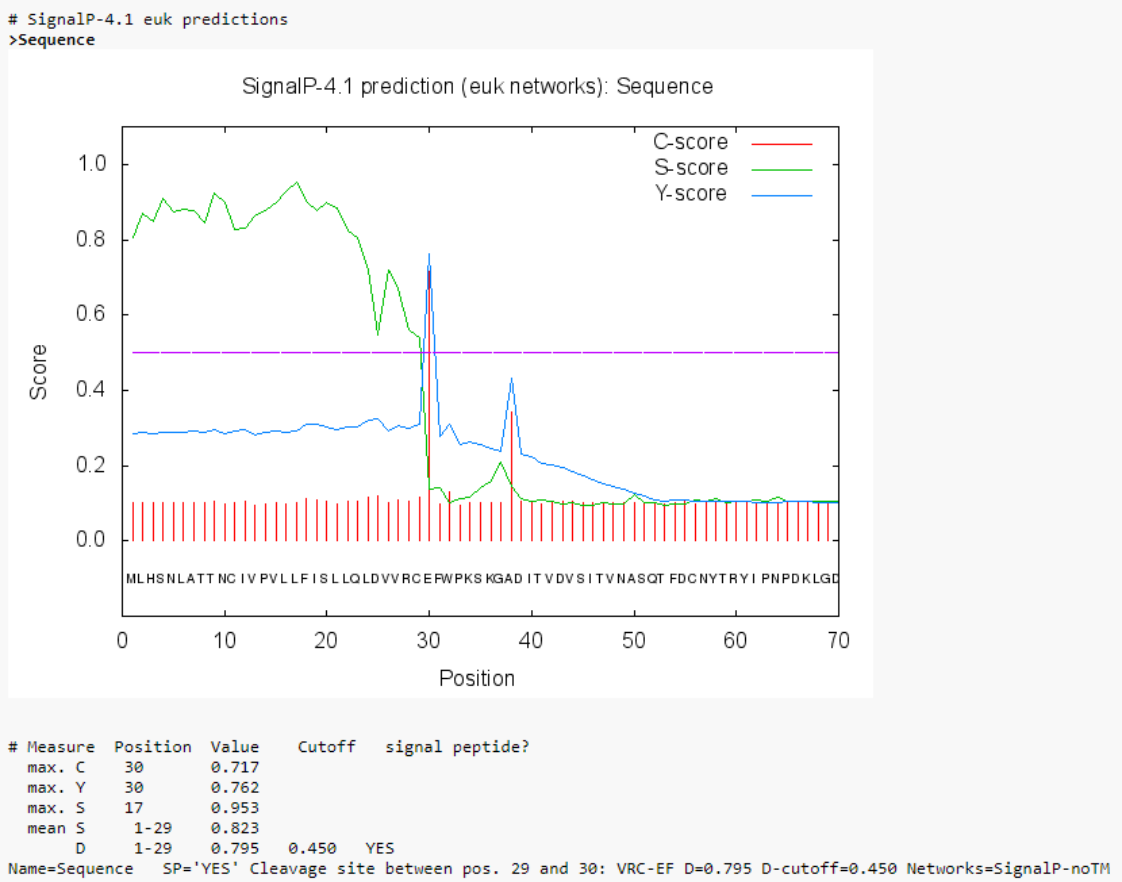

Figure 3.14. SignalP 4.1 analysis of the *Pt-PL* amino acid. Red, green and blue lines indicate C-score (raw cleavage site score), S-score (signal peptide score) and Y-score (combined cleavage site score).

```
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# WEBSEQUENCE Number of predicted TMHs:  0
# WEBSEQUENCE Exp number of AAs in TMHs: 5.66351000000000001
# WEBSEQUENCE Exp number, first 60 AAs:  5.49852
# WEBSEQUENCE Total prob of N-in:        0.25234
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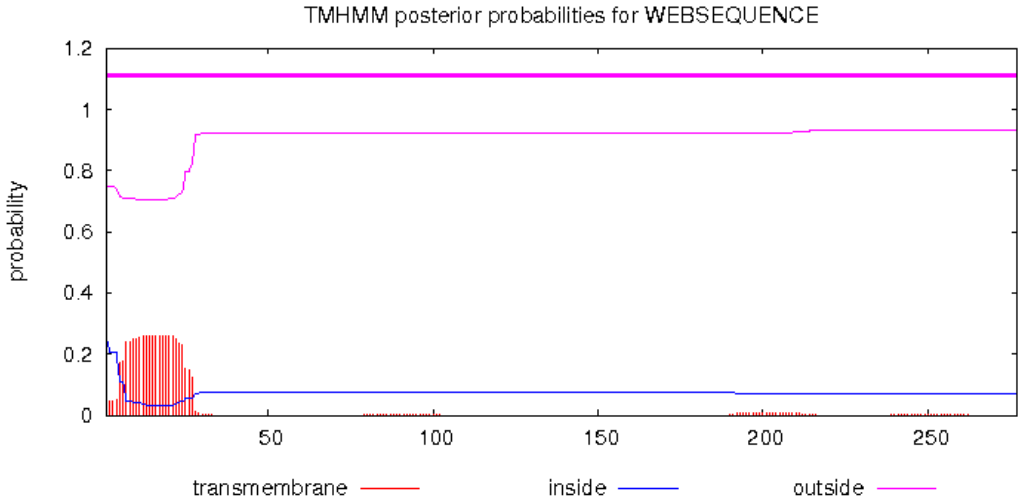
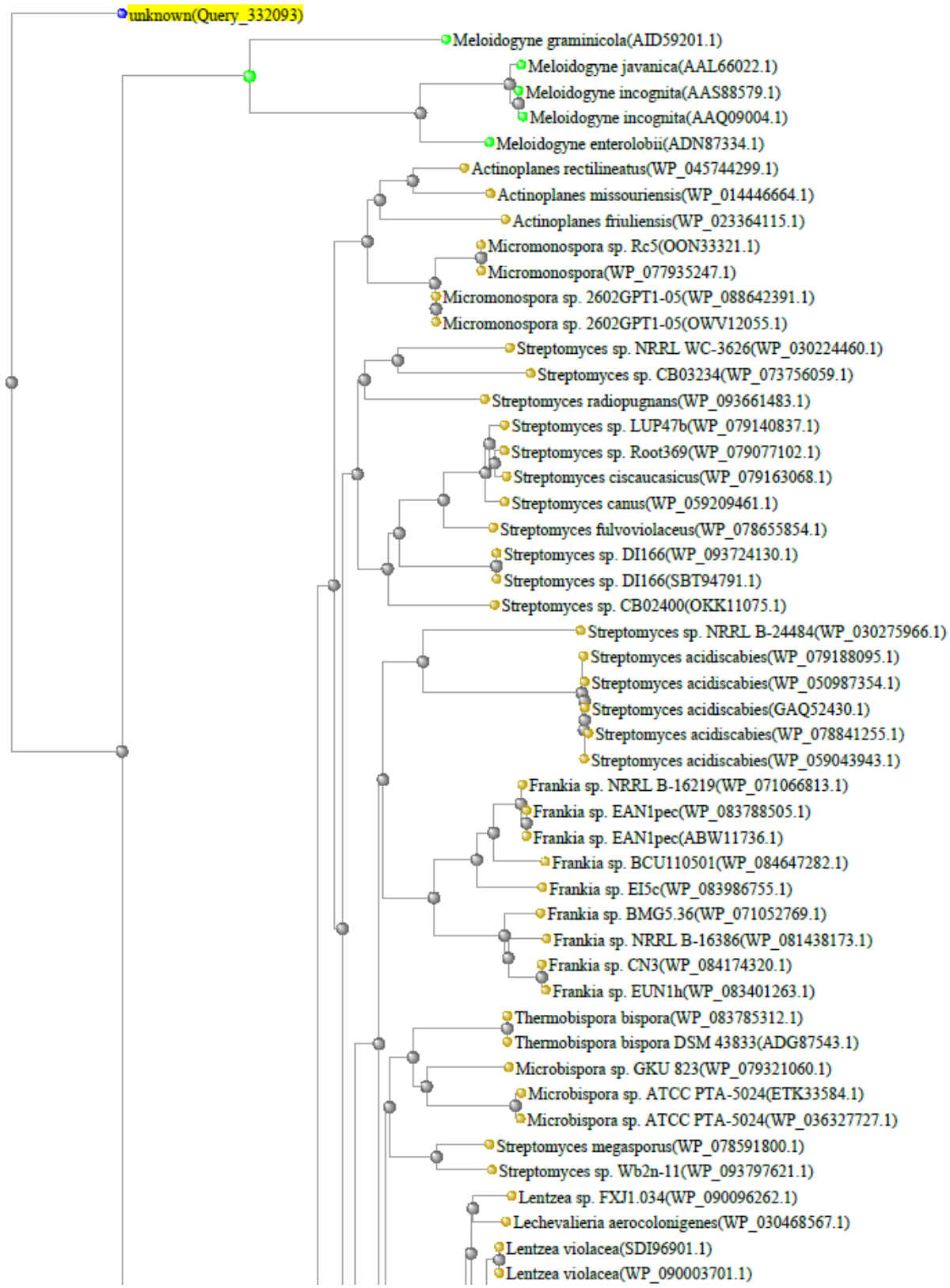


Figure 3.15. TMHMM analysis for prediction of transmembrane helices in *Pt-PL* amino acid sequence.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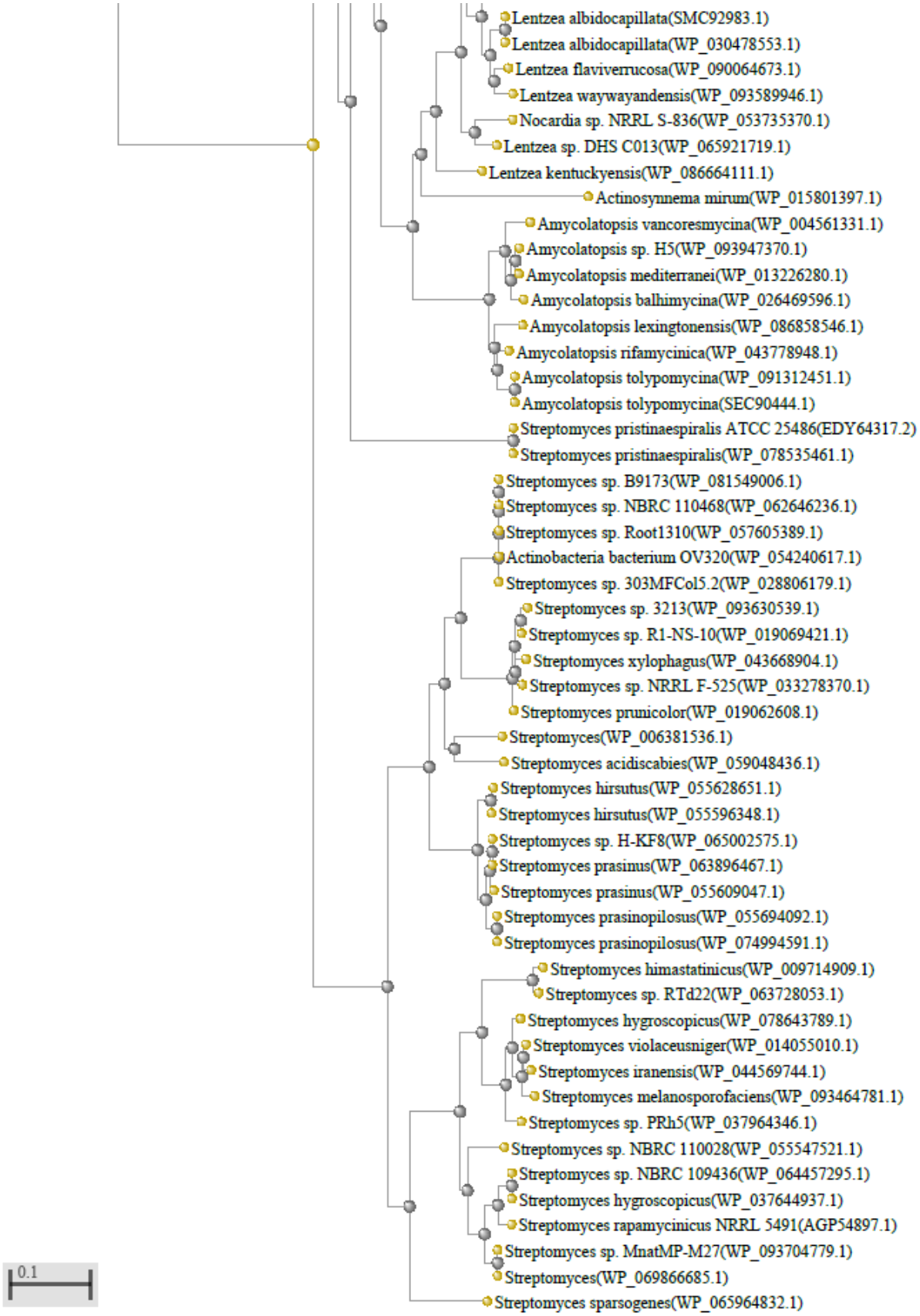
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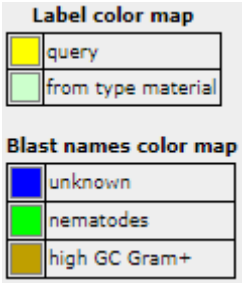


Figure 3.16. Phylogenetic tree representing the position of *Pt-PL* query as a result of BLAST pairwise alignment with respect to its 100 closest homologous amino acid sequences. The horizontal branches represent evolutionary lineages changing over time whilst the horizontal bar of 0.1 at the bottom of the figure provides a scale for the genetic change.

3.3.4.5 Characterisation and validation of *Pt-GST*

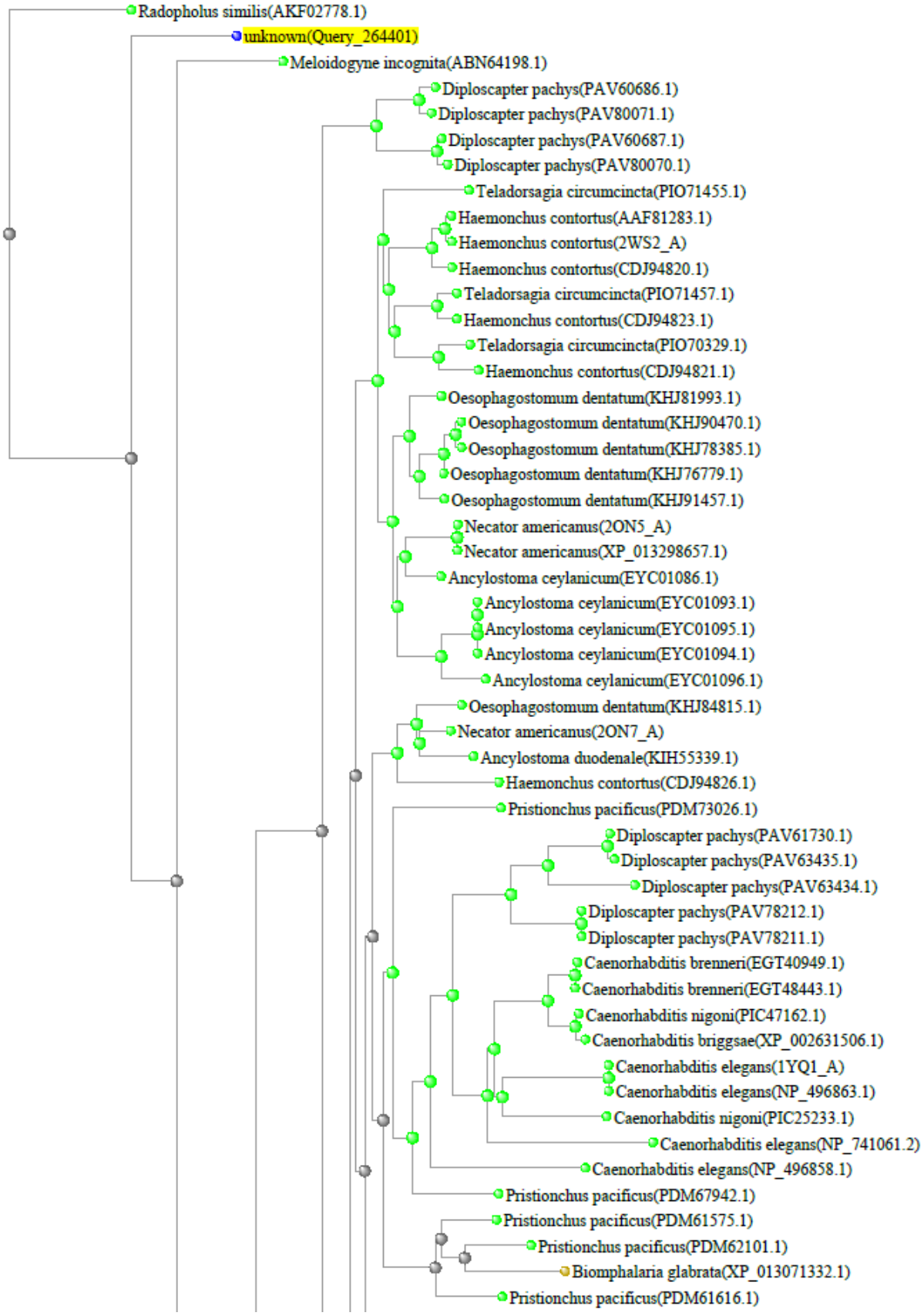
Whilst both 5' and 3' RACE PCRs were done, only 3' RACE PCR resulted in a clear band at approximately 900 bp gel band, and so only a partial sequence was available. Sequencing results used as query in BLASTX showed hits from eukaryotic and bacterial genera. Amongst the eukaryotic matches, most hits were from free-living nematodes, APNs and only two from PPNs. The PPN matches were *GSTs* of *M. incognita* (ABN64198) and *R. similis* (AKF02778) and these two were also the first two amongst the top five matches based on lowest e-values and highest Hsp bit scores. The remaining three were, sigma class glutathione S-transferase of the APN, *Baylisascaris schroederi* (AJH66211), a roundworm, and two hypothetical proteins, CAEBREN 16327 and 08009 of *C. brenneri* (EGT52651 and EJT40878). BLASTX results also showed the presence of the C- terminal alpha helical domain of class alpha GST between 307 to 516 nt within which lies the GST C-terminal domain that begins at 286 nt and ends at 519 nt.

The 179 aa virtual protein derived from ExPasy used as a query in BLASTP also resulted in the same five top hits as the BLASTX results. The taxonomy BLAST report showed that the maximum number of hits that matched the query belonged mostly to APNs, free-living nematodes and only two PPNs *M. incognita* (ABN64198) and *R. similis* (AKF02778). The BLASTP results also showed a conserved C- terminal alpha helical domain of class alpha GST between 58 to 163 aa and GST C-terminal domain between 79 to 175 aa (Figure 3.17). Within the C-terminal alpha helical domain of class alpha GST sequence, seven essential and conserved

residues for each; a dimer interface, substrate binding pocket (an H site), and an N-terminal domain interface were found on the query sequence (Figure 3.17). The seven residues each present in the dimer interface were W_{58}, E_{60}, S_{64}, E_{65}, D_{68}, F_{69}, D_{72} (blue triangles in Figure 3.17), the substrate binding pocket were K_{71}, G_{74}, N_{75}, F_{78}, P_{79}, F_{138} and L_{140} (yellow arrows in Figure 3.17) and the N-terminal domain interface were S_{64}, A_{67}, K_{71}, Y_{130}, M_{134}, Q_{137}, L_{140} (orange circles in Figure 3.17). The substrate binding pocket contains the conserved histidine (H) site at H_{107} of the query also found amongst the other aligning sequences (red star in Figure 3.17). Two more conserved glutathione-binding moieties, Q_{38} and D_{72}, were also found in the query sequence. When the virtual amino acid query of *Pt-GST* was analysed using Interpro, the results indicated the presence of N-terminal GST domain (IPR00405) and C-terminal GST (IPR010987) at 1 to 54 aa and 52 to 179 aa, respectively.

Multiple alignment using COBALT for the amino acid sequence of *Pt-GST* query against the top five hits resulted in similarity of several contiguous sequences and conserved domains across the five matches (Figure 3.17). Due to the absence of a 5'*Pt-GST* RACE product, the sequence information obtained was only partial and hence a methionine residue at the beginning of the sequence could not be identified. The phylogenetic tree produced using BLAST pairwise alignments of 100 closest homologous sequences that matched to *Pt-GST* query (shown in yellow) as a result of BLASTP showed its proximity various GSTs of free-living, APNs and was closest to two PPNs *M. incognita* and *R. similis* (Figure 3.18).

Figure 3. 17. Multiple sequence alignment of the 179 aa sequence of *Pt-GST* query against the five PPN effector proteins as a result of BLASTP. The solid and the dotted horizontal lines indicates C- terminal alpha helical domain of class alpha glutathione S-transferase (GST) and GST C-terminal domain, respectively. Seven residues of the dimer interface, substrate binding pocket, and N-terminal domain interface each are shown with blue triangles, yellow arrows, and orange circles, respectively. The histidine site with the substrate binding pocket is shown by a red star, whilst the two blue stars indicate the presence of glutathione-binding moieties.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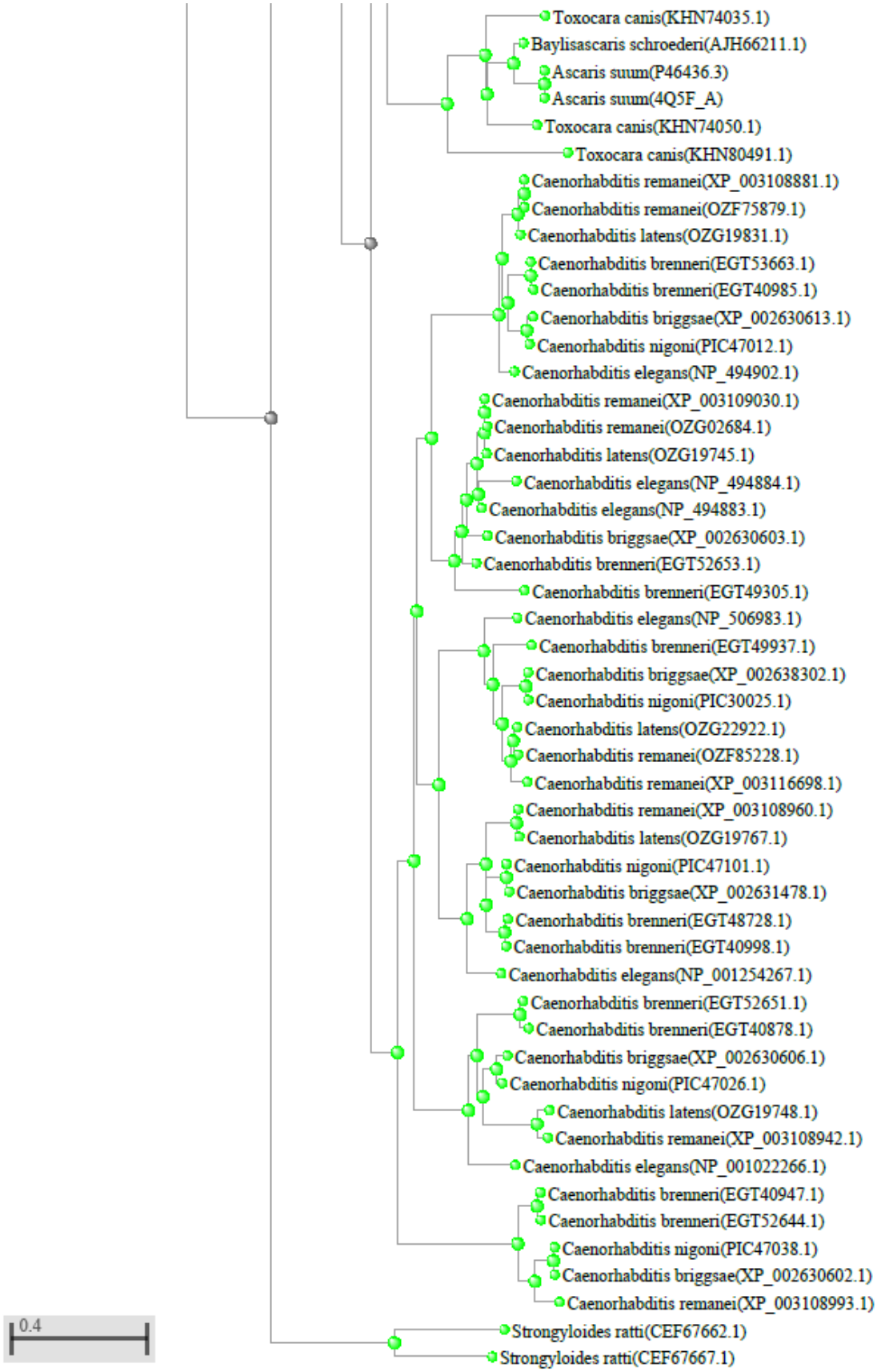
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Caenorhabditis latens(OZG19767.1)
Caenorhabditis nigoni(PIC47101.1)
Caenorhabditis briggsae(XP_002631478.1)
Caenorhabditis brenneri(EGT48728.1)
Caenorhabditis brenneri(EGT40998.1)
Caenorhabditis elegans(NP_001254267.1)
Caenorhabditis brenneri(EGT52651.1)
Caenorhabditis brenneri(EGT40878.1)
Caenorhabditis briggsae(XP_002630606.1)
Caenorhabditis nigoni(PIC47026.1)
Caenorhabditis latens(OZG19748.1)
Caenorhabditis remanei(XP_003108942.1)
Caenorhabditis elegans(NP_001022266.1)
Caenorhabditis brenneri(EGT40947.1)
Caenorhabditis brenneri(EGT52644.1)
Caenorhabditis nigoni(PIC47038.1)
Caenorhabditis briggsae(XP_002630602.1)
Caenorhabditis remanei(XP_003108993.1)
0.4
Strongyloides ratti(CEF67662.1)
Strongyloides ratti(CEF67667.1)

Figure 3.18. Phylogenetic tree representing the position of *Pt-GST* query as a result of BLAST pairwise alignment with respect to its 100 closest homologous amino acid sequences. The horizontal branches represent evolutionary lineages changing over time whilst the horizontal bar of 0.3 at the bottom of the figure provides a scale for the genetic change.

3.4 Discussion

Parasitism effectors that play a significant role during the early stages of infection thereby promoting host entry or invasion are critical in the success of *P. thornei* as a pest. RNAi targeting of such effectors can provide useful information that can be utilised in crop protection strategies. However, one of the factors for achieving successful RNAi is the selection of suitable effector targets and this requires data mining to identify them prior to testing. Hence, in this chapter the primary goals were to undertake comparative bioinformatics using *in silico* tools to identify orthologues of known parasitism effectors in *P. thornei* that could be advanced to RNAi studies.

The selection of target genes in this research made use of existing information on previously reported parasitism effectors of PPNs with RNAi phenotypes. The list of 88 parasitism effectors studied here mainly belonged to sedentary PPNs, and provided a useful starting point to selected effectors in *P. thornei* that may contribute to damage during migration and feeding (Abad et al., 2008; Haegeman et al., 2013; Haegeman et al., 2011b; Nicol et al., 2012). Of the 21 effectors selected that might be involved in host invasion, seven effector sequences were identified *in silico* that matched to *P. thornei* TSA contigs. *P. thornei* sequence that matched to the *Tpx* gene encoding peroxiredoxin of *G. rostochiensis* was not advanced for further because of inconsistencies in PCR amplification. The reason could have been low transcript levels at the time when mixed-stage nematodes were used for RNA extraction. The *P. thornei* were

collected from carrot discs using a mistifier, over 1-3 days. This treatment, in the absence of host tissues, may have reduced such transcript levels. Low expression of peroxiredoxin in non-invasive stages such as eggs and pre-parasitic juveniles of *M. incognita* was reported previously, suggesting it has a role in plant invasion (Dubreuil et al., 2011).

The parasitism effector gene sequences selected as reference for *in silico* searches of the best matching *P. thornei* contigs have been reported to play important roles in host invasion (Abad et al., 2008; Haegeman et al., 2011b; Haegeman et al., 2013; Nicol, 2012). Their known roles, structure and functions have been discussed here with respect to the findings on orthologous sequences of *P. thornei* identified and characterised. It is important to note that all the putative target effector gene sequences that were advanced to RNAi studies included their conserved domains, and four of them were partially sequenced by RACE PCRs, validated and annotated.

Plant cell walls are a major barrier to nematode penetration and migration, and nematodes solve this issue mechanically and using their repertoire of cell wall modifying enzymes (Gao et al., 2004). Although *Pratylenchus* spp. can use their stylet to exert mechanical force to pierce plant cell walls during migration and feeding, the same structure is also responsible for delivering CWDEs that aid in modifying plant cell walls components. Of these CWDEs, β-1, 4-endoglucanases (cellulases) were the first parasitism genes to be cloned from the sedentary PPNs, *H. glycines* and *G. rostochiensis* (Smant et al., 1998; Yan et al., 2001; Yan et al., 1998). It has since been cloned from other sedentary PPNs such as *M. incognita, G. tabacum, G. rostochiensis*, *H. schachtii,* as well as migratory nematodes such as *P. penetrans*, *P. vulnus*, *D. africanus* and *R. similis,* and studied further (Chen et al., 2005a; De Meutter et al., 1998; De Meutter et al., 2001; Fanelli et al., 2014; Goellner et al., 2000; Kyndt et al., 2008; Rehman et al., 2009; Rosso et al., 1999; Uehara et al., 2001). These enzymes hydrolyse the β-1, 4-glucan backbone of polysaccharides such as cellulose and xyloglucan in cell walls during nematode penetration and intracellular migration in roots (Smant et al., 1998; Wang et al., 1999). Whilst all of these enzymes belong to GHF5, those belonging to GHF45 and GHF12 have also been reported in *B. xylophilus* and *X. index* (Jones et al., 2005; Kikuchi et al., 2004). All cellulases of GHF5 in PPNs have a catalytic domain, with some that may or may not have a carbohydrate binding module (CBM) in addition to a linker.

In the current study, the *Pt-Eng-1* identified and characterised using RACE PCRs had a signal peptide for secretion and a catalytic domain. It had a translation start codon, conserved cellulase GHF5 and CBM-2 domains separated by a linker region. This can be supported by evidence of the signature sequences within the GHF5 domains. For example, the conserved site of I-I-Y-E-T-F-P-E-P residues present in the catalytic GHF5 domain of *Pt-Eng-1* was also found in the β-1, 4-endoglucanase sequences of RLNs, *Meloidogyne*, *Heterodera*, *Ditylenchus* and *Aphelenchoides* that were used for multiple alignments thus providing an evidence of its identity (Yan et al., 1998). Similarly, the F-V-T-E-Y-G-T sequence was also conserved amongst the alignments, and is characterised by the presence of nucleophilic glutamic acid (E) (Yan et al., 1998). The *Pt-Eng-1* sequence showed the presence of aryl-phospho-beta-D-glucosidase BglC GHF1 which was not surprising because β-glucosidases have been classified into GH 1, 3, 5, 9, and 30 based on their amino acid sequences. Moreover, GH 1, 5 and 30 represent GH clan A, thus explaining the similarities. β-glucosidases that play a role in metabolism of glycolipids and exogenous glycoside in animals (Cairns and Esen, 2010). The linker region in nematodes has short sequences of glycine, serine, threonine, alanine, proline and lysine with proline/serine/threonine-rich, threonine-rich or glycine-rich linkers in RLNs (Gilkes et al., 1991; Kyndt et al., 2008). The *Pt-Eng-1* sequence studied here also showed a threonine-rich linker region was similar to the other RLN sequences of *P. penetrans* and *P. vulnus* when compared in multiple alignment. Within its CBD-II, two tryptophan residues which are known to be involved in cellulose binding (Béra-Maillet et al., 2000; Tomme et al., 1995) were also found in the *Pt-Eng-1* sequence. It also showed remarkable similarities to the β-1, 4-endoglucanases of *P. penetrans* and *M. incognita.* Finally, the clustering of *Pt-Eng-1* with sequences of *Pratylenchus* suggested the conservation of endoglucanases within Pratylenchidae. The close phylogenetic relation of endoglucanases of RKNs, CNs, and RLNs found here probably suggests horizontal gene transfer from a common ancestor (Haegeman et al., 2011b; Jones et al., 2005).

In the current study, sequence information obtained from 5' and 3' *Pt-Eng-1* RACE PCRs helped in deducing a virtual amino acid sequence based on the overlapping regions. However, using more primer sets in RACE and long-distance PCRs based on the existing *Pt-Eng-1* sequence could have provided data on the missing residues (X) or nucleotides (N). These missing sequences may have contributed to the presence of two peaks at 19th and 21st residues reported in SignalP analysis. In any case, the SignalP results suggested that *Pt-Eng-1* showed

the presence of a cleavage site followed by a signal peptide at the N-terminus. Interestingly, whilst the TMHMM analysis suggested no transmembrane helices in *Pt-Eng-1*, the short segment of approximately 25 aa that appeared as a transmembrane helix, although at a low probability, may have been due to the presence of a signal peptide (Krogh et al., 2001; Sonnhammer et al., 1998). Remarkably, the cleavage site that was predicted at the 24th residue from the N-terminal using SignalP also complemented the pre-sequence TPlen of 23 that precedes the cleavage site using TargetP.

Peptidases collectively referred to as proteases are important proteins that enable all parasites to survive and propagate aiding the infection process (McKerrow et al., 2006; Sajid et al., 2009). They play important biological roles such as contributing to degradation of cellular and tissue barriers, digestion of host proteins, and manipulating host defences to avoid immune responses (Dixit et al., 2008; Goldberg, 2005). Cysteine proteases, synthesised as zymogens, belong to subfamily C1 of clan A possess catalytic activity due to the presence of extra shell electron on the sulphur of thiol group (Atkinson et al., 2009; Silva et al., 2004). The cysteine protease cathepsins include B, H, L, S, C, K, O, F, V, X and W whereas, aspartic protease cathepsins include D and E. In nematodes, cathepsin B, C, D, F, L, S and Z are known so far, of which cathepsin L is functionally an endopeptidase that is constitutively expressed at high levels in many tissues (Guha and Padh, 2008). Intestinal cathepsin L-like cysteine proteases may play a role in extracellular digestion of dietary proteins indicating direct involvement in digestion (Lilley et al., 1996; Neveu et al., 2003; Shingles et al., 2007; Urwin et al., 1997).

In the current study, because there was sequence information for 3'-*Pt-CLP* and not for 5'-*Pt-CLP*, only a partial sequence was obtained. The histidine (H) residue within the signature conserved site of all cysteine cathepsins was also identified in the partial 189 aa sequence of *Pt-CLP* between 133 to 143 aa (Guha and Padh, 2008). The important C, H and N residues that form the catalytic triad of the active site were found in the multiple alignment with seven sequences of cathepsin proteases from various PPNs (Neveu et al., 2003; Shingles et al., 2007). Amongst these residues, the conserved histidine (H) and aspargine (N) residues were found in *Pt-CLP*, however, due to limited RACE sequence information the cysteine (C) residue could not be identified. Nevertheless, the presence of two other residues of the catalytic triad support the identity of *Pt-CLP.* The phylogenetic analysis using partial *Pt-CLP* seemed to be an outgroup

in comparison to the clustered cysteine proteases of free-living nematodes, APNs and PPNs. If more sequence information was derived from 5'-*Pt-CLP* and a comprehensive amino acid sequence data were available, this would provide a better phylogenetic relationship with respect to other PPNs.

The major polysaccharides in the primary wall of plants are cellulose, hemicelluloses and pectin. Whilst cellulose and hemicellulose are composed on 1,4-linked β-D-glucan and 1,4-linked β-D-hexosyl residues, respectively, pectin is a complex polysaccharide that contains 1,4-linked α-D-galacturonic acids (Barras et al., 1994). Pectin, is located in the middle lamella and primary cell wall and acts as a matrix anchoring cellulose and hemicellulose fibres (Carpita and Gibeaut, 1993; Tamaru and Doi, 2001). Pectate, a highly methylated form of pectin, can be degraded by pectin degrading genes encoding pectate lyases, which have been found in PPNs such as *Heterodera*, *Globodera*, *Meloidogyne* and the migratory nematode *B. xylophilus* (De Boer et al., 2002; Doyle and Lambert, 2002; Huang et al., 2005; Kikuchi et al., 2006; Kudla et al., 2007; Popeijus et al., 2000a; Popeijus et al., 2000b; Vanholme et al., 2007). Pectate lyases class are classified as polysaccharide lyases and are found in five of the fifteen families (1, 2, 3, 9 and 10) (Henrissat, 1991; Henrissat and Davies, 1997; Kikuchi et al., 2006). Pectate lyases are produced in the oesophageal gland cells and secreted by the stylet when nematodes invade host tissue. Pectate lyases are defined by their homology to known pectate lyases and some examples in PPNs are: the *Bx-Pel-1* and *Bx-Pel-2* gene of *B. xylophilus*, *Mi-Pel-1* and *Mi-Pel-2* of *M. incognita*, *Mj-Pel-1* of *M. javanica*, *Hg-Pel-1* of *H. glycines*, *Pel-3, 4, 6* and 7 of *H. glycines*, *Gr-Pel-1* and *Gr-Pel-1* of *G. rostochiensis*, *Pel-2* of *G. rostochiensis, G. pallida* and *G. tabacum* (De Boer et al., 2002; Doyle and Lambert, 2002; Huang et al., 2005; Kikuchi et al., 2006; Peng et al., 2016; Popeijus et al., 2000a; Popeijus et al., 2000b; Stare et al., 2011). In addition, *in silico* 30 pectate lyases have been identified in the genome of *M. incognita* (Abad et al., 2008; Bellafiore et al., 2008; Caillaud et al., 2008)

In the current study, the partial 282 aa *Pt-PL* contained a putative start codon and signatures of the pectate lyase domain. Within its catalytic domain, the *Pt-PL* had the conserved residues aspartic acid (D_{158}), lysine (K_{159} and K_{166}) and arginine (R_{84}) that also aligned with other pectate lyases used in multiple alignments. The presence of these residues indicates a BF domain that is typically found in pectate lyase family 3 (Hatada et al., 2000; Kudla et al., 2007). These are

amongst the seven residues that known to be active and calcium binding sites in the class III *Pel-2* of *G. rostochiensis* (Kudla et al., 2007). Similarly, four other conserved regions that are hallmarks of family 3 or class III pectate lyases containing the BF domain (Huang et al., 2005) were found in the partial *Pt-PL*. The two closely spaced cysteine residues (C186, C189) found in the partial *Pt-PL* have also been found in the other class III pectate lyases such as *Bx-Pel-1* and *Bx-Pel-2* gene of *B. xylophilus*, *Mj-Pel-1* of *M. javanica, Mi-Pel-1* and *Mi-Pel-2* of *M. incognita*, *Hg-pel-1, 2, 3, 4, 6,* and *7* of *H. glycines.* The conserved cysteines appear to play a role in supporting two disulphide bonds and catalysis (Huang et al., 2005; Kikuchi et al., 2006; Peng et al., 2016; Popeijus et al., 2000a; Popeijus et al., 2000b). Similarly, an N-glycosylation site (N_{201}) found in the partial *Pt-PL* has been reported in other class III pectate lyases such as *Hg-Pel-1* of *H. glycines*, *Mj-Pel-1* of *M. javanica*, which, in fact has one more in addition to the this (De Boer et al., 2002; Doyle and Lambert, 2002). All these data support the identity of the partial *Pt-PL* to be a class III pectate lyase. If there were sequence data available from *3'Pt-PL*, then perhaps a full-length amino acid sequence could have been generated which would have additional hallmarks of class III pectate lyase such as two more cysteine residues towards the carboxyl end of the amino acid sequence that have been found in *Hg-Pel-1* and *Hg-Pel-2* described by de Boer (2002) and Gao (2003), and also found in the alignment of family 3 pectate lysases of *Hg-Pel-1* to *7*, *Hs-Pel-1* of *H. schachtii*, *Gr-Pel-1* and *2* of *G. rostochiensis*, *Gp-Pel-2* of *G. pallida*, *Aa-Pel-2* of *A. avenae*, *Bx-Pel-1* and *Bx-Pel-2* gene of *B. xylophilus*, *Bm-Pel-1* and *Bm-Pel-2* gene of *B. mucronatus* and *Mi-Pel-1* and *Mi-Pel-2* of *M. incognita* (De Boer et al., 2002; Gao et al., 2003; Peng et al., 2016). Interestingly, although the last cysteine residue towards the C-terminal was present, the cysteine preceding it was not identified in the query.

The phylogenetic tree indicated that *Pt-PL*, although an outgroup, was closely related to pectate lyases of *Meloidogyne* and Gram positive bacteria. The Tylenchida group consist of CNs and RKNs and hence the phylogenetic relationship with *Meloidogyne* was not surprising. Similarly, clustering of bacterial sequences was not surprising because pectate lyases are thought to be of bacterial origin *via* horizontal gene transfer (Jones et al., 2005). They are also secreted by plant pathogenic bacteria that cause devastating diseases and maceration of tissues in various plants (Keen et al., 1984; Kelemu and Collmer, 1993; Marín-Rodríguez et al., 2002; Perombelon and Kelman, 1980). Similarly, horizontal gene transfer of other PPN genes is thought to have occurred from bacterial and fungi, and this is particularly the case for the

cell wall degrading enzymes like cellulases, pectate lyases, polygalacturonase, xylanase, chorismite mutase, and even expansins (Haegeman et al., 2011a; Jones et al., 2005).

The use of consensus sequence to modify the two missing residues in proximity to the methionine at the N-terminal of *Pt-PL* provided important information on the signal peptide, cleavage sites suggesting a non-classical secretory pathway. As with *Pt-Eng-1*, 5'-*Pt-PL* also had a short signal peptide sequence that may have resulted in the transmembrane segment that was detected with low probabilities in TMHMM. The cleavage site at the 30^{th} residue complemented the pre-sequence TPlen of 29 that precedes the cleavage site using SignalP and TargetP, respectively. Nonetheless, the current information on obtained for *Pt-PL* indicates its strong likelihood to be a family 3 or class III pectate lyases.

The GSTs are evolutionarily conserved major cellular detoxification enzymes and they catalyse the conjugation of various electrophiles with glutathione (GSH) thus detoxifying endogenously and exogenously produced toxic compounds (Jerina and Bend, 1977; Ketterer and Meyer, 1989; Leiers et al., 2003; Wilce et al., 1995). GSTs are thought to play a role in reducing oxidative stress as a result of the plant defence system by scavenging reactive oxygen species such as hydrogen peroxide (Kuźniak and Urbanek, 2000; Melillo et al., 2006; Torres-Rivera and Landa, 2008). Seven species-independent soluble GSTs are known and these are Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega (Board et al., 2000). In *C. elegans*, for example, there are 50 putative GSTs of which some are Alpha, Pi and Sigma whilst others are nematode-specific GSTs (Campbell et al., 2001). GSTs have been found in free-living nematodes, APNs and PPNs. Amongst the free-living nematodes, *Ce-GST-p24* of *C. elegans* has been reported (Leiers et al., 2003). Similarly, in APNs, *Na-GST-1* to *3* of *Necator americanus, Bsc-GST-sigma* of *B. schroederi*, GST of *Heligmosomoides polygyrus* have also been reported (Brophy et al., 1995a; Brophy et al., 1995b; Xie et al., 2015). A few PPN GSTs such as *Mi-GSTS-1* of *M. incognita*, *Rs-GST* of *R. similis* and *Gsts* of *R. reniformis* have been reported (Dubreuil et al., 2007; Espada et al., 2016; Hou, 2013; Sherratt and Hayes, 2002; Van Rossum et al., 2001a; Zhan et al., 2010).

The *Pt-GST* in this study was a 179 aa partial sequence which showed the presence of the GST domain containing signature conserved residues each for a dimer interface (W_{58}, E_{60}, S_{64}, E_{65}, D_{68}, F_{69}, D_{72}), substrate binding pocket with an H_{107} site (K_{71}, G_{74}, N_{75}, F_{78}, P_{79}, F_{138} and L_{140}),

and an N-terminal domain interface (S_{64}, A_{67}, K_{71}, Y_{130}, M_{134}, Q_{137}, L_{140}). There are two tyrosine (Y) residues associated with the stabilisation of GSH and are known to be conserved in the N-terminal end closer to the start codon (Y4 and Y8) (Torres-Rivera and Landa, 2008). These two residues were found in at least three of the five top hits, the PPN, *M. incognita* (ABN64198), the APN, *B. schroederi* (AJH66211), and the free-living nematode *C. brenneri* (EGT52651 and EJT40878). Unfortunately, due to incomplete sequence information for *5'-Pt-GST*, the two tyrosine residues were not evident. Several GSH-binding moieties are conserved in the GST domain, of which glutamine (Q_{63}) and aspartic acid (D_{97}) have been reported in *B. schroederi* and were found in the query sequence as Q_{38} and D_{72}. Interestingly, whilst the glutamine was found to be common amongst all five hits, aspartic acid residue was only common in *B. schroederi* and *C. brenneri* but not in the two PPNs (Jerina and Bend, 1977). There is less information on GSTs of PPNs thus limiting the comparisons. However, the conserved GST domain-related data from the two PPNs, APNs and free-living nematodes, supports the identity of the partial *Pt-GST-1*. This also reflected in the phylogenetic tree where *Pt-GST-1* clustered with the GSTs of two PPNs, and several APNs and free-living nematodes, but most importantly, it showed close relation with the migratory burrowing PPN, *R. similis* and the RKN, *M. incognita.*

Whilst it would have been very interesting to have complete amino acid sequences as a result of *3'-Pt-PL*, *5'Pt-CLP* and *5'Pt-GST* RACE PCRs as well as identifying all residues of the reported sequences, there were some practical difficulties. First, several primer sets and sequencing rounds were required to identify unknowns or missing bases and to amplify the lacking RACE-ends if missing. This would have been not only time-consuming but also required resources, which were in fact limited. Second, the number of reactions in a RACE kit were limited, requiring additional components thereby increasing the cost of carrying out RACE PCRs. The use of full-length sequences in RNAi seemed exciting however, due to the limitations described above, only best matching *P. thornei* sequences within the conserved domains were selected, cloned into suitable vectors based on the best option of restriction enzymes, and advanced to RNAi studies. Finally, this research focused on studying the effects of *in vitro* and *in planta* RNAi in two different hosts. Time was a major limiting factor to pursue full-length gene sequences through more RACE PCRs. However, there were enough gene sequences to proceed to RNAi studies.

CHAPTER 04

Effects of *In Vitro* RNAi on *Pratylenchus thornei*.

4.0 Introduction

Following the discovery and development of target gene silencing in *C. elegans* (Fire et al., 1998) RNAi has offered a functional genomics tool to specifically and transiently silence target PPN genes, including the parasitism genes that encode secreted effector proteins (Dalzell et al., 2011; Davis et al., 2008; Gheysen and Vanholme, 2007; Rosso et al., 2009).

The efficacy of target gene silencing *via* RNAi depends on the delivery of dsRNA complementary to the mRNA of the gene to be silenced. There are several options for delivery of dsRNA, such as microinjection, soaking, and feeding bacteria expressing dsRNA. Soaking of PPNs in dsRNA solution is the most frequent method used because PPNs are obligate parasites and do not normally feed until they have infected a host plant, and microinjection is a difficult mode of delivery for PPNs. Soaking solutions often include octopamine to stimulate oral uptake and fluorescein isothiocyanate (FITC) to provide a visual marker for uptake of dsRNA. RNAi effects using soaking were first demonstrated by Urwin et al., (2002) in J2s of *H. glycines* and *G. pallida*, where targeting cysteine proteases resulted in alteration of sexual fate in favour of males (Urwin et al., 2002). Since then, RNAi *via* soaking has been achieved for infective stages of boths sedentary and migratory PPNs, including *M. artiellia*, *M. incognita*, *M. javanica*, *M. graminicola*, *H. schachtii*, , *B. xylophilus*, *R. similus*, *P. coffeae*, *P. thornei P. zeae* and *P vulnus* (Fanelli et al., 2005; Fanelli et al., 2014; Gheysen and Vanholme, 2007; Gleason et al., 2008; Haegeman et al., 2009; Huang et al., 2005; Joseph et al., 2012; Park et al., 2008; Tan et al., 2013).

For *P. thornei*, so far there is less information on silencing. For example, two essential body-wall muscle component genes, *unc-87* and *pat-10* have been silenced using *in vitro* RNAi, although these genes are involved in motility rather than host parasitism. Tan et al., (2013), reported paralysis and uncoordinated movement, and a significant reduction in the reproduction of 77% to 81% on carrot mini discs over a period of five weeks after infecting with dsRNA-fed nematodes. However, in her Ph.D. thesis (Tan, 2015) she also studied *in vitro* RNAi of some parasitism effector genes such as those involved in cell wall modification, protein digestion, reactive oxygen species scavenging enzymes and putative parasitism. In other published reports of *in vitro* RNAi of parasitism effectors of other *Pratylenchus* species, in *P. vulnus* the effects of RNAi silencing of two β-1, 4-endoglucanase genes (*Pv-eng-1* and *Pv-eng-*

2) was studied through mRNA expression and phenotypic assays (Fanelli et al., 2014). The authors reported a significant reduction in *Pv-eng-1* mRNA expression, but no significant reduction of *Pv-eng-2* mRNA, and no apparent effects on motility of the nematodes for either gene. *In vitro* RNAi *via* soaking thus provides an opportunity to screen target genes, particularly parasitism effector genes, and study their effects on growth and infectivity before advancing promising targets to plant-mediated RNAi.

4.1 Aims and objectives of Chapter 4

The aims of research in this chapter were to test the effects of silencing target *P. thornei* parasitic effector sequences by soaking and to assess their suitability for *in planta* RNAi. Specific objectives were: synthesis of dsRNA for five target *P. thornei* parasitic effector sequences, soaking nematodes in dsRNA solution, studying immediate effects on phenotype or behaviour, assessing transcript levels after soaking, and long-term effects on reproduction of *P. thornei* on two hosts, soybean and alfalfa.

4.2 Materials and methods

4.2.1 Sucrose gradient separation, cellulase activity, and temporal expression of target genes in eggs and mixed stages of *P. thornei*.

A density gradient-based separation of *P. thornei* life-stages was undertaken to separate eggs from mixed stages of *P. thornei* before undertaking *in vitro* RNAi. This was done to obtain a homogenous collection of eggs to check the temporal expression of target genes before *in vitro* RNAi studies, and also to use them to assess possible cellulase enzyme activity.

A simple, effective technique for separating eggs from mixed stages of *P. thornei* was adapted from Acedo and Dropkin (1982) with slight modifications. Nematodes from callusing carrot discs were collected in a 50 mL sterile Falcon tube as described in section 2.2, the only difference being that a coffee filter paper was not used to ensure the greatest number of nematodes at different stages were collected. Nematodes collected after 24 h on the mistifier were sterilised as described in section 2.2, and an aliquot of 500 µL (approx. 10,000 mixed-stage nematodes) was used for sucrose density separation. In a sterile 50 mL round-bottom

clear polypropylene tube, 10 mL layers of 70%, 50%, 40% and 20% pre-chilled sucrose were poured successively, followed by the nematode sample, which was loaded carefully to slide along one wall of the tube.

A subsequent separation of mixed life-stages from the 20% sucrose sample was done by resuspending the nematodes in water, followed by washing and centrifugation at 800 g for 1 to 2 minutes to obtain a clean nematode pellet. After this, a 500 µL aliquot of the sample was introduced into a tube containing 10 mL layers of 50%, 30%, 10% and 5% sucrose (poured sequentially). This was done very carefully by allowing the liquid to slide down the wall of the tube. The tubes were centrifuged at 800 g for 5 minutes after which different developmental stages were separated. The stages were confirmed under a dissecting microscope by carefully withdrawing an aliquot of each sucrose layer using a 200 µL pipette. Subsequently, separated *P. thornei* nematodes and eggs were washed thoroughly with water, cleaned as described in section 2.2., and confirmed under a dissecting microscope (to ensure no juveniles and/or adults were present). The cleaned eggs were used to stud cellulose activity and temporal target gene expression.

A carboxymethylcellulose (CMC) plate assay was used with slight modifications from Rosso et al. (1999), Ray (2001) and Saha and Ray (2015). Briefly, CMC and agar at concentrations of 0.5% and 1.5% were used to prepare media plates to study cellulase activity in eggs and mixed stages of *P. thornei*. Samples were tested in homogenised and unhomogenised (intact) forms. Homogenisation was done by adding 2-3 steel beads to a 2 mL tube containing eggs or mixed life-stage pellet and lysed in a tissue lyser at 25 strokes/sec for 3 minutes. A total of 2 µL sample was loaded in triplicates on one CMC plate, and three plates per sample were done. The water for cleaning was used as a control. Plates were incubated overnight at room temperature, subsequently 0.1% Congo Red was used to stain the plates for 30 minutes after which the stain was drained off and plates were gently washed with 1 M sodium chloride. Any remaining stain was washed off. The zone of clearing around the extracts was then measured.

To study the expression pattern of selected target gene sequences in eggs and mixed life-stages of *P. thornei*, a temporal analysis represented by varying PCR cycles was undertaken. For this, RNA was extracted from cleaned samples and cDNA was synthesised as described in

section 2.3. Semi-quantitative PCRs as described in section 2.6.2 were carried out using the GSPs shown in Table 2.1. PCRs were done at 20, 25, 30 and 35 cycles, after which the products were electrophoresed on an agarose gel as described in section 2.7.

4.2.2 Synthesis of dsRNA

The *in vitro* transcription vector, pDoubler containing the target sequences was used to synthesise dsRNA. After confirmation of the cloned sequences, the sequences with T7 promoter regions were digested from the vector using either *NotI* or *EcoRI* and electrophoresed on agarose gel. Products were checked for their expected sizes, cleaned using a Wizard SV Gel and PCR Clean-up kit, quantified with a NanoDrop spectrophotometer and used as a template for *in vitro* transcription reactions. The Hi-Scribe T7 *in vitro* transcription kit (NEB, Genesearch, Australia) was used to generate dsRNA as per the manufacturer's protocol. A total of 1 µg template, 1 X reaction buffer, 10 mM of ATP, GTP, CTP and UTP, 2 µL T7 RNA polymerase mix, and the appropriate volume of RNase-free water was used in a 20 µL reaction. Reactions were incubated at 37°C for 16 h in a thermal cycler. Reactions were treated with DNaseI to remove any template DNA. This was done by adding 2.5 µL RNase-free DNase, 10 µL RDD buffer (Qiagen, Australia), and the appropriate volume of RNase-free water was added to make the volume to 200 µL. Reactions were incubated at 20 to 25°C for 10 minutes.

DNA-free dsRNA was precipitated using chloroform followed by sodium acetate and ethanol precipitation described in section 2.3. The RNA pellet was washed with 70% ethanol, dissolved in 20 to 30 µL RNase-free water, quantified with a NanoDrop spectrophotometer and stored at -80°C until further use.

4.2.3 *In vitro* feeding of dsRNA to *P. thornei*

Nematodes extracted from callusing carrots were cleaned as described in section 2.2 and a mixed-stage population of 8000 *P. thornei* were soaked in M9 buffer (43 mM disodium phosphate Na_2HPO_4, 22 mM monopotassium phosphate KH_2PO_4, 2 mM sodium chloride NaCl, 4.6 mM ammonium chloride NH_4Cl) containing 1 mg/mL dsRNA, 50 mM octopamine hydrochloride (Sigma Aldrich), 3 mM spermidine trihydrochloride (Sigma Aldrich) and 0.05% gelatine. All stock solutions were prepared in sterile M9 buffer. Tubes containing the soaking

medium were incubated at 25°C for 16 h in the dark. Control of 1 mg/mL dsGFP and no dsRNA were also set up. Another soaking medium containing 1 mg/mL FITC isomer I (Fluka 46952) without dsRNA was set up to assess uptake of solution. Two replicates of soaking experiments were set up. Nematodes were washed with sterile water twice after which their behaviour and activity was checked under a photomicroscope (Olympus BX-51) with filter for fluorescein wavelength to observe nematodes fed on FITC. To assess any changes in the integrity 500 ng/uL dsRNA before and after feeding was checked on 1.2% agarose gel.

4.2.4 Target gene expression after *in vitro* RNAi

Changes in gene expression were assessed in nematodes fed on dsRNA for 16 h. Two thousand nematodes from each sample were washed, flash-frozen in liquid nitrogen and stored at -80°C until further use. RNA extraction and cDNA synthesis from 100 ng of total RNA was done as described in section 2.3. A 1:10 dilution of cDNA was used for semi-quantitative PCRs with gene-specific primers were carried out as described in section 2.6.2. *P. thornei* 18S rRNA primers were used as normalisation control and the products along with a 100 bp DNA marker was electrophoresed on 1.5% agarose gel. In addition to visual analysis, densitometric analysis of band intensities was done using ImageJ as described in section 2.8. and expressed as percent reduction of target gene expression relative to 18S rRNA.

4.2.5 Long-term effects of *in vitro* RNAi on reproduction of *P. thornei*

To study the effects of *in vitro* RNAi on reproduction and infectivity of *P. thornei*, cleaned nematodes after 16 h of soaking were used to inoculate soybean and alfalfa plants. Seeds of soybean (*Glycine max*) cv. Williams 82 were kindly donated by Dr. Brett Ferguson, CILR, School of Agriculture and Food Sciences, University of Queensland, Australia. Alfalfa (*Medicago sativa*) seed ('Sprouts Alive', Mr. Fothergills Seeds Pty Ltd., NSW, Australia) were purchased from Bunnings, Australia.

A preliminary susceptibility test of both hosts was done to ensure infections by *P. thornei.* For this, seeds were surface-sterilised with 2% sodium hypochlorite containing Tween (1 drop per 50 mL solution) for 5 to 10 minutes, washed thoroughly with sterile water, and blot dried on Whatman filter paper in a sterile 90 mm Petri dish. Seeds were then planted in a 620 mL clear plastic cups containing washed and pasteurised Brunnings propagating sand (Bunnings,

Australia). Two-week-old plants were infected with approximately 500 cleaned nematodes and placed in the glasshouse (25 ± 5°C) for four weeks. For the first two days plants were not watered after which they were watered regularly. Two weeks after inoculation, some plants were gently uprooted, checked for any visual symptoms of infection (lesions), and presence of nematodes by staining as described in section 2.16. Twenty-one days after inoculations plants were finally uprooted, roots excised and placed together with the soil in the misting chamber (section 2.2) for nematode collection at 24 and 48 h. Since *P. thornei* is a migratory nematode, including soil in the misting chamber was done to ensure achieve maximum extraction of nematodes.

The same procedure was also used to study long-term effects of *in vitro* RNAi on reproduction and infectivity of *P. thornei*. Ten plants of soybean and alfalfa each for every sample (dsRNA of five target genes and *GFP*, and no dsRNA) were inoculated with approximately 500 nematodes cleaned after 16 h soaking experiments. Presence of nematodes was confirmed by staining two weeks after inoculation, and a final count of total number of nematodes was recorded after 21 days.

4.2.6 Statistical analysis

All data were analysed in Microsoft Excel using the functions, formulae and data analysis tool package. This involved the Shapiro-Wilk test to determine normality and Levene's test for homoscedasticity (homogeneity of variances) followed by one-way analysis of variance (ANOVA) to compare means of groups in an experiment was done. Wherever necessary, a post-hoc test was done to determine significant differences ($p < 0.05$). A Tukey's honest significance difference (HSD) test appropriate for equal samples sizes was done to achieve pairwise comparisons amongst all groups within an experiment. Bar charts were made with error bars representing standard error of means for each treatment.

4.3 Results

4.3.1 Sucrose gradient separation, cellulase activity, and temporal expression of target genes in eggs and mixed stages of *P. thornei*

Density-based sucrose gradient separation was used to separate eggs from mixed stages of *P. thornei.* After centrifugation, there were no motile nematodes or carrot particles in the lower-most 70% sucrose layer. However, some remaining carrot particles were suspended in the 50% sucrose layer.

In the 40% sucrose layer, an obvious band of motile nematodes was visible at the meniscus of 20% and is indicated by a blue arrow in Figure 4.1. This was followed by another band immediately below, and is indicated by grey arrow in Figure 4.1. Aliquots of both of these bands contained a combination of eggs, juveniles and adults. However, the proportion of eggs in comparison to juveniles and adults differed. In the meniscal layer between 20% and 40% sucrose, there were 40% eggs as compared to juveniles and adults, while in the band immediately below, eggs accounted for only 20% of the life-stages. In the 20% sucrose layer, there was also mixture of eggs, juveniles and adults, and is indicated by a red arrow in Figure 4.1. In this layer, the percentage of eggs (80%) was much greater than juveniles and adults than that found in the 40% sucrose layer.

Overall, given the higher proportion of eggs found in the 20% sucrose, this layer was used to purify eggs in gradients of 50%, 30%, 10% and 5% sucrose. The results of gradient separation were: in the 5% sucrose solution there were no motile nematodes or eggs, in the 10% sucrose layer there were >90% eggs, in the 30% sucrose layer there were 5% eggs and the remainder were juveniles and adults, and in 50% sucrose layer there were no life-stages detected. Eggs were collected carefully from the 10% sucrose layer and used for CMC plate assays and temporal target gene expression.

CMC plate assays were done to test if eggs and mixed-stage nematodes exhibited cellulase activity, which was visualised as a zone of clearing or halo around the site of inoculation. Both nematode stages were tested in their homogenised and unhomogenised forms, and a control consisting of the water used for washing the nematode stages was also checked. While the control plates showed no halo formation, both eggs and mixed-stage nematodes displayed a halo around the sites of inoculation (Figure 4.2). It was evident that the zone of clearing measured around mixed-stage nematodes was greater than that around the eggs. Between the two forms (homogenised or intact) of eggs tested for cellulase activity, the diameter of the

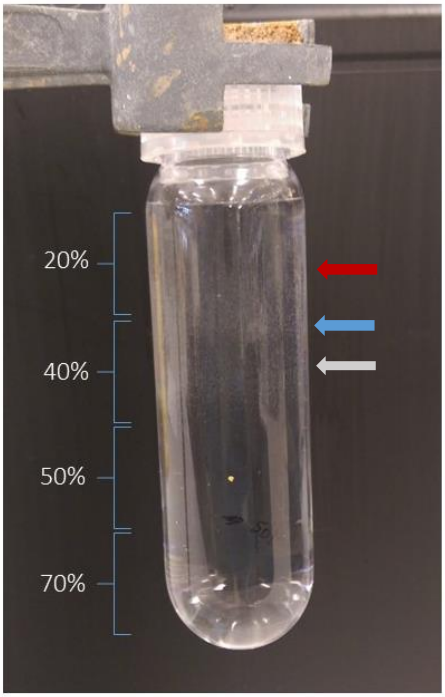

Figure 4.1. Density-based sucrose gradient separation showing life-stages of *P. thornei* separated in varying percentages of sucrose layers.

clearing around the homogenised eggs (0.98 cm ± 0.20 cm) was slightly greater than for the unhomogenised eggs (0.95 cm ± 0.21 cm), but this was not significantly different. Similarly, the diameter of the zone of clearing measured around the homogenised mixed-stage nematodes (1.7 cm ± 0.20 cm) was slightly greater than for unhomogenised mixed-stage nematodes (1.6 cm ± 0.20 cm), although this was also not significantly different.

Temporal patterns of target gene expression were assessed using semi-quantitative PCRs for eggs and mixed-stage nematodes. Semi-quantitative PCRs done at different PCRs cycles on cDNA synthesised from extracted RNA indicated that while the constitutively produced housekeeping gene control, 18s rRNA was expressed consistently in both eggs and mixed-stage nematodes, the expression of target genes varied between eggs and motile stages (Figure 4.3).

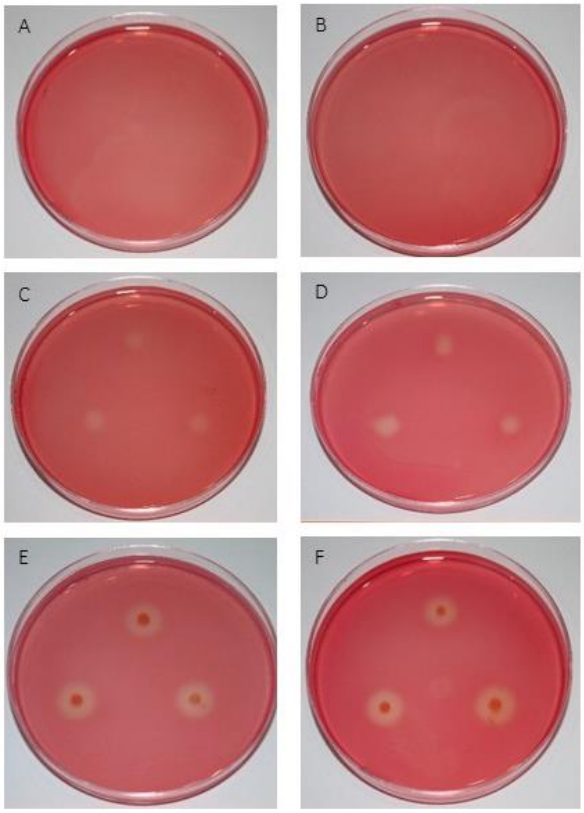

Figure 4.2. CMC plate assay. Images of CMC plates showing no cellulase activity indicated by the absence of a halo from the washing water used to clean nematodes and eggs (Figure A and B, respectively). Presence of a halo indicated cellulase activity from washed whole and homogenised eggs (Figure C and D, respectively). Similar but larger diameter halos are present around washed whole and homogenised mixed stages of nematodes (Figure E and F, respectively).

In eggs, *Pt-Eng-1* and *Pt-PL* were detectable at the 35th PCR cycle while none of the other genes was detected. On the other hand, in mixed-stages of *P. thornei*, all five target genes were expressed at all PCR cycles except at the 20th PCR cycle, where *Pt-UEP* and *Pt-GST* were generated very faint bands compared to the other three genes. The intensities of the DNA bands on the gels clearly increased from 25 PCR cycle onwards, and this was consistent for all the target genes. This information was valuable in determining the number of PCR cycles to be used for semi-quantitative PCRs analyses for target gene expression after *in vitro* and *in planta* RNAi.

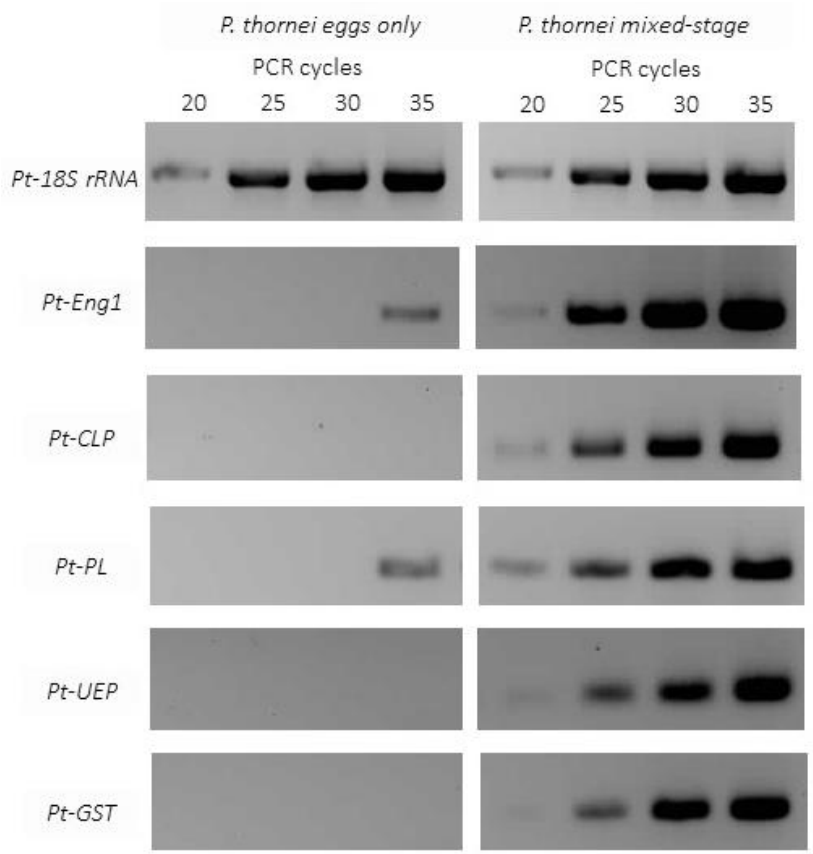

Figure 4.3. Temporal expression of target genes in eggs and mixed-stages of *P. thornei* by semi-quantitative PCR at 20, 25, 30 and 35 cycles. Visible expression of the positive control, 18S rRNA at all PCR cycles in eggs and mixed-stages of nematode shown above. Visible expression of *Pt-Eng-1* and *Pt-PL* in eggs detected at 35 cycles, whereas, expression of all five target genes sequences detected in mixed-stages of *P. thornei* at all cycles.

The results of the CMC plate assays suggested that both eggs and mixed-stage nematodes exhibit cellulase activity. The results of temporal expression assays confirmed that the genes *Pt-Eng-1* and *Pt-PL* were expressed in eggs. However, these gene transcripts were only detected at the 35th PCR cycle, indicating low transcript abundance. Because target gene expression at 20 PCR cycles was more variable, 25, 30 and 35 PCR cycles were used for semi-quantitative PCRs after *in vitro* and *in vivo* RNAi studies.

4.3.2 Quality of dsRNA before and after feeding

To assess the integrity of dsRNA, 500 ng/uL of the feeding solution before and after nematode soaking was electrophoresed on agarose gels. No obvious degradation in the form of smearing was seen after 16 h soaking with nematodes, but a slight smear was visible in the lane containing ds*GFP* (Figure 4.4).

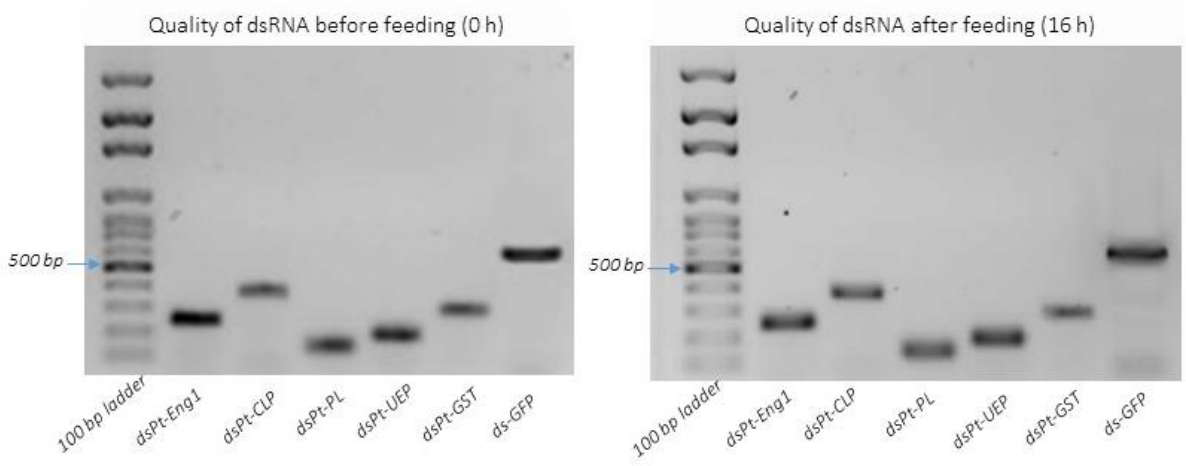

Figure 4.4. Inverse images of agarose gel electrophoresis showing the quality of dsRNA in the feeding solution before and after soaking nematodes for 16 h.

Spectrophotometric analysis provided information on the purity of nucleic acids based on the two ratios: 260/280, and 260/230 (Table 4.1). The measure for purity of RNA at A260/280 is acceptable at 2.0 and close to this value was found for all the samples before and after feeding for 16 h, again indicating that there was no reduction in the purity of dsRNA was evident. Similarly, the secondary measure for the purity of RNA at A260/230 is acceptable between 2.0 - 2.2, and this measure was also found for the dsRNA tested. These results indicate that dsRNA was not degraded during the 16 h feeding.

Table 4.1. Concentrations and absorbance ratios of dsRNA samples before and after 16 h feeding experiments.

Sample	At 0 h			At 16 h		
	ng/µL	A260/280	A260/230	ng/µL	A260/280	A260/230
ds*Pt*Eng-1	508.10	2.02	1.92	500.00	2.09	1.99
ds*Pt*CLP	498.25	2.00	1.98	495.55	2.16	2.05
ds*Pt*PL	498.35	2.02	1.95	494.10	2.07	2.01
ds*Pt*UEP	500.05	2.00	1.92	495.53	2.05	2.00
ds*Pt*GST	500.10	2.01	1.95	495.00	2.05	2.02
dsGFP	510.00	2.02	1.92	497.21	2.09	1.99

4.3.3 Phenotypic effects of *in vitro* RNAi on mixed stages of *P. thornei*

Behavioural changes of *P. thornei* after 16 h soaking with dsRNA and no dsRNA control were examined under an Olympus BX-51 photomicroscope. Nematodes soaked in 1 mg/mL FITC showed the presence of fluorescence in the stylet and the gut with an overall fluorescence within the body, indicating uptake of dye through ingestion and/or through the body wall. Control nematodes soaked with no dsRNA were found to be active with normal sinusoidal body movement (Figure 4.5). Nematodes treated with all other dsRNAs except for ds*Pt-PL* also maintained the normal sinusoidal movement, with only a few inactive. Nematodes fed on ds*Pt-PL* did not have a sigmoid shape and seemed straight or slightly curved.

4.3.4 Effects on target gene expression after *in vitro* RNAi

Changes in transcript levels and percent reduction in target gene expression relative to the internal 18S rRNA control were assessed in nematodes fed on target dsRNA after 16 h of soaking using semi-quantitative PCRs. Amplification of target genes was visualised as DNA bands after agarose gel electrophoresis, and their intensities were compared by densitometric analysis. The housekeeping gene, 18S rRNA, gene was amplified from cDNA of nematodes soaked with dsRNA for all five target genes. The transcript accumulation of 18S rRNA was not affected in nematodes fed on no dsRNA control, ds*GFP* and target dsRNAs (Figure 4.6A). On the other hand, the target transcript accumulation was noticeably reduced in nematodes fed with the target dsRNA, but not for the no dsRNA control and ds*GFP* (Figure 4.6B). Amongst these, the changes in transcript accumulation of *Pt-Eng-1*, *Pt-CLP*, and *Pt-PL* were most obvious

at all PCR cycles as compared to *Pt-UEP* and *Pt-GST,* which also showed reduction but less so at the 35th PCR cycle (Figure 4.6B).

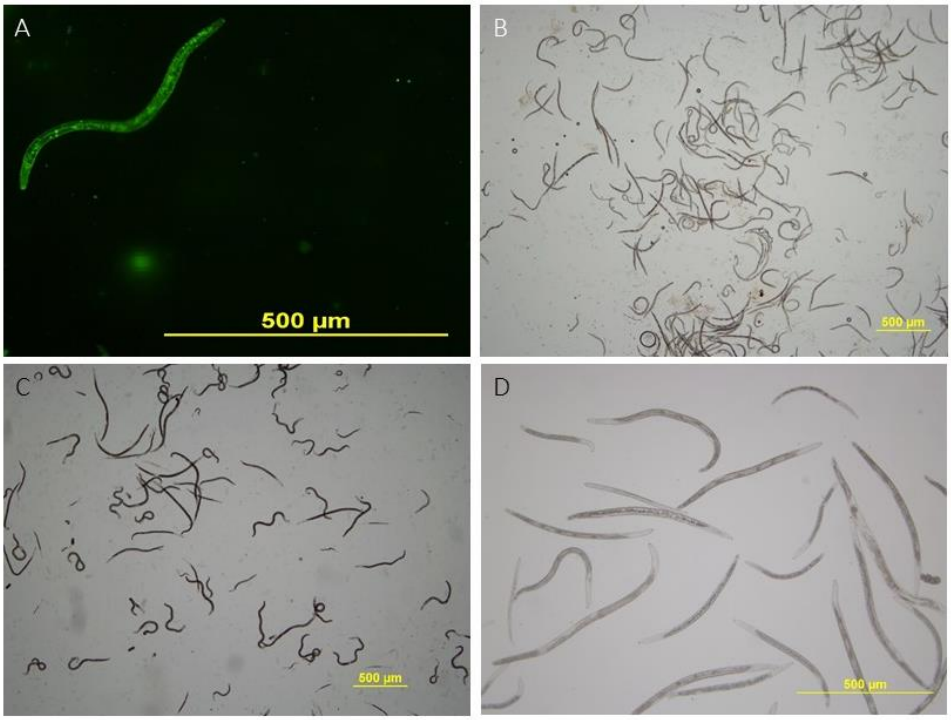

Figure 4.5. Microscopy images of *P. thornei* mixed stages after 16 h *in vitro* RNAi. Uptake of FITC visualised inside the nematode at 20X (A), nematodes soaked in no dsRNA at 4X (B) and ds*GFP* at 4X (C) exhibiting normal sigmoid movement, and nematodes soaked in ds*Pt-PL* that appeared straight or slightly curved at 10X.

The changes in transcript accumulation for each of the target genes was studied by densitometric analysis using pixel intensity for each band from inversed gel electrophoresis images. By applying ImageJ, the difference in relative densities between control and samples was calculated and expressed as a percentage of reduction relative to 18S rRNA. The percentage of reduction was higher at 25 cycles and lowest at 35 cycles for all target genes, and this also complemented visual observations of transcript accumulation. The percent reductions of transcript accumulation of target genes at 25, 30 and 35 PCR cycles relative to 18S rRNA were as follows.

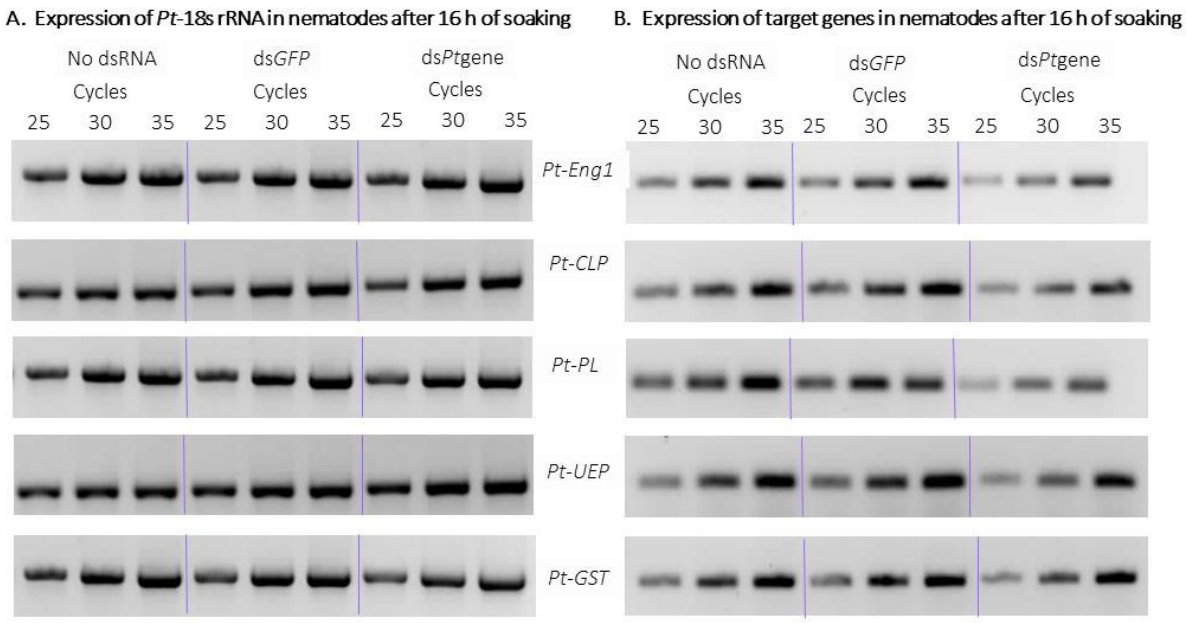

Figure 4.6. Inverse images of agarose gel electrophoresis for semi-quantitative PCRs after 16 h of soaking *P. thornei* in dsRNA.

For *Pt-Eng-1*, the reduction was 66.4%, 59.1% and 50.1% (Figure 4.7A); for *Pt-CLP*, it was 55.2%, 50.4% and 42.2% (Figure 4.7B); for *Pt-PL*, a reduction of 59.2%, 52.5% and 44% was seen; for *Pt-UEP* it was found to be 53.4%, 46.3% and 39.5%; while for *Pt-GST,* it was 35.4%, 28.9% and 18.5% which was also the greatest reduction achieved. One-way ANOVA was used to test the hypothesis of equal percent reduction of target transcript accumulation relative to 18S rRNA at each time-point (PCR cycle). At each PCR cycle, highly significant differences in reduction of transcript accumulation were found between target gene and controls (no dsRNA and ds*GFP*), and are denoted by asterisks (Tukey's HSD, $p < 0.001$).

4.3.5 Long-term effects of *in vitro* RNAi on reproduction of *P. thornei*

To assess the long-term effects of *in vitro* RNAi on reproduction, the nematodes fed without dsRNA and with dsRNA were inoculated on to the roots of wild-type soybean and alfalfa seedlings, and the mean number of nematodes present were counted after 21 days. After the first two weeks post-inoculation, to confirm root infections with *P. thornei*, staining of roots with red food colour was done. The images below show the presence of nematodes in soybean and alfalfa (Figure 4.8 A and B, respectively).

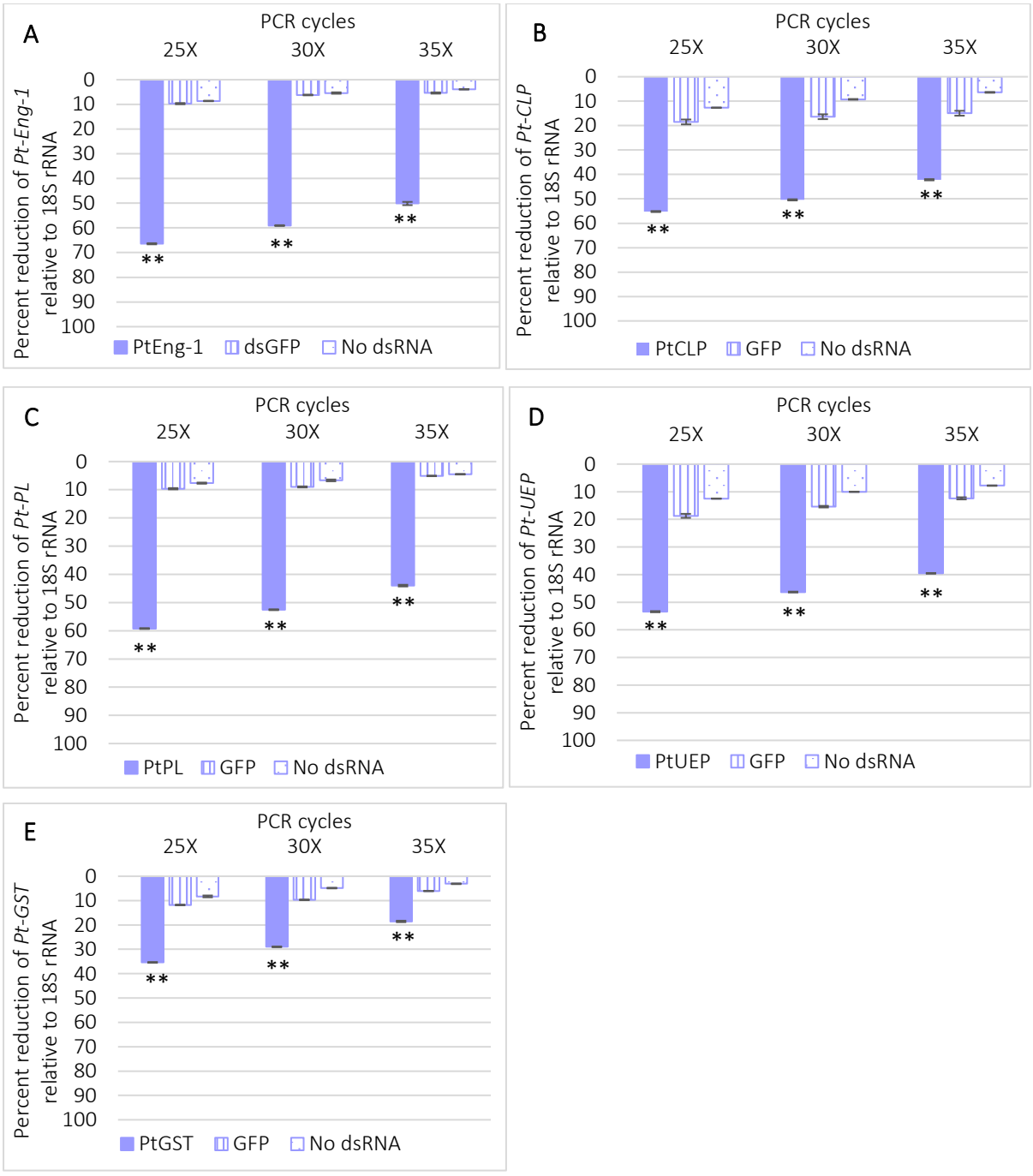

Figure 4.7. Percentage of reduction of *P. thornei* target genes relative to 18S rRNA after *in vitro* RNAi *via* dsRNA soaking for 16 h. Data represent ± standard errors of mean with $n = 3$ per group. Significance with respect to each control at an experimental time-point (PCR cycle) is denoted by asterisk(s). ** indicates $p < 0.001$.

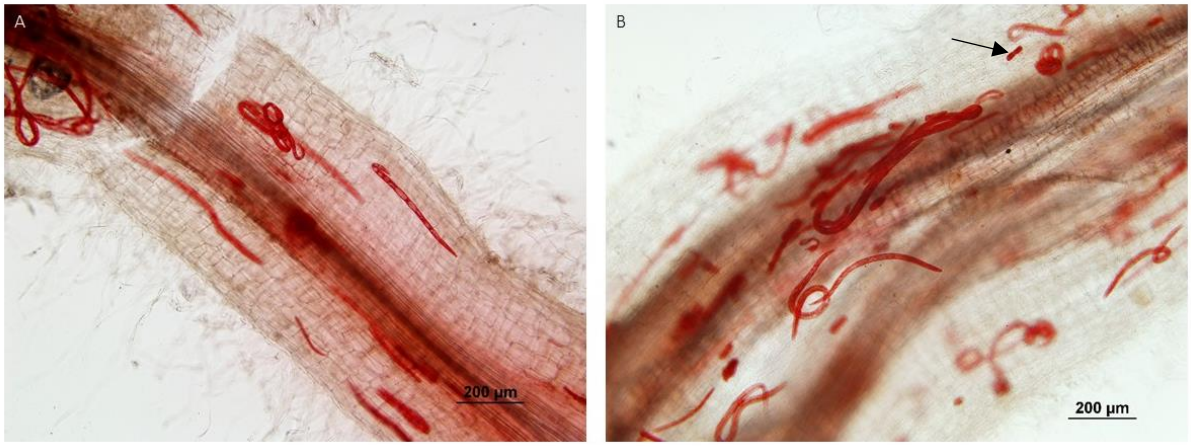

Figure 4.8. Staining of soybean (A) and alfalfa (B) roots two weeks post-inoculations with no dsRNA fed *P. thornei,* confirming the presence of nematodes as observed under a microscope at 10X magnification. Arrow indicates the presence of eggs.

A comparison of the mean number of nematodes found in soybean and alfalfa from each treatment is given in Figure 4.9. In both hosts, the mean number of *P. thornei* recorded 21 dpi for target genes was significantly lower in comparison to both no dsRNA and ds*GFP* controls. One-way ANOVA was done to test the hypothesis of equal means of nematode numbers found on soybean after 21 dpi with nematodes fed on dsRNA and controls. Similarly, this was also done for alfalfa.

In soybean, highly significant differences in mean number of nematodes were found on roots of all five target dsRNA treatments as compared to no dsRNA and ds*GFP*, and are denoted by green asterisks (Tukey's HSD, $p < 0.001$). A pairwise comparison between the following treatments indicated no significant differences in mean number of nematodes fed on: no dsRNA and ds*GFP*, ds*Pt-Eng-1* and ds*Pt-PL*, ds*Pt-CLP* and ds*Pt*-PL, ds*Pt-CLP* and ds*Pt-GST*, ds*Pt-UEP* and ds*Pt-GST*.

Similarly, amongst the treatments on alfalfa, highly significant differences in average number of nematodes were found on roots of all five target dsRNA treatments as compared to no dsRNA and ds*GFP*, and these are denoted by orange asterisks (Tukey's HSD, $p < 0.001$). A pairwise comparison between the following treatments indicated no significant differences in

mean number of nematodes fed: on no dsRNA and ds*GFP*, ds*Pt-Eng-1* and ds*Pt-CLP*, ds*Pt-Eng-1* and ds*Pt-PL*, ds*Pt-Eng-1* and ds*Pt-UEP*, ds*Pt-Eng-1* and ds*Pt-GST*, ds*Pt-CLP* and ds*Pt-PL*, ds*Pt-CLP* and ds*Pt-UEP*, ds*Pt-CLP* and ds*Pt-GST*, ds*Pt-UEP* and ds*Pt-GST*.

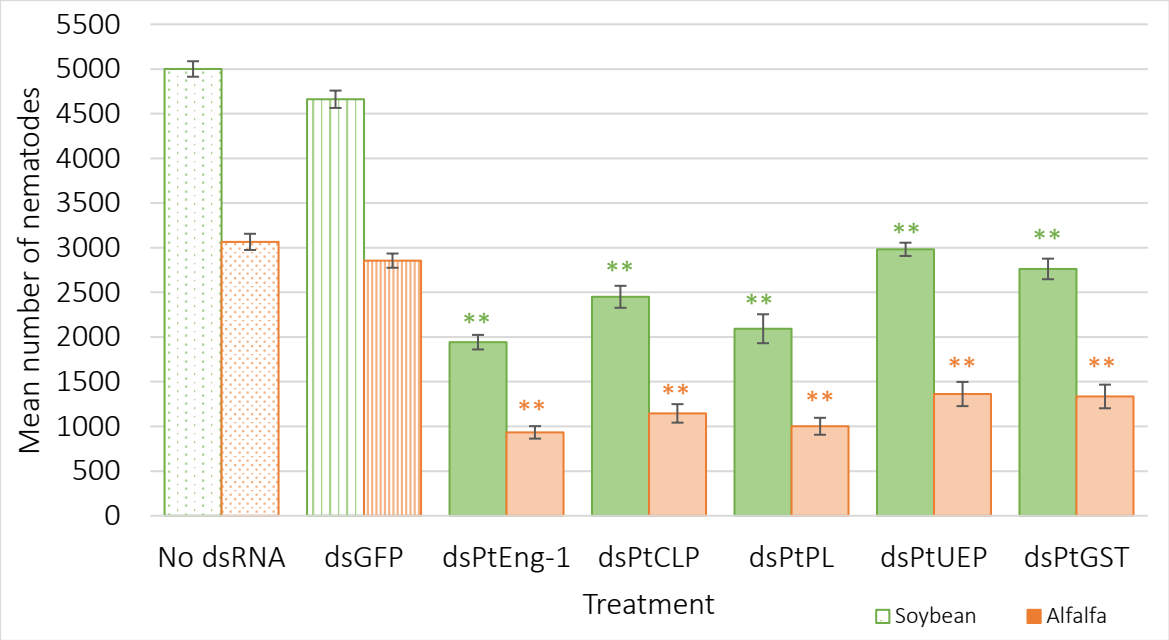

Figure 4.9. Mean number of *P. thornei* extracted from wild-type soybean and alfalfa 21 days after inoculating with nematodes treated for 16 h with dsRNA and no dsRNA. Data represent ± standard errors of mean with $n = 10$ per group. Significance with respect to each control in each host is denoted by asterisks. ** indicates $p < 0.001$.

Overall, the numbers of *P. thornei* recovered from alfalfa were lower than for soybean for both controls and treatments. Moreover, the percent reduction in mean number of nematodes between target dsRNA and no dsRNA control was consistently greater in alfalfa than soybean. For example, the percent reduction in mean number of *P. thornei* found in soybean infected with nematodes fed on ds*Pt-Eng-1*, ds*Pt-CLP*, ds*Pt-PL*, ds*Pt-UEP* and ds*Pt-GST* was 61.1%, 51.0%, 58.1%, 40.3% and 45.0% respectively, as compared to no dsRNA. Whereas, the percent reduction in mean number of *P. thornei* found in alfalfa infected with nematodes fed on ds*Pt-Eng-1*, ds*Pt-CLP*, ds*Pt-PL*, ds*Pt-UEP* and ds*Pt-GST* was 70.0%, 63.0%, 67.3%, 55.5% and 56.4% respectively, as compared to no dsRNA. In other words, more *P. thornei* were found in soybean

roots infected with nematodes fed on ds*Pt-Eng-1*, ds*Pt-CLP*, ds*Pt-PL*, ds*Pt-UEP* and ds*Pt-GST* was 39.0%, 49.0%, 42.0%, 60.0%, and 55.2% respectively, compared to no dsRNA control. Whereas, fewer *P. thornei* were recovered from alfalfa infected with nematodes fed on ds*Pt-Eng-1*, ds*Pt-CLP*, ds*Pt-PL*, ds*Pt-UEP* and ds*Pt-GST:* 30.4%, 37.4%, 33.0%, 44.5%, and 44.0%, respectively.

4.4 Discussion

In vitro RNAi provides a relatively convenient method of assessing the suitability of a gene as a potential candidate for nematode control based on resulting effects such as reduced survival or reproduction, impaired growth and development, and or reduced establishment in a host. In this study, mixed-stage *P. thornei* soaking with target dsRNA for 16 h resulted in reduced target transcript accumulation and also negatively affected reproduction.

A sucrose gradient density-based separation provided an efficient method to separate eggs which otherwise would have been time consuming to isolate manually. This technique had not been applied before to separate life-stages of *P. thornei,* although it has been used to isolate eggs of *P. penetrans* from alfalfa callus (Dunn, 1973). It has also been used to separate eggs, juveniles and adults in *H. glycines* (Acedo and Dropkin, 1982) and *Meloidogyne* (McClure et al., 1973). Acedo and Dropkin (1982) reported the distribution of *H. glycines* eggs in the 40% sucrose gradient, however, the majority of *P. thornei* eggs in this study were distributed in the 20% fraction, with some at the meniscus of 20% and 40% sucrose. This difference in distribution across sucrose concentrations in the two studies could be attributed to differences in egg sizes. Eggs of *H. glycines* (approximately 100 µ x 48 µ) are twice the length and width of most *Pratylenchus* species eggs (approximately 56 µ x 24 µ), and are also potentially denser than *Pratylenchus* eggs (Acedo and Dropkin, 1982; Dunn, 1973; Thistlethwayte, 1968).

The CMC plate assay provided a reliable, rapid and an inexpensive method of checking for cellulase activity in eggs or motile-stages of nematodes. The water control used in the assay did not induce a clear zone, which indicated that there was no contaminating bacterial cellulases, and that the zone of clearing around the eggs and mixed-stage nematodes was therefore of nematode origin. A zone of clearing was present in the CMC plates for both

homogenised and unhomogenised forms of eggs and mixed-stage nematodes, indicating cellulase activity. This activity results from degradation of β-1,4-glucan cellulose polysaccharides by cellulases produced endogenously by *P. thornei*. This finding agrees with that of Rosso et al. (1999), in which cellulase activity in total nematode homogenate and stylet secretions of *M. incognita* J2 was evident as a zone of clearing on CMC agar plates (Rosso et al., 1999). Likewise, Kikuchi et al. (2004) also validated cellulase activity in *B. xylophilus* using total homogenates and nematode secretions using a CMC plate test (Kikuchi et al., 2004). Similarly, Smant et al. (1998) characterised cellulase genes from two cyst nematodes, *G. rostochiensis* and *H. glycines*, and also reported endogenous production of the β-1,4-endoglucanases using CMC cup plate assay (a variation of the same principle) of affinity-purified heterologous fusion proteins (Smant et al., 1998). In the current study, the CMC plate assay provided visual evidence of the presence of cellulase activity in mixed-stage nematodes and eggs, and this has not been shown before for *P. thornei*. The cellulase activity in the CMC plate assays could result from several cellulolytic proteins of glycosyl hydrolase family (GH 5) genes, and so this assay does not provide information on the exact genes responsible for the activity. However, it did allow relative comparisons and qualitative assessments of cellulase activity in *P. thornei*.

Temporal expression of the five target genes visualised as transcript accumulation appeared consistently from the 25th PCR cycle and subsequently at 30 and 35 cycles in mixed-stage nematodes, indicating that these PCR cycles could be used for assessing gene expression after *in vitro* and *in planta* experiments using the same amount of RNA and cDNA from nematodes. Temporal expression of target genes in eggs revealed that *Pt-Eng-1* and *Pt-PL* were detectable at varying levels in semi-quantitative RT-PCRs, however, *Pt- CLP, Pt-UEP* and *Pt-GST* were not detected.

The observation that β-1,4-endoglucanase was expressed in eggs of *P. thornei* is in line with the study done by Gao et al. (2004) in which β-1,4-endoglucanases were found in eggs of *H. glycines*. In that study, high transcript abundance of two endoglucanases, *Hg-eng-1* and *Hg-eng-4* was reported whereas, *Hg-eng-5* had significantly lower transcripts in eggs (Gao et al., 2004). Rosso et al. (1999) and Ledger et al. (2006) also reported the presence of β-1,4-endoglucanase, *Mi-eng-1* and *Mi-eng-2* transcripts in *M. incognita* eggs. Similarly, Hu et al.

(2013) found endoglucanase *Mj-eng-3* transcripts in eggs of *M. javanica* (Hu et al., 2013). While most of the expression studies reported the presence of β-1,4-endoglucanase in sedentary PPNs, Haegeman et al. (2008) found significantly higher transcript levels of *Rs-eng3* compared to the other β-1,4-endoglucanase *Rs-eng1B* expressed in the eggs of the migratory PPN, *R. similus* (Haegeman et al., 2008).

The observation that pectate lyase was expressed in eggs of *P. thornei* also agrees with the report on developmental expression of *Mi-pel-1* and *Mi-pel-2* pectate lyases in eggs of *M. incognita* (Huang et al., 2005). A similar expression pattern for both *GrPEL1* and *GrENG1* was also reported in eggs of *G. rostochiensis* (Ali et al., 2015). Another study recently found expression of *Hg-pel-3, Hg-pel-4, Hg-pel-6* and *Hg-pel-7* in eggs of *H. glycines* (Peng et al., 2016). Interestingly, by contrast, transcripts for *Hs-eng1*, *Hs-pel1* and *Hs-pel2* endoglucanase and pectate lyases were not found in eggs of *H. schachtii* (Vanholme, 2007). The presence of transcripts for both β-1,4-endoglucanases and pectate lyases in PPN eggs may be because some developing embryos in eggs were at more advanced stages of development, in which the embryo would soon differentiate into a pre-parasitic juvenile, and so would require effectors for plant invasion and migration (Gao et al., 2004; Haegeman et al., 2008; Huang et al., 2005; Long et al., 2013; Roman and Hirschmann, 1969a). In certain RKNs the presence of endoglucanases in the egg matrix could be associated with their production by rectal glands during egg extrusion on to the plant root surface, however, this is unlikely to be the case for *P. thornei* (Hu et al., 2013; Rosso et al., 1999).

The expression of *Pt-CLP* was not detected in eggs at any of the PCR cycles, and this agrees with results on *M. incognita* eggs for *Mi-clp-1* (Neveu et al., 2003). Similarly, the observation that *Pt-UEP* was not detected in eggs of *P. thornei* agrees with finding that transcripts for the ubiquitin extension protein encoded by *Hs-UBI* in *H. schachtii* were not detected in egg using quantitative RT-PCRs (Tytgat et al., 2004). However, there is a report that *GrUBCEP-12 from G. rostochiensis,* a gene encoding ubiquitin carboxyl extension protein belonging to the family of ubiquitins, was detected in eggs using quantitative RT-PCR (Chronis et al., 2013). No data for temporal expression of glutathione S-transferase in other PPNs is available on expression of *Pt-GST* in eggs.

The semi-quantitative PCR analysis showed that there was a significant reduction in transcripts of all five target genes after soaking *P. thornei* in dsRNA for 16 h. This assessment was based on visual and densitometric analysis relative to the housekeeping gene 18S rRNA, and is discussed below for each gene together with the long-term effects on *P. thornei*.

Pt-Eng1 transcript levels were significantly reduced compared to no dsRNA and ds*GFP* controls after dsRNA soaking for 16 h. This observation is in line with that reported by Chen et al. (2005), in which the first demonstration of RNAi to analyse the function of a pathogenicity factor in PPNs was done. In that study, the authors reported significant decrease in levels of mRNAs of three endoglucanases, *eng-1*, *eng-3* and *eng-4* compared to controls, using RT-PCR after 24 h soaking *G. rostochiensis* J2s in dsRNA solutions (Chen et al., 2005b). Similarly, when *Pc-eng* of *P. coffeae* was targeted by *in vitro* RNAi for 24 h, the transcript levels were decreased compared to controls on gels after semi-quantitative PCRs (Joseph et al., 2012). In addition, in a migration assay, these authors did not find any aberrant phenotype in nematodes treated with dsRNA of *Pc-eng* compared to no dsRNA treated nematodes. This result is supported, since no phenotypic or behavioural changes were seen in *P. thornei* treated with ds*Pt-Eng-1.*

Fanelli (2014) reported significant reduction of 88% and 98% in *Pv-eng-1* mRNA expression after 12 h and 24 h soaking respectively, using quantitative PCRs with 28S rRNA as an internal control (Fanelli et al., 2014). In the current study semi-quantitative PCRs were done to estimate the percent reduction in transcript accumulation relative to 18S rRNA using densitometry, and the results obtained were similar to those of *Pv-eng-1* mRNA expression by Fanelli (2014). The percent reduction of *Pt-Eng-1* transcript accumulation relative to 18S rRNA was 66.4%, 59.1% and 50.1% at 25, 30 and 35 PCR cycles respectively, which was slightly less than that reported by Fanelli (2014). This difference may result from differences in the methods used to measure transcript abundance (semi-qPCRs vs. qPCRs). In addition, sequences of the endoglucanases in the two organisms share less similarity, and factors such as the target region chosen or the fact that endoglucanases belong to a a large family of cell wall modifying genes and there may be other transcripts to compensate the effect may also account for the differences reported. There is good evidence that levels of silencing are influenced strongly by differing target regions, for example, pectate lyase of *H. glycines*, endoxylanase of *R. similis*, and *Pv-eng-2* of *P. vulnus* (Haegeman et al., 2009; Sukno et al., 2007).

Long-term effects (21 d) on the reproduction of *P. thornei* treated with ds*Pt-Eng-1* showed 61.1% and 70.0% reduction in nematode numbers on soybean and alfalfa respectively, compared to their corresponding no dsRNA controls. A similar reduction in reproduction of ds*Pv-eng*-1-treated *P. vulnus* was found on carrot discs; 54% and 34% lower reproduction at 33 dpi and 60 dpi, respectively, compared to untreated controls (Fanelli et al., 2014).

The transcript accumulation of *Pt-CLP* in this study measured after semi-quantitative PCR was significantly less than no dsRNA and ds*GFP* controls upon dsRNA soaking for 16 h. This agrees with a similar observation for *M. incognita* in which *in vitro* RNAi silencing of *Mi-cpl-1* led to an apparent reduction in transcript level compared to no dsRNA after semi-quantitative PCRs (Shingles et al., 2007). The reduction of *Pt-CLP* transcript accumulation relative to 18S rRNA at 25, 30 and 35 PCR cycles was 55.2%, 50.4% and 42.7% respectively, than no dsRNA and dsGFP controls.

The reduction in *Pt-CLP* transcript accumulation was also reflected in the significant reduction in *P. thornei* reproduction after 21 dpi on roots of soybean and alfalfa by 51.0% and 63.0% respectively, compared to corresponding no dsRNA controls. A similar reduction of up to 60.0% for *M. incognita* numbers was found at 21 dpi compared to no dsRNA controls when roots of adzuki bean (*Vigna angularis)* were infected with ds*Mi-cpl-1* treated *M. incognita* (Shingles et al., 2007). It is worth noting that in the study done by Shingles et al (2007) higher levels (2 to 5 mg/mL) of ds*Mi-cpl-1* were used which may have also contributed to the greater reduction in reproduction; whereas, only 1 mg/mL ds*Pt-CLP* was used in this study.

The *Pt-PL* transcript levels were also significantly reduced compared to controls after soaking in dsRNA for 16 h. Similar results were found by Vanholme et al. (2007), who reported a reduction in transcript level of *Hs-pel-2* in *H. schachtii* compared to controls (Vanholme, 2007). Similarly, Peng et al. (2016) reported a significant reduction in transcript levels of *Hg-pel-6* compared to no dsRNA controls using semi-quantitative PCRs (Peng et al., 2016). The abnormal 'non-sigmoid' phenotype found here for nematodes soaked with ds*Pt-PL* was not described for RNAi studies on pectate lyase of *H. schachtii* and *H. glycines* (Bakhetia, 2007; Sukno, 2007; Vanholme, 2007). Compared to controls, the densitometric analysis showed that there was a significant reduction of *Pt-PL* transcript accumulation relative to 18S rRNA: 59.2%,

52.5% and 44% at 25, 30 and 35 PCR cycles, respectively, and this agreed with the visual observations of faint DNA bands on agarose gels.

The reduction in transcript of *Pt-PL* also clearly mirrored the reduced *P. thornei* reproduction on soybean and alfalfa which was 58.1% and 67.3% respectively, compared to controls. Peng et al. (2016) reported similar results for *H. glycines* with reduced numbers on wild-type soybean after inoculation with dsRNA fed nematodes (Peng et al., 2016). Likewise, a 50% reduction in reproduction of *H. schachtii* was found after *in vitro* RNAi of *Hs-pel-2* compared to no dsRNA controls in 6 d-old *A. thaliana* roots (Vanholme, 2007). However, in this study on *P. thornei*, the time frame was longer (21 days) than that studied by Vanholme et al. (2007). Differences in these results can be explained by difference in hosts and nematode species (sedentary endoparasites *versus* migratory), and to mimic natural infections, the soybean and alfalfa challenged in this study were not grown under sterile conditions, but in cups containing pasteurised soil, which allowed more natural root growth and area for *P. thornei* to access. In the study by Vanholme et al. (2007), *A. thaliana* was grown in Petri dishes under sterile conditions where the nematode migration to the roots from the point of inoculation has fewer challenges. Quantifying nematode numbers is also more difficult for RLNs. In the current study, nematodes were extracted using a mistifier then counted microscopically, whereas, in the study by Vanholme (2007) nematodes stained inside root tissues were counted directly (Vanholme, 2007).

After dsRNA soaking for 16 h, *Pt-UEP* and *Pt-GST* transcript accumulation in *P. thornei* was only slightly reduced, especially at 35 cycles, compared to no dsRNA and ds*GFP* controls. However, this transcript accumulation was not as obvious as it was for *Pt-Eng-1*, *Pt-PL* and *Pt-CLP* suggesting that there was lesser knockdown of *Pt-UEP* and *Pt-GST*.

The densitometric analysis showed that the reduction in transcript accumulation of *Pt-UEP* relative to 18S rRNA was 53.41%, 46.31% and 39.54% at 25, 30 and 35 PCR cycles, respectively. For *Pt-UEP*, the knockdown level correlated with greater reproduction of *P. thornei* in at least one host. There was 40.3% reduction in nematode numbers compared to the no dsRNA control on soybean; by contrast, in alfalfa, there was 55.5% reduction. This difference could be attributed to different host genotypes and is discussed later in this section.

On the other hand, the reduction in transcript accumulation of *Pt-GST* relative to 18S rRNA was 35.4%, 28.9% and 18.5% at 25, 30 and 35 PCR cycles, respectively. This transcript reduction again mirrored the reduced percentages of nematode numbers in host roots (45.0% and 56.4% in soybean and alfalfa, respectively). The reduction in *P. thornei* reproduction is supported by a similar finding in *M. incognita* where reproduction and fertility on tomato roots six weeks post-inoculation was significantly reduced after *in vitro* RNAi of *Mi-gsts-1* (Dubreuil et al., 2007). In the current study, once again, the difference in nematode numbers relative to their respective controls on soybean and alfalfa did not correlate and are discussed in context to the differences in the two host genotypes at a later point.

One explanation for reduced knockdown of *Pt-UEP* and *Pt-GST* could be that their roles in parasitism may be mainly to protect against host defences, reactive oxygen species, modulate host responses triggered by pathogen attack and or regulate signalling pathways for long-term establishment as found in sedentary PPNs (Davis et al., 2004; Dubreuil et al., 2007; Gao et al., 2003). Given the migratory nature of *P. thornei*, it is possible that it may not require these effectors to the same extent as some other parasites, or perhaps the timing of their expression would be regulated depending on the stage of parasitic interaction with the host. Another possibility could be that there was only a transient reduction in expression of these genes and that the nematodes were able to overcome the effects of *in vitro* RNAi to some extent. Alternatively, there were other transcripts that may have compensated for the effects of RNAi.

Carrot discs are often used to maintain *Pratylenchus* species, and have been used to study the effects of *in vitro* RNAi on long-term reproduction (Boisseau and Sarah, 2008; Chitambar and Raski, 1985; Fanelli et al., 2014; Tan, 2015; Verdejo-Lucas and Pinochet, 1992). However, in this study, soybean and alfalfa seeds grown in pasteurised soil were infected with *P. thornei* to mimic natural infections more closely. Testing plants grown in non-sterile conditions for example, in growth pouches, has been done in many *in vitro* RNAi studies on sedentary nematodes such as RKNs and CNs (Bakhetia et al., 2005a; Bakhetia et al., 2007; Lilley et al., 2005a; Urwin et al., 2002). Both soybean and alfalfa are hosts of *P. thornei* and the soybean cv. Williams-82 has been used for studies on *P. penetrans* (Collins, 2015; Owen et al., 2015; Owen et al., 2017; Owen et al., 2016; Petersen et al., 1991; Vieira et al., 2015). Findings of the

preliminary susceptibility tests also supported the use of these two hosts for infections after *in vitro* RNAi.

Soybean appears to be a better host for *P. thornei* than alfalfa, and the reduction in transcript levels and nematode reproduction was lower for alfalfa. One reason may be that the density of root mass available for infection in alfalfa was less than that of soybean even though the two hosts were initially infected at the same time-point of two weeks. Over a period of 21 d in which infections were allowed to progress, the root mass of soybean remained greater than that of alfalfa because of the higher number of lateral roots, offering more surface area for nematode entry. Another reason may be that the overall root phenotype of alfalfa and soybean differ thus affecting the number of nematodes that infect. Roots of alfalfa are thin and slender with few lateral roots at the time of nematode infections. It is also possible that root exudates of soybean may be more effective in attracting *P. thornei* nematodes than alfalfa.

In fact, for all the target genes tested, there were 9% to 16% fewer nematodes (compared to their respective controls) on alfalfa than on soybean. This suggests that that there may be host genotypic differences which affect the reproduction of nematodes. For example, for *Pt-Eng-1* there were 39% nematodes in soybean or 2.6 times fewer nematodes than no dsRNA control, whereas, in alfalfa there were 30% nematodes or 3.3 times fewer nematodes than no dsRNA controls: this observation suggest that reproduction of ds*Pt-Eng-1* fed nematodes was affected more in alfalfa than in soybean. This may reflect differences in genotype, root exudates attracting nematodes, host preference or endogenous host defence responses that eventually affect nematode reproduction.

Overall, percent reduction in transcript accumulation correlated with *P. thornei* numbers recorded on soybean and alfalfa at least for three of the target genes tested, and these can be ranked as follows beginning with the greatest level of knockdown observed: *Pt-Eng-1*, *Pt-PL*, *Pt-CLP*, *Pt-UEP*, and *Pt-GST*. Similarly, *Pt-Eng-1*, *Pt-PL* and *Pt-CLP* can also be ranked in the same order beginning with the lowest reproduction recorded on soybean and alfalfa, so indicating a positive correlation between the level of knockdown and reduced reproduction. In soybean, however, the percentage of reproduction observed for these genes were followed

by *Pt-GST* and *Pt-UEP*. For alfalfa, the latter two genes did not differ significantly with respect to the percentage of reproduction.

Staining of *P. thornei* to visualise their presence in roots using red food dye was an inexpensive, reliable and non-toxic method that is preferable to use over acid fuschin and phloxine B, both of which are widely used for nematode staining. Use of the food dye reduces user risk and concerns on waste disposal.

Quantitative real-time PCRs or digital PCRs are regarded as gold standards for studying gene expression and permit absolute quantitation of transcripts after knockdown experiments. However, the costs for using such kits can limit their use, thus making semi-quantitative PCRs the 'default' method. The use of semi-quantitative PCRs to visualise changes in transcript accumulation as reflected by the band intensities is valid and is still used in many research publications. For example, Upadhyay et al., 2011 assessed changes in transcript levels after RNAi of five target genes of whitefly, *Bemicia tabaci*, after dsRNA and siRNA *via* oral feeding, using semi-quantitative RT-PCRs at varying PCR cycles (Upadhyay et al., 2011). In another study, knockdown of cyp35a2, a stress-response gene in *C. elegans* was investigated through RNAi and semi-quantitative RT-PCRs to assess changes in transcript levels (Roh et al., 2010). Similarly, transcript abundance of cathepsin L-protease of *Brugia malayi* was studied using semi-quantitative PCRs, and visualisation of DNA band intensities was done to assess gene knockdown after injecting siRNA and dsRNA into *Aedes aegypti*, the host parasitised by the filarial nematode (Song et al., 2010). In addition to semi-quantitative PCRs, some publications use densitometric analysis of gel images to assess changes in transcript levels relative to a control gene.

Densitometric analysis using ImageJ is widely used in nucleic acid and protein analysis studies across various fields such as agriculture, veterinary science and medicine. For example, recently, ImageJ was applied to study digital chemiluminescent images of Western blots in research to investigate the association between myofibroblasts and the ability to assemble fibronectin in idiopathic pulmonary fibrosis (Torr et al., 2015). Similarly, the densitometric analysis approach using ImageJ was used to study the efficiency of siRNA through semi-quantitative RT-PCR in research on spatial segregation of brain-derived neurotrophic factor

(Baj et al., 2011). ImageJ has also been applied to assess persistence and approximate percentage of knockdown of glutathione S-transferase in the parasitic mite, *Varroa destructor* after semi-quantitative RT-PCR (Campbell et al., 2010).

In the current research, semi-quantitative PCRs provided a visual evidence for changes in transcript accumulation, and densitometric analysis using ImageJ further provided a quantitative estimate of percent reduction of transcript accumulation relative to 18S rRNA that also complemented visual observations. Conditions were kept constant and stringent to achieve high level of accuracy, and included preparation of 1X TAE for gels, the volume of agarose gel poured, comb size, amount of SYBR® safe stain, manufacturer for stain and agarose, electrophoresis unit and the voltage applied, and the thermocycler used. Additionally, PCR products amplified from cDNA of nematodes fed on no-dsRNA, ds*GFP*, and target dsRNA was electrophoresed on the same gel with the same default settings (exposure etc.) each time on a Gel Documentation System (Fisher Biotech, Australia). All these conditions that were met have also been recommended and explained elaborately to achieve reliable and accurate measurement of DNA quantities based on gel images using ImageJ (Antiabong et al., 2016).

CHAPTER 05

Effects of host-mediated RNAi on *Pratylenchus thornei.*

5.0 Introduction

The focus of the previous Chapter was on the effects of externally introduced dsRNA: in this Chapter the focus is on delivery of dsRNA *in planta*, also known as host-mediated RNAi or Host Induced Gene Silencing (HIGS).

As described earlier, RNAi is a cellular mechanism in which presence of dsRNA drives post-transcriptional silencing of genes with homologous sequences, and it has been used widely as a tool to manipulate gene expression and study gene function (Fire et al., 1998). Although RNAi can be induced in PPNs using number of *in vitro* dsRNA delivery methods, the obligatory parasitic nature of PPNs points towards the use of *in planta* dsRNA delivery as a more convincing functional assay for PPN target genes.

The first examples of *in planta* RNAi to investigate PPN genes were in 2006 (Huang et al., 2006a; Yadav et al., 2006). Huang et al. (2006) reported a reduction in gall formation by RKNs on transgenic Arabidopsis expressing dsRNA for the parasitism gene, 16D10 (Huang et al., 2006a). Similarly, *in planta* RNAi has now been used to investigate gene function in migratory PPNs as well as sedentary species. Most work has been done on the sedentary species, for example, tobacco expressing *MjTis11* transcription factor gene from *M. javanica*, soybean expressing major sperm protein gene from *H. glycines*, Arabidopsis expressing four parasitism genes of CNs or two house-keeping genes of RKNs, oil-seed rape expressing a conserved region of the gene *8H07* encoding an important secretory protein of oesophageal glands in *H. schachtii* (Huang et al., 2006a; Sindhu et al., 2009; Steeves et al., 2006; Tsygankova et al., 2014; Yadav et al., 2006). More recently, *in planta* RNAi in *Pratylenchus* species has been shown to decrease reproduction of *P. thornei* in hairy root cultures (Fosu-Nyarko and Jones, 2016; Jones and Fosu-Nyarko, 2014; Tan, 2015), and to be effective in hosts such as walnut, expressing the *C. elegans* orthologue *Pv010* in *P. vulnus*, and soybean expressing fatty acid- and retinol-binding *Pp-far-1* gene, and two locomotion genes *Pp-pat-10* and *Pp-unc-87* of *P. penetrans* (Vieira et al., 2015; Vieira et al., 2017; Walawage et al., 2013). In most of these studies, *Agrobacterium*-mediated plant transformation was used to achieve *in planta* RNAi silencing that resulted in decreased levels of target gene transcripts and a reduction of PPN reproduction.

The use of *in planta* RNAi lays a foundation for potential crop protection strategies against several economically important PPNs. *In planta* delivery of dsRNA through transgenic plants expressing target nematode genes in the roots offers a 'natural' mode of feeding with long term delivery of dsRNA, hence this approach appears ideal for plant protection against PPNs. To generate plants expressing target dsRNA requires plant transformations using hairpin RNAi constructs in which the target gene fragment in sense and antisense orientation separated by an intronic region, with a a constitutive, root- or wound specific promoter. On transcription, the resulting intron spliced hairpin RNA with sense and antisense fragments forms dsRNA molecules that subsequently trigger the RNAi pathway in the pest when it feeds on such a transgenic plant and ingests the dsRNA (Helliwell and Waterhouse, 2003). To illustrate a nematicidal RNAi-based approach it is essential to demonstrate the efficiency of target parasitism effectors using host-mediated RNAi. The first demonstration of HIGS for RLNs was the silencing of *pat-10* and *unc-87* in *P. thornei* using *Agrobacterium rhizogenes*-mediated transformation to generate carrot hairy roots expressing dsRNA to these genes. This led to a reduction in the target nematode gene expression and reduced nematode reproduction (Tan, 2015). This work provided a basis to further investigate the effects of host-mediated RNAi silencing of various *P. thornei* parasitism effectors.

5.1 Aims and objectives of Chapter 5

Thus, the aims of this Chapter were to study the effects of silencing *P. thornei* target parasitic effector genes by host-mediated RNAi and to assess the degree of resistance conferred in two hosts. The work undertaken in this Chapter included: cloning of hairpin expression cassettes into a binary vector, generation of axenic soybean and alfalfa hairy roots, generation of non-axenic hairy roots by establishing 'composite' soybean plants, molecular analyses of transgenic hairy roots, and to study the effects of knockdown on target gene expression and nematode reproduction. This Chapter provides the first evidence of *in planta* RNAi of putative effectors of *P. thornei* in both axenic and non-axenic (composite) plant systems.

5.2 Materials and methods

5.2.1 Generation of binary vectors

Five target gene sequences, *Pt-Eng-1*, *Pt-CLP*, *Pt-PL*, *Pt-UEP* and *Pt-GST*, and *GFP* were used to construct binary vectors for plant transformation as described in section 2.10. Briefly, the sequences were first cloned into the transcription vector, pDoubler, after which the sense and antisense strands were digested out and cloned sequentially into the hairpin vector, pCleaver Nos-T, and finally, the hairpin cassette was digested out and cloned into the binary vector, pART27. All clones were confirmed *via* PCRs, restriction digestions and Sanger sequencing as described in sections 2.14.2 and 2.15, respectively.

5.2.2 Hairy root transformation

A. rhizogenes-mediated transformation was done to generate axenic and non-axenic hairy root systems. The axenic method of hairy root development was applied to soybean and alfalfa whereas, a non-axenic method was applied to soybean only to develop composite plants with wild-type shoots and transgenic roots.

5.2.2.1 *A. rhizogenes* cultures

An aliquot of 100 µL from glycerol stocks of the transformed *A. rhizogenes* (K599) containing the recombinant binary vector was used to initiate cultures. Inoculations were done with untransformed K599 and K599 transformed with the binary vector containing null (no gene, *i.e.* only empty vector), *GFP*, and target genes. Culture stocks were added to 15 mL LB media in a 50 mL Falcon tube, containing 100 mg/L spectinomycin and 25 mg/L rifampicin, and cultures were grown for 16 h at 28°C on a shaker set at 225 rpm. Cell cultures were centrifuged at 3,000 g for 10 minutes at room temperature and the pellet was resuspended to a final OD_{600} of 0.5-0.6 in fresh LB media with the same antibiotics. This liquid cell culture was used to inoculate axenic and non-axenic soybean. For inoculating axenic alfalfa, this cell culture was plated on LB agar containing the same antibiotics, incubated in the dark at 28°C for 48 hours after which the bacterial mat (paste) was used in the inoculation process.

5.2.2.2 Seed sterilisation

Soybean (*Glycine max*) cv. Williams 82 seeds used were kindly donated by Dr. Brett Ferguson, CILR, School of Agriculture and Food Sciences, University of Queensland, Australia. Healthy

seeds were surface-sterilised using 70% ethanol for 30 seconds followed by 4% (v/v) sodium hypochlorite containing 0.1% Tween 20 for 15 minutes. After surface disinfestation, the seeds were rinsed thoroughly for five times in sterile distilled water, allowed to soak in sterile distilled water for 20 minutes before placing them between layers of sterile Whatman™ filter paper No. 1 in sterile Petri dish of 100 mm diameter. The filter paper was then soaked with sterile distilled water. Three seeds were kept in a single Petri Dish to allow aeration and optimal growth. Petri dishes were sealed using Parafilm® and kept at 25°C under 18:6 light:dark cycle for up to seven days until seedlings with good radical and plumules emerged from healthy green cotyledons, which were excised eventually (described in section 5.2.2.3). The seeds were kept moist by adding sterile distilled water to the filter paper every two days. Within a week, when the cotyledons were flaccid and green they were moistened by placing them in a Petri dish containing sterile distilled water to aid in removal of the attached seed coat. After removal of seed coats, the cotyledons were used for transformations.

Alfalfa (*Medicago sativa*) seeds used ('Sprouts Alive', Mr. Fothergills Seeds Pty Ltd., NSW, Australia) were purchased from Bunnings, Australia. Seeds were surface-sterilised with 95% ethanol for 1 hour followed by washing in a mixture of 0.2% mercuric chloride and 4% (v/v) sodium hypochlorite for 15 minutes. After surface-disinfestation, the seeds were rinsed five times in sterile distilled water and excess water was removed by pat-drying on sterile Whatman™ filter paper No. 1. Approximately 20 seeds were placed on 1% agar plates. Petri dishes were kept at 18°C in dark until initiation of seed germination after which they were placed at 21 to 22°C under 18:6 light:dark cycle until healthy green cotyledons developed with a radical length of approximately 2 to 3 cm (up to 30 to 48 hours). All the procedures were done in a laminar chamber.

5.2.2.3 Axenic hairy root transformation of soybean

Axenic hairy root cultures of soybean were established using a combination of protocols from Cho *et al.* (2000) and Hernandez-Garcia (2010) with modifications to reduce the time of callus formation (Cho et al., 2000; Hernandez-Garcia et al., 2010).

For plant transformation, any development of the plumule or innate (native/original) roots from the radical end of the cotyledons were excised prior to inoculation. Seeds were then

inoculated with *A. rhizogenes* culture, which involved uniformly wounding the abaxial surface of the excised cotyledon at 1 to 2 cm distance from the hypocotyl end using a sterile needle (27 G x ½ 0. 40 X 13 mm, Terumo) tip dipped in LB agar media covered with bacteria. After the cotyledons were inoculated, they were transferred to a Petri dish lined with layers of sterile Whatman™ filter paper No. 1 soaked with sterile distilled water and kept in the dark for co-cultivation at 25°C for three days. After co-cultivation, cotyledons were transferred to a fresh Petri dish lined with layers of sterile Whatman™ filter paper soaked in liquid MXB media (Murashige Skoog (MS) basal nutrient salts, Gamborg Vitamins, 3% sucrose, pH 6.4) containing kanamycin (100 mg/L) and timentin (500 mg/L) or carbenicillin disodium 500 mg/L. This step created a humid environment which promoted callus and hairy root formation. The Petri dishes were maintained at 25°C under 18:6 light:dark cycle for up to 8 to 10 days, during which cotyledon surfaces showed callusing accompanied by initiation of hairy roots. Mock inoculations without *A. rhizogenes* were used as controls to confirm that root growth was inhibited in the presence of antibiotics. Another control of only untransformed K599 was included with timentin (500 mg/L) or carbenicillin disodium 500 mg/L in the MXB media. Once hairy roots emerged and were more than 2 cm in length they were excised (referred to as individual events at this stage) and transferred to solid MXB media with the above antibiotics, their growth monitored and root lengths measured. In this time, at least three passages onto fresh media plates with antibiotics were carried out (21 days). Six events per construct, each arising from different seeds, were established.

5.2.2.4 Axenic hairy root transformation of alfalfa

Axenic hairy root cultures of alfalfa were established using a combination of protocols from Boisson-Dernier *et al.* (2001) Limpens *et al.* (2004) and Caetano-Anolles *et al.* (1990) with modifications (Boisson-Dernier et al., 2001; Caetano-Anollés et al., 1990; Limpens et al., 2004).

To transform alfalfa, seedlings with radical length of approximately 2 to 3 cm were placed in a Petri dish containing sterile water to prevent desiccation of the radical. The radical was excised approximately 3 mm from the root tip with a sterile blade while holding the root tip with forceps. The cut surface was coated with *A. rhizogenes* culture by lightly dipping or scraping on the surface of the bacterial plate.

The seedling was then placed on a layer of Whatman™ filter paper soaked with MXB media supplemented with suitable antibiotics and co-cultivated for three days in dark at 21°C. Up to 10 seedlings were placed side by side on a single Petri dish containing MXB agar supplemented with kanamycin (25 mg/L) and timentin (500 mg/L). Petri dishes were sealed with Parafilm® and several incisions were made on the upper edge of the Parafilm® to allow gas exchange. Petri dishes were initially placed at an angle of 45° for 2 to 3 days, and then kept vertical for further 4 to 5 days at 21°C and 18:6 light:dark cycle. Controls inoculated with untransformed *A. rhizogenes* were included to check that root development was inhibited in the presence of kanamycin. Another control of only untransformed K599 was included with timentin (500 mg/L) or carbenicillin disodium 500 mg/L in the MXB agar media. After 7 days, co-transformed roots appeared from the inoculated end of the radical. Once hairy roots emerged and were about 2 cm long they were excised (referred to as individual events) and transferred to solid MXB media with the above antibiotics, their growth monitored, and root lengths measured. As done for soybean, alfalfa hairy roots were also given three passages onto fresh media plates with antibiotics (21 days).

5.2.2.5 Non-axenic hairy root transformation of soybean

Soybean seeds were surface-sterilised with 70% ethanol for 30 seconds followed by 4% (v/v) sodium hypochlorite containing 0.1% Tween 20 for 15 minutes. After surface-disinfestation, the seeds were rinsed thoroughly five times in sterile distilled water and planted in 620 mL clear plastic cups containing pasteurised Hortico® all-purpose potting mix supplemented with Yates Thrive® all-purpose soluble fertiliser. Pots were placed in a tray and kept in a growth chamber at 18:6 light:dark cycle with 70% humidity. After the appearance of the first true-leaf, inoculations with *A. rhizogenes* (untransformed K599-only, null, *GFP*, target genes) were carried out at the cotyledonary node using a sterile syringe needle (27 G x ½ 0. 40 X 13 mm, Terumo). Seedlings were covered with 620 mL clear plastic cups to maintain high humidity. Plants were watered regularly as required. Hairy roots began to appear about 8 days post-inoculation (dpi) and by 16 dpi a dense mass of hairy roots appeared. Soil was topped-up in the cups to ensure that the hairy root system was covered completely and the set-up was placed in the growth chamber for another week. After one week, plants were uprooted from the cup, the innate root system was carefully excised, plants were re-potted in the cups, and allowed to establish with their transgenic roots for another week in the growth chamber

without the inverted cup on top. Six cups per treatment were prepared and each cup was treated as an event.

5.2.3 Molecular analysis of transgenic hairy roots

To confirm the transgenicity of axenic and non-axenic hairy roots, molecular analyses of genomic DNA (gDNA) and cDNA for insertion and expression of target genes respectively, was done. During the 21 days of root growth, samples of hairy root events were excised for molecular analysis. Root tissue from six events each of axenically generated hairy roots of soybean and alfalfa were tested. RNA extraction, cDNA synthesis and genomic DNA extraction was done as described in section 2.3 and 2.4. PCRs using cDNA and gDNA were done as described in section 2.6.1. Integration of T-DNA in the gDNA of hairy roots was confirmed through the amplification of the 356 bp-long fragment of the neomycin phosphotransferase (*nptII*). The expression of target genes in the cDNA was confirmed through the amplification of the expected target gene sizes.

Transformation efficiency of non-axenically generated soybean hairy roots was confirmed through molecular analysis after the dense mass of hairy roots appeared by day 16. Sections of about 10 roots from each event were excised and the number of hairy roots that tested positive for the insertion of T-DNA were recorded. Expression of target genes in cDNA was also confirmed by pooling roots that tested positive for T-DNA insertion.

5.2.4 Nematode infection of transgenic hairy root

After 21 days of monitoring root growth and molecular confirmation of transgenicity, hairy roots were prepared for nematode infection. For axenically grown soybean and alfalfa roots, each of the six events were cut into 10 replicates, and placed in a sterile 24-well plate containing 0.5% water agar supplemented with MS media without antibiotics (Figure 5.1). Hairy roots were allowed to grow for 10 days after which they were inoculated with cleaned mixed-stage nematodes (50 per well). The set-up was kept in the dark at room temperature for 21 days. If any contamination post-inoculations were observed, fresh 24-well plates were prepared.

For non-axenically grown soybean, plants were inoculated with mixed-stage nematodes (500 per cup) and the set-up was kept in a growth chamber at 18:6 light:dark cycle with 70% humidity for 21 days (Figure 5.2). Plants were not watered for the following five days after which regular watering was resumed as required. Two weeks after inoculation, staining with red food dye as per section 2.16 was done to check for the presence of nematodes in the roots.

5.2.5 Nematode development on transgenic hairy roots

Twenty-one dpi, ten replicates of axenic hairy roots were transferred to a mistifier, nematodes were collected at 24 h and 48 h, pooled together and number of nematodes were recorded. Similarly, for non-axenic soybean, hairy roots for each event was cut into 10 replicates and these were transferred to a mistifier along with all of the soil. Nematodes were collected at 24 h and 48 h, and the total number of nematodes on replicates of hairy root events of soybean- and alfalfa-expressing targets genes, null, *GFP*, and untransformed K599 were recorded. Soil was included in the extraction procedure to ensure the recovery of *P. thornei* that may have migrated outside the root.

5.2.6 Target gene expression after *in planta* RNAi

Changes in nematode target gene expression were assessed after 21 dpi on axenic and non-axenic transgenic hairy roots. Nematodes were collected from replicates of an event, pooled, cleaned, and RNA was extracted for cDNA synthesis from 100 ng of total RNA as described in section 2.3. Two thousand nematodes from each sample were washed, flash-frozen in liquid nitrogen, and stored at -80°C until further use. A 1:10 dilution of cDNA was used for semi-quantitative PCRs with gene specific primers were carried out as described in section 2.6.2. *P. thornei* 18S rRNA primers were used as normalisation control and the products plus a 100 bp DNA marker was electrophoresed on 1.5% agarose gel. In addition to a visual analysis, densitometric analysis of band intensities was done using ImageJ as described in section 2.8. and expressed as percent reduction of target transcript accumulation relative to 18S rRNA.

5.2.7 Statistical analysis

All data were analysed in Microsoft Excel using functions, formulae and data analysis tool package. A Shapiro-Wilk was done test to determine normality and Levene's test for homoscedasticity (homogeneity of variances) followed by one-way analysis of variance

(ANOVA) to compare means of groups in an experiment. Where necessary, a post-hoc test was done to determine significant differences ($p < 0.05$). A Tukey's honest significance difference (HSD) test appropriate for equal samples sizes was done to achieve pairwise comparisons among all groups within an experiment. Bar charts were made with error bars representing standard error of means for each treatment.

5.3 Results

5.3.1 Axenic hairy roots of soybean and alfalfa

The processes of production and culture of axenic transgenic hairy roots of soybean and alfalfa are presented in Figure 5.1. Six events per construct each arising from different seeds were established.

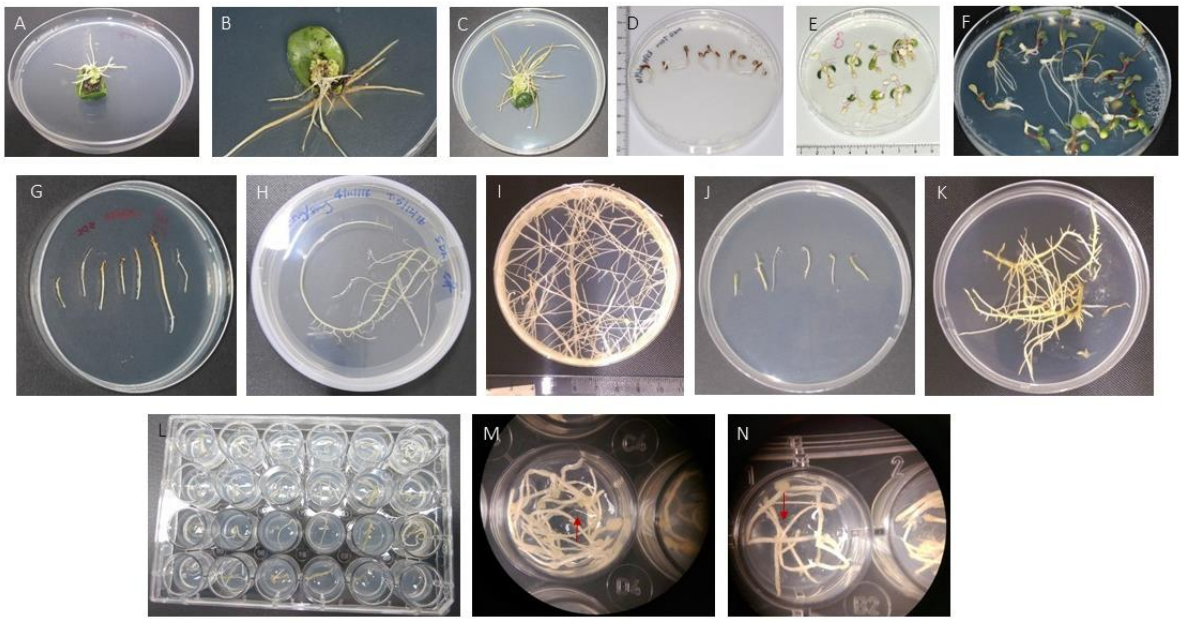

Figure 5.1. Hairy root transformation of axenic soybean and alfalfa. Hairy roots growing from the point of infection after inoculation with *A. rhizogenes* in soybean (A-C) and alfalfa (D-F). Hairy roots of soybean (G-I) and alfalfa (J and K) growing on MS medium with antibiotics. Replicates of hairy root events in a 24-well plate containing MS medium (L) and inoculated with *P. thornei*, indicated by arrows, on soybean (M) and alfalfa (N) roots as observed under a dissecting microscope.

After inoculation with transformed and untransformed *A. rhizogenes* K599, the number of hairy roots growing out from the point of inoculations were recorded for six seeds of soybean and alfalfa (one seed per plate) over a period of 16 days at regular intervals (Figure 5.3). One-way ANOVA was done to test the hypothesis of equal number of transgenic events for the eight treatments (five target genes, null, *GFP* and K599-only) at each experimental time-point. The results suggested that there were highly significant differences in the mean number of hairy roots that emerged from soybean and alfalfa treatments at an experimental time-point. Subsequently, a Tukey's HSD was done to identify the differences.

No hairy root events emerged at 8 dpi from soybean whereas, between 4 to 6 events emerged from all eight treatments of alfalfa. By 12 dpi, between 8 to 10 events from all soybean treatments emerged compared to 6 to 7 events of alfalfa. By 14 dpi, between 14 to 15 events had emerged in soybean whereas, only between 7 to 9 events from alfalfa. By 16 dpi, 19 t- 20 events emerged from soybean and 10 to 12 events for alfalfa (Figure 5.2). While a statistically significant difference amongst the mean number of hairy root events emerging from soybean treatments alone or alfalfa treatments alone was not seen, a clear difference between the mean number of hairy root events emerging from soybean and alfalfa was evident, and this was statistically significant at each time-point (Tukey's HSD, $p < 0.05$). Hence, although hairy roots emerged sooner from alfalfa than soybean, the number of events on soybean was greater than on alfalfa at all the subsequent time-points.

The excised hairy root events of soybean and alfalfa measuring about 2 cm were placed on MXB agar medium and the root lengths of six events per treatment were recorded every three days over a period of 21 days (Figure 5.3). The root length increased over time, and after 21 days the root mass was too dense to measure the length as it twisted around inside the Petri dish. One-way ANOVA was done to test hypothesis of equal mean length of transgenic hairy root events for the eight treatments at each experimental time-point. In both soybean and alfalfa, the root lengths of untransformed K599, null and *GFP* seemed slightly greater than all other transformed K599 hairy roots at each experimental time-point, however this difference was not statistically significant ($p < 0.05$). No morphological differences were observed between hairy root events arising from untransformed and transformed K599 in both hosts.

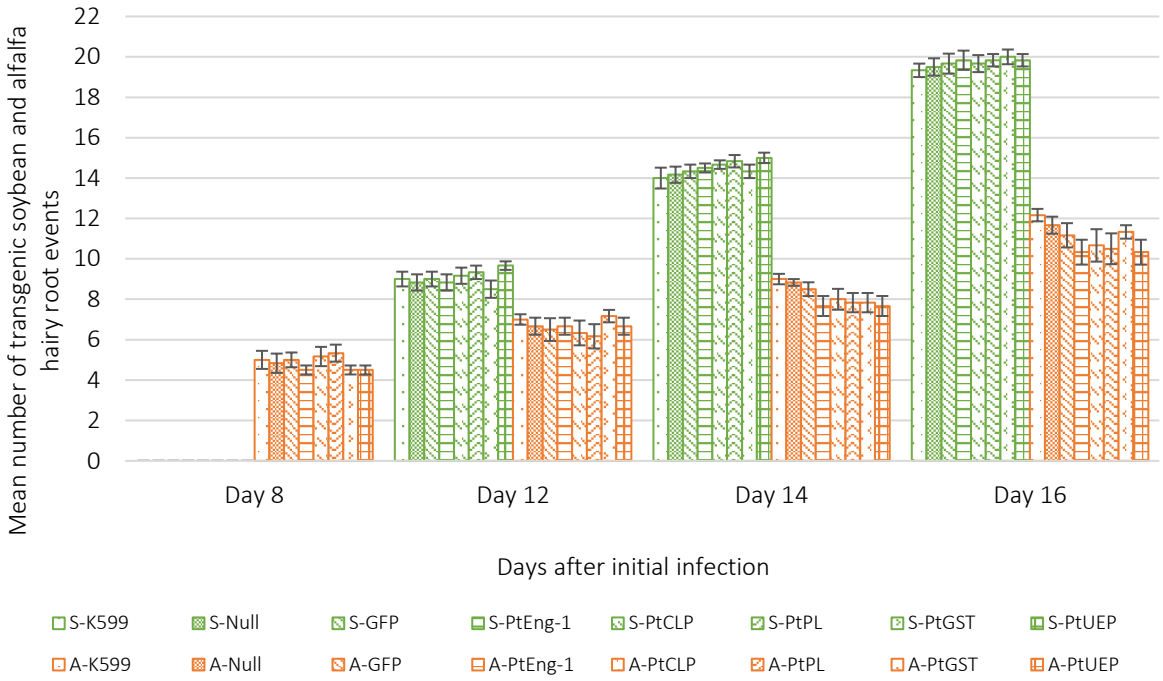

Figure 5.2. Mean number of hairy root events from soybean and alfalfa. Data represents ± standard errors of mean with *n* = 6 per treatment. S- and A- stand for soybean and alfalfa, respectively.

Based on the above data of mean root length of transgenic hairy roots, the rate of root elongation was calculated and is expressed in Figure 5.4. It increased over time as root lengths increased and for both, soybean and alfalfa, the rate of root elongation was highest at day 15 after which the elongation rate reduced, but was still greater than day 3, 6, 9, and 12 as roots continued to grow. Hairy roots of soybean and alfalfa arising from untransformed K599 showed no growth and died in the presence of kanamycin in MXB medium.

5.3.2 Molecular analysis of transgenic hairy roots

The transgenicity of axenic and non-axenic hairy roots was confirmed by molecular analyses of gDNA and cDNA for insertion and expression of target genes, respectively. Successful amplification of the 356 bp-long *nptII* fragment indicated the integration of T-DNA in the gDNA of axenic soybean and alfalfa as well as non-axenic soybean hairy roots (Figure 5.5) in all six events tested.

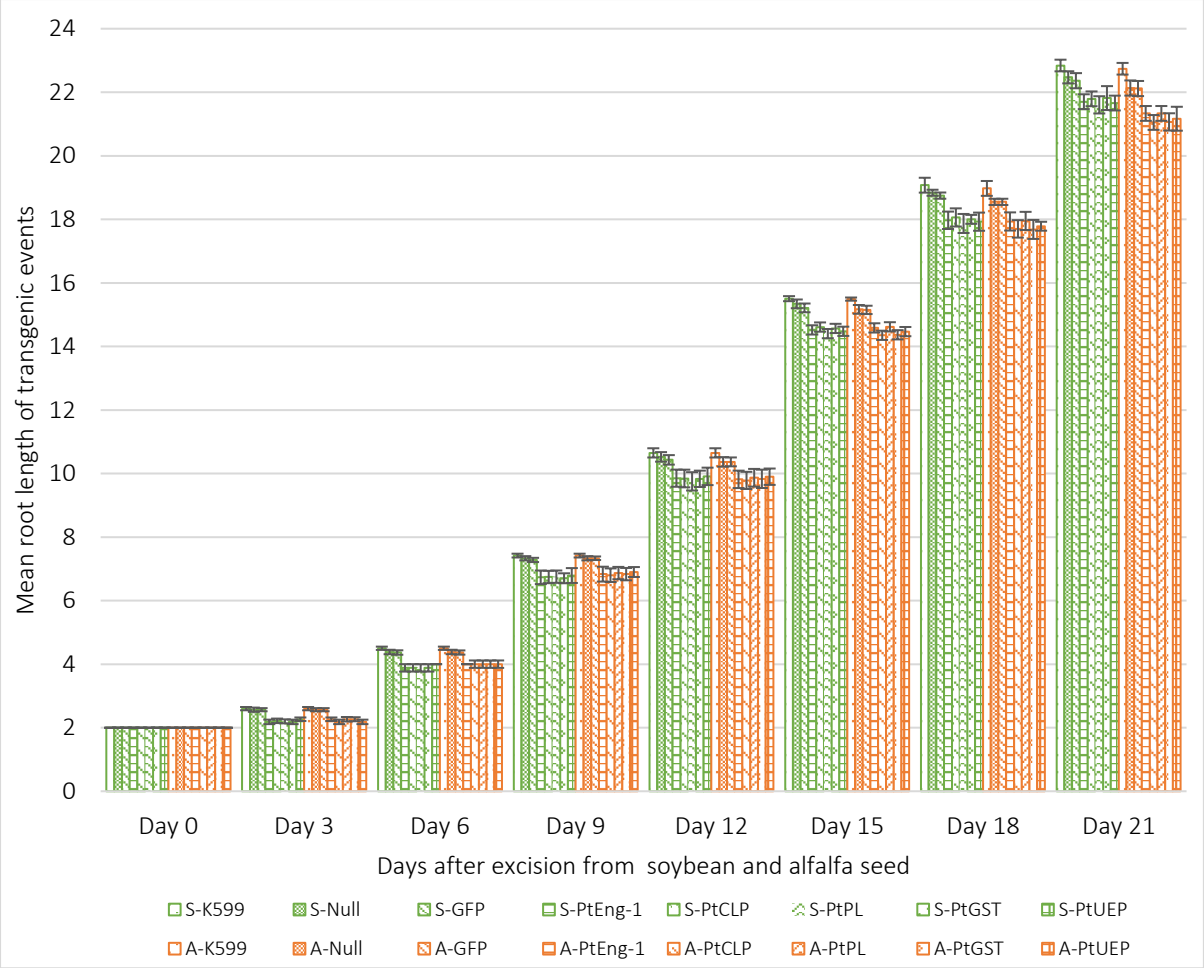

Figure 5.3. Mean root length of soybean and alfalfa hairy roots on MXB agar medium. S- and A- stand for soybean and alfalfa, respectively.

A high transformation efficiency expressed as the percentage of non-axenic hairy roots that tested positive for T-DNA integration in the six events of null, *GFP*, *Pt-Eng-1* and *Pt-CLP* is shown in Table 5.1. The average percentage of hairy roots positive for the presence of the *nptII* fragment across events of null, *GFP*, *Pt-Eng-1* and *Pt-PL* were 97%, 95%, 93%, and 92%, respectively.

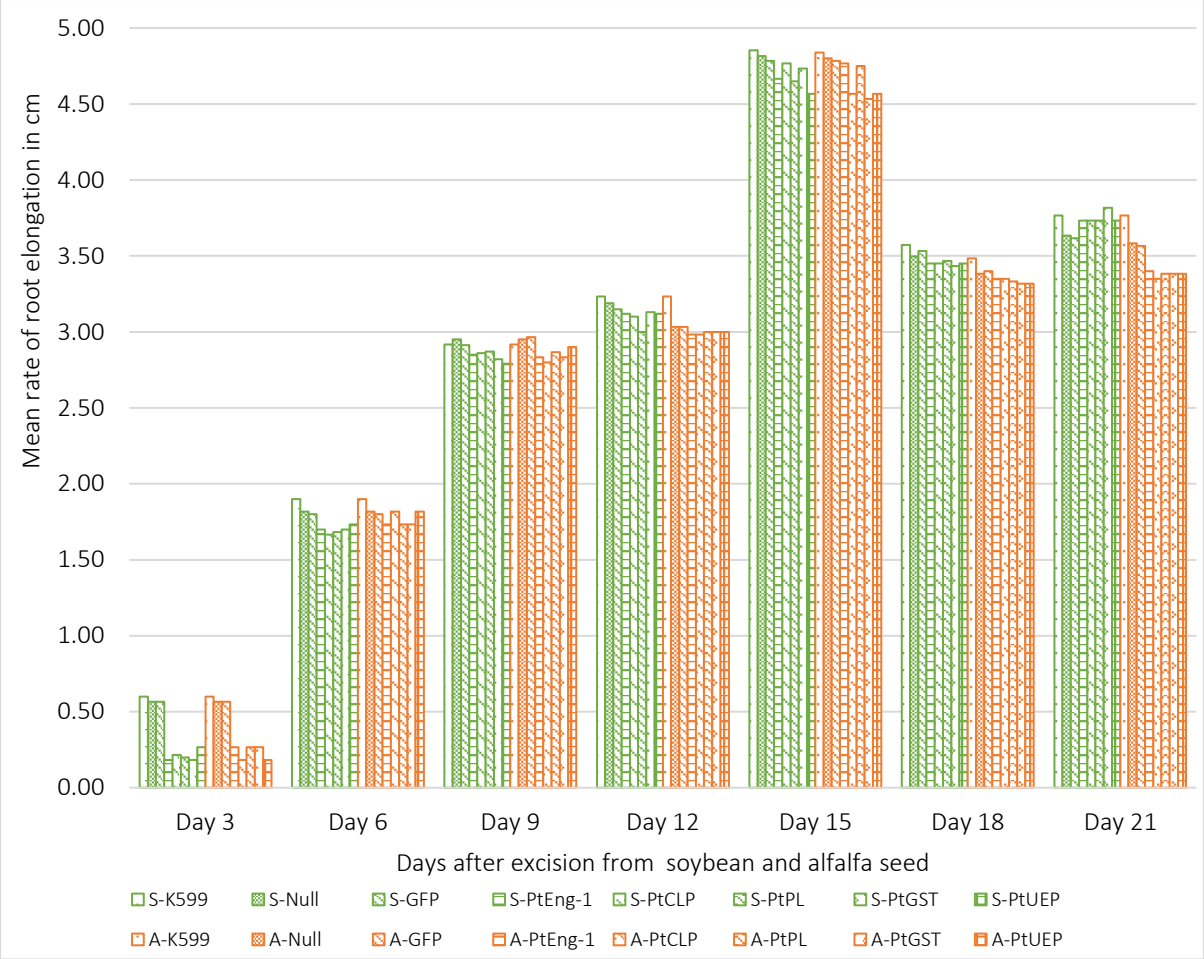

Figure 5.4. Mean rate of root elongation of soybean and alfalfa hairy roots on MXB agar medium.

S- and A- stand for soybean and alfalfa, respectively.

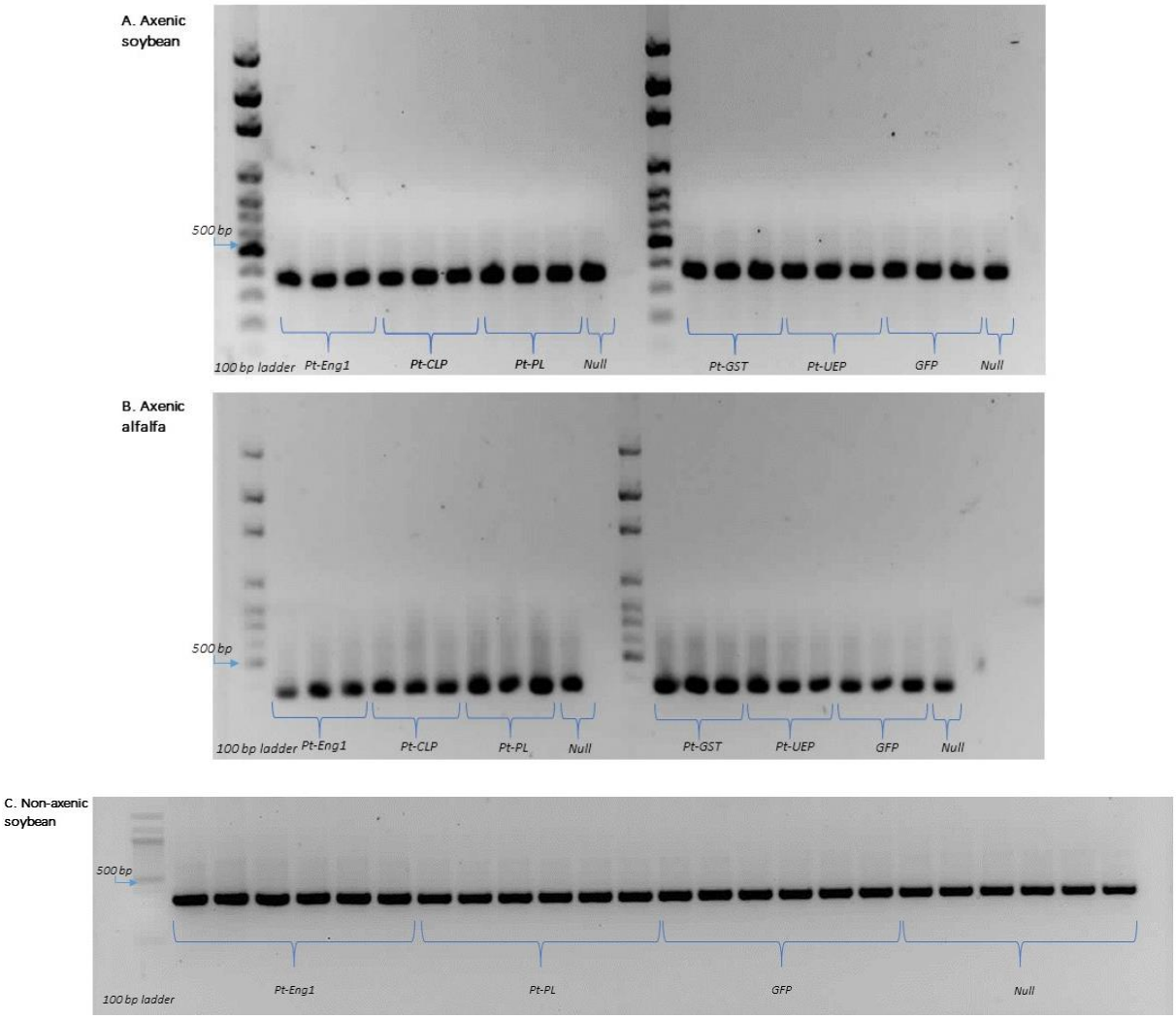

Figure 5.5. Molecular analysis of genomic DNA of axenic soybean (A) and alfalfa (B), and non-axenic soybean (C). Inverse images of agarose gels showing amplification of the 356 bp-long *nptII* fragment in the genomic DNA of three transgenic soybean (A) and alfalfa events (B), E1, E2 and E3 of *Pt-Eng-1*, *Pt-CLP*, *Pt-PL*, *Pt-GST*, *Pt-UEP* and GFP, and only E1 and E2 of null. The *ptII* gene was present in non-axenic transgenic soybean hairy root events E1 to E6 (C).

Table 5.1. Transformation efficiency of non-axenic soybean hairy root confirmed after molecular analyses of genomic DNA insertion of *nptII* fragment in ten selected hairy roots.

Non-axenic events	No. of hairy roots for molecular analysis	No. of hairy roots positive for *nptII*	Percentage of positive hairy roots	Non-axenic events	No. of hairy roots for molecular analysis	No. of hairy roots positive for *nptII*	Percentage of positive hairy roots
Null E1	10	10	100	*PtEng-1* E1	10	10	100
Null E2	10	9	90	*PtEng-1* E2	10	9	90
Null E3	10	10	100	*PtEng-1* E3	10	10	100
Null E4	10	10	100	*PtEng-1* E4	10	10	100
Null E5	10	10	100	*PtEng-1* E5	10	8	80
Null E6	10	9	90	*PtEng-1* E6	10	9	90
GFP E1	10	10	100	*PtPL* E1	10	10	100
GFP E2	10	9	90	*PtPL* E2	10	8	80
GFP E3	10	10	100	*PtPL* E3	10	10	100
GFP E4	10	10	100	*PtPL* E4	10	10	100
GFP E5	10	9	90	*PtPL* E5	10	8	80
GFP E6	10	9	90	*PtPL* E6	10	9	90

5.3.3 Reproduction of *P. thornei* on axenic soybean and alfalfa hairy roots, and non-axenic soybean

The steps in non-axenic transgenic hairy root development for soybean are shown in Figure 5.6. Two weeks after inoculation, the presence of nematodes was checked by staining samples of axenic and non-axenic hairy roots with red food dye (Figure 5.7). In all the treatments, the three stages of nematode development were evident in the roots: eggs, juveniles and adults. Twenty-one dpi with live mixed-stage nematodes, *P. thornei* reproduction was studied by recording the total number of nematodes collected at 24 h and 48 h for axenic soybean and alfalfa, and non-axenic soybean. One-way ANOVA was done to test the hypothesis of equal means of nematode numbers recovered after 21 dpi from soybean hairy roots of untransformed K599 and transformed K599 (null, *GFP*, and target genes).

Figure 5.6. Hairy root transformation of non-axenic soybean. *A. rhizogenes* inoculation of the cotyledonary node (A), inoculated plant in a moisture chamber (B), hairy root development (C to F), set-up placed in a growth chamber (G), excision of the innate root system below the hairy root system (H and I), re-establishment of the composite plant with hairy roots only (J), and inoculation with *P. thornei* for challenge assays (K).

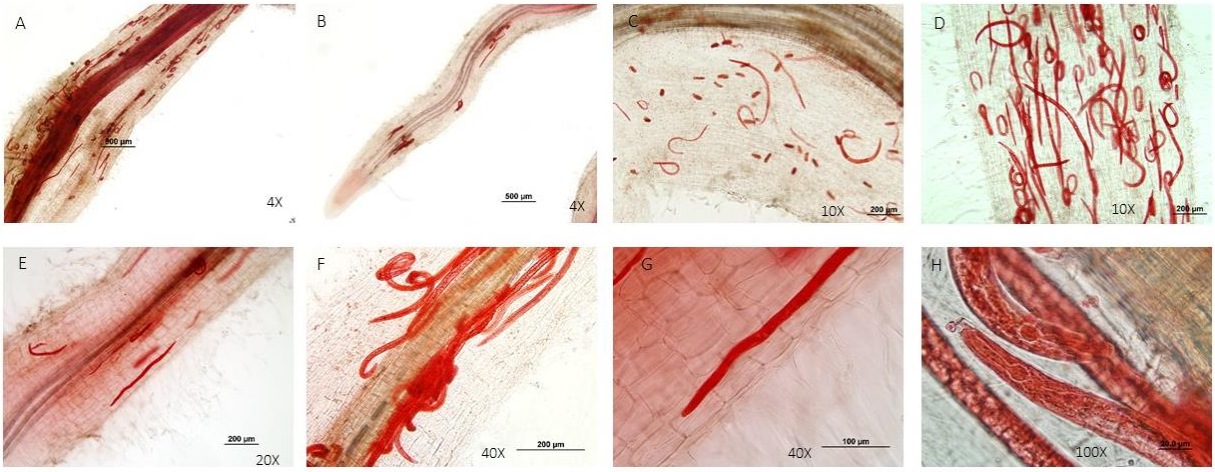

Figure 5.7. Staining of hairy roots confirming the presence of nematodes 21dpi. *P. thornei* nematodes in non-axenic hairy roots of K599 infected soybean (A) vs. *PtEng-1* (B) at a

magnification of 4X. Presence of eggs in axenic hairy root of soybean (C) and nematodes in axenic hairy roots of alfalfa at 10X (D). Nematodes inside axenic soybean hairy root at 20X (E) and in axenic alfalfa hairy roots at 40X (F). Image of a nematode inside an axenic soybean hairy root at 40X (G) and at 100X showing the stylet and median bulb of the oesophagus in a nematode feeding on axenic soybean hairy roots (H).

Amongst the axenic hairy root events of soybean, highly significant differences in mean number of nematodes were found on events expressing all five target genes compared to untransformed K599 and the transformed K599 controls: null and *GFP* (Figure 5.8). A pairwise comparison between the following treatments indicated no significant differences in mean number of nematodes fed on events of: untransformed K599 control and null, untransformed K599 control and *GFP*, null and *GFP*, *Pt-Eng-1* and *Pt-CLP*, *Pt-CLP* and *Pt-PL*, and *Pt-UEP* and *Pt-GST* (Tukey's HSD, $p < 0.001$). Of the five target genes, events expressing *Pt-Eng-1* showed the lowest number of nematodes and ranged between 196.5 (±10.69) to 219 (±14.40); these were *Pt-Eng-1* E6 and *Pt-Eng-1* E1, respectively.

Similarly, amongst the axenic hairy root events of alfalfa, highly significant differences in mean number of nematodes were found on events expressing all five target genes as compared to untransformed K599 control, and the transformed K599 controls: null and *GFP* (Figure 5.9). A pairwise comparison between the following treatments indicated no significant differences in mean number of nematodes fed on events of: untransformed K599 control and null, untransformed K599 control and *GFP*, null and *GFP*, *Pt-Eng-1* and *Pt-CLP*, *Pt-Eng-1* and *Pt-PL*, *Pt-CLP* and *Pt-PL*, and *Pt-UEP* and *Pt-GST* (Tukey's HSD, $p < 0.001$). Of the five target genes, events expressing *Pt-Eng-1* showed the lowest number of nematodes and ranged between 59 (±6.27) to 78 (±6.0); these were *Pt-Eng-1* E6 and *Pt-Eng-1* E1, respectively.

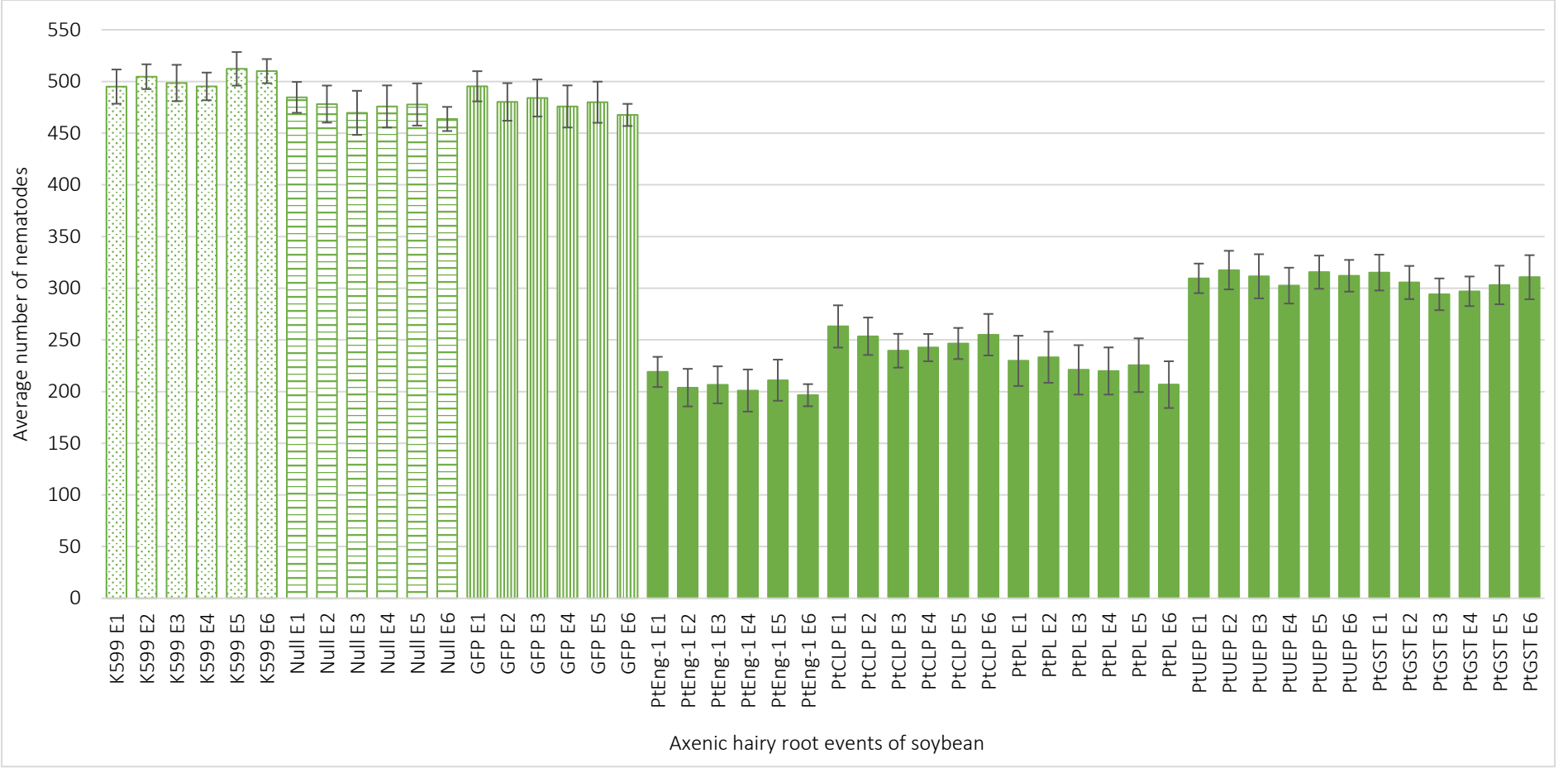

Figure 5.8. Mean number of *P. thornei* extracted from axenic hairy roots of soybean. Data represents ± standard errors of mean with *n* = 10 per group.

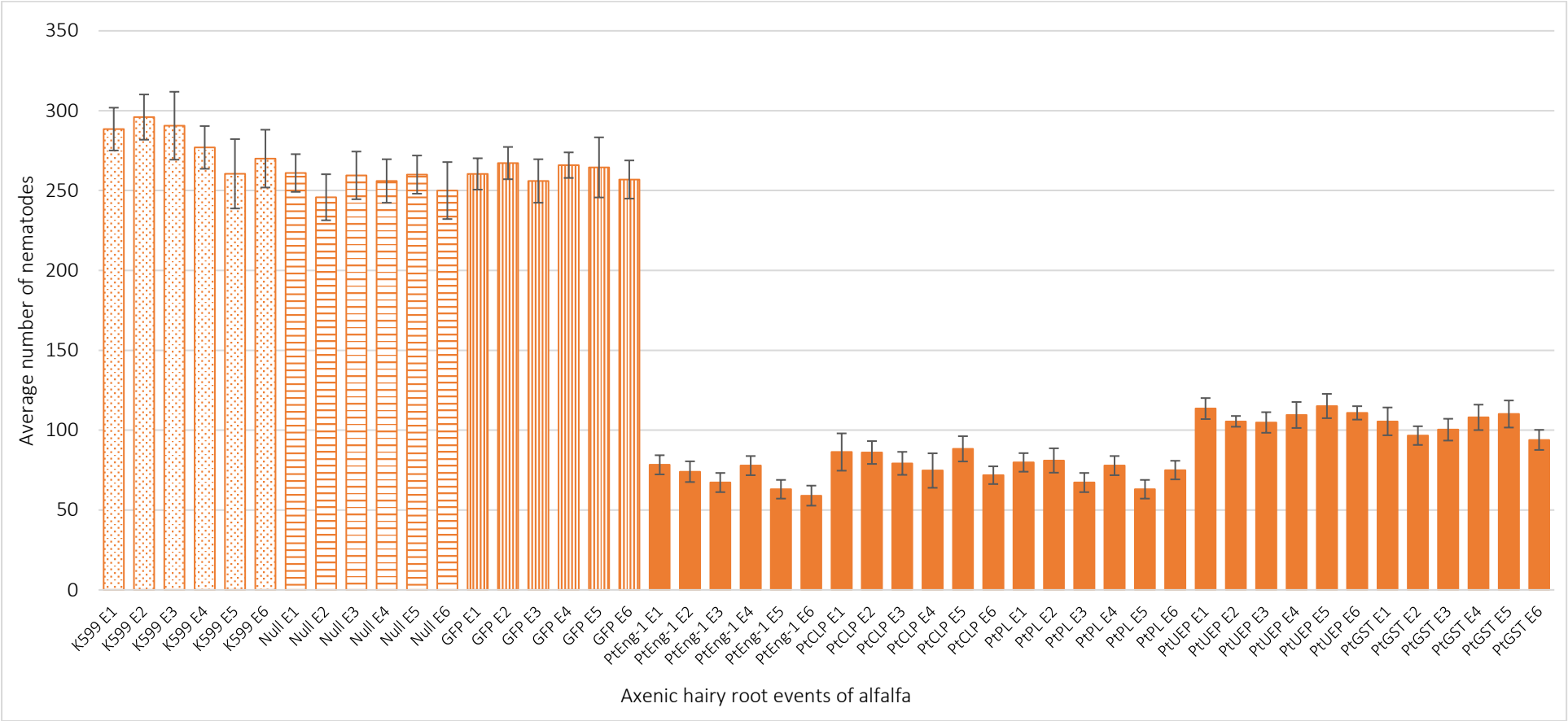

Figure 5.9. Mean number of *P. thornei* extracted from axenic hairy roots of alfalfa. Data represents ± standard errors of mean with *n* = 10 per group.

The mean number of nematodes recovered from non-axenic soybean events expressing *Pt-Eng-1* and *Pt-PL* were also found to be significantly different compared to untransformed K599 and the transformed K599 controls: null and *GFP* (Figure 5.10). A pairwise comparison between the following treatments indicated no significant differences in mean number of nematodes fed on events of: untransformed K599 control and null, untransformed K599 control and *GFP*, null and *GFP*, and *Pt-Eng-1* and *Pt-PL* (Tukey's HSD, $p < 0.001$). Of the two target genes, events expressing *Pt-Eng-1* showed the lowest number of nematodes and ranged between 2087 (±232.57) to 2413 (±146.08); these were *Pt-Eng-1* E5 and *Pt-Eng-1* E6, respectively. On the other hand, number of nematodes on events expressing *Pt-PL* ranged between 2384 (±137.89) to 2613 (±110.59); these were *Pt-PL* E4 and *Pt-PL* E5, respectively.

5.3.4 Effects on target gene expression in *P. thornei* after *in planta* RNAi

Changes in transcript levels and reduction in target gene expression relative to the internal control, 18S rRNA were assessed in nematodes fed for 21 days on transgenic events of axenic soybean and alfalfa, and non-axenic soybean, using semi-quantitative PCRs. Amplification of target genes was visualised as DNA bands by agarose gel electrophoresis and their intensities were compared by densitometric analysis. The changes in transcript accumulation for each of the target genes was studied using pixel intensity of each band from inversed gel electrophoresis images. By applying ImageJ, the difference in relative densities between control and sample was calculated and expressed as a percentage of reduction relative to 18S rRNA. Results of transcript accumulation as seen on agarose gels, and reduction of target transcript accumulation in axenic soybean and alfalfa, and non-axenic soybean are described below as separate sections.

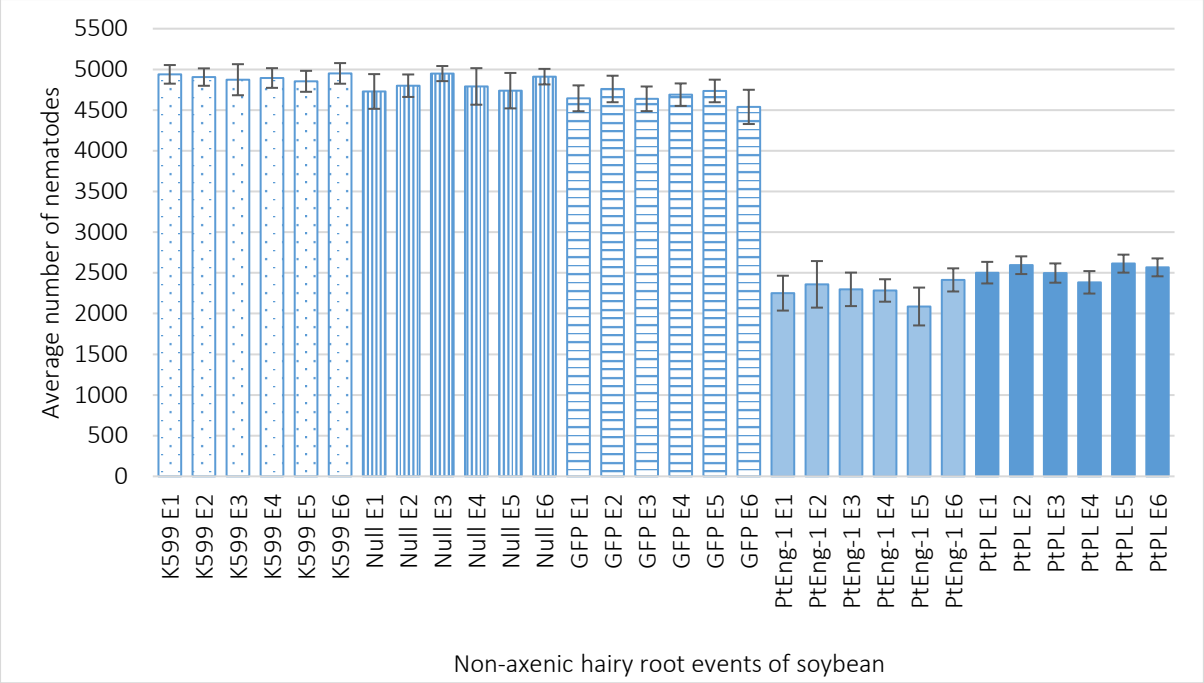

Figure 5.10. Mean number of *P. thornei* extracted from non-axenic hairy roots of soybean. Data represents ± standard errors of mean with *n* = 10 per group.

5.3.4.1 Effects on target gene expression in *P. thornei* after feeding on axenic soybean hairy root events

The housekeeping gene, 18S rRNA gene, was successfully amplified from cDNA of nematodes fed on soybean hairy roots expressing the five target genes and the controls: untransformed K599, *GFP*, null (5.11A). Similarly, transcripts all five target genes were also amplified successfully in nematodes that fed on soybean hairy roots expressing untransformed K599, *GFP*, null and target genes itself (5.11B). However, there was an obvious reduction in the amplification of each target gene in nematodes that fed on soybean hairy roots expressing the target genes compared to the three respective controls, which were visually brighter with constant expression. For all target genes, the target transcript accumulation at each PCR cycle tested was visibly lower than in their three respective controls. Amongst these genes, an obvious reduction in transcript accumulation at all PCR cycles was evident for *Pt-PL* and *Pt-Eng-1*. In *Pt-CLP* and *Pt-UEP* there was a more visible reduction at 25 and 30 PCR cycles than at 35 PCR cycle. For *Pt-GST*, while the intensity of transcript accumulation appeared greater than other genes, the visible reduction was apparent only at the 25 PCR cycle in comparison to its respective control.

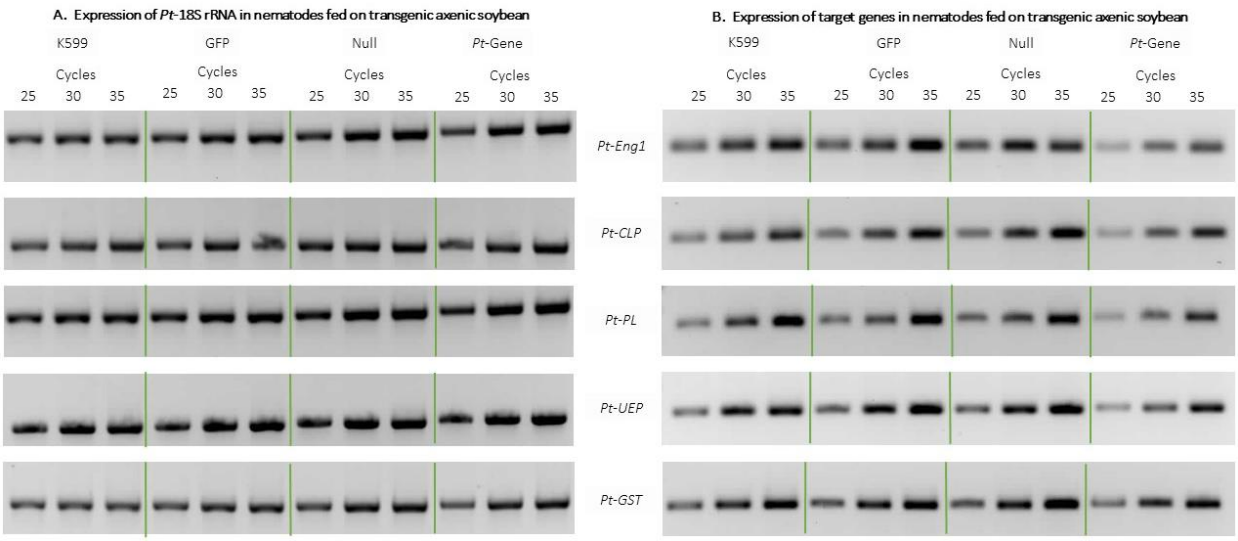

Figure 5.11. Inverse images of agarose gel electrophoresis for semi-quantitative PCRs on cDNA of *P. thornei* fed on transgenic hairy roots of axenic soybean for 21.

The reductions in transcript accumulation relative to 18S rRNA at 25, 30 and 35 PCR cycles were as follows. For *Pt-Eng-1*, the reduction was 60.8%, 50% and 36.8% which was also the greatest (Figure 5.12A); followed by *Pt-PL*, which had a reduction of 57.7%, 47.4% and 40.7% (Figure 5.12C); after which was *Pt-CLP*, having a reduction of 55.6%, 46.3% and 36.5% (Figure 5.12B); while for *Pt-UEP* it was found to be 50.7%, 40.6% and 31.3% (Figure 5.12D); finally, for *Pt-GST,* it was 42.5%, 32.3% and 22.1% which was also the lowest compared to other target genes indicating a less knockdown compared to the others (Figure 5.12E).

The obvious reduction in transcript accumulation of *Pt-Eng-1* followed by *Pt-PL* observed visually, was reflected in the densitometry-based measurement of reduction in transcript accumulation. Similarly, *Pt-GST* transcript accumulation, which appeared greater than for the other genes, also had the lowest reduction.

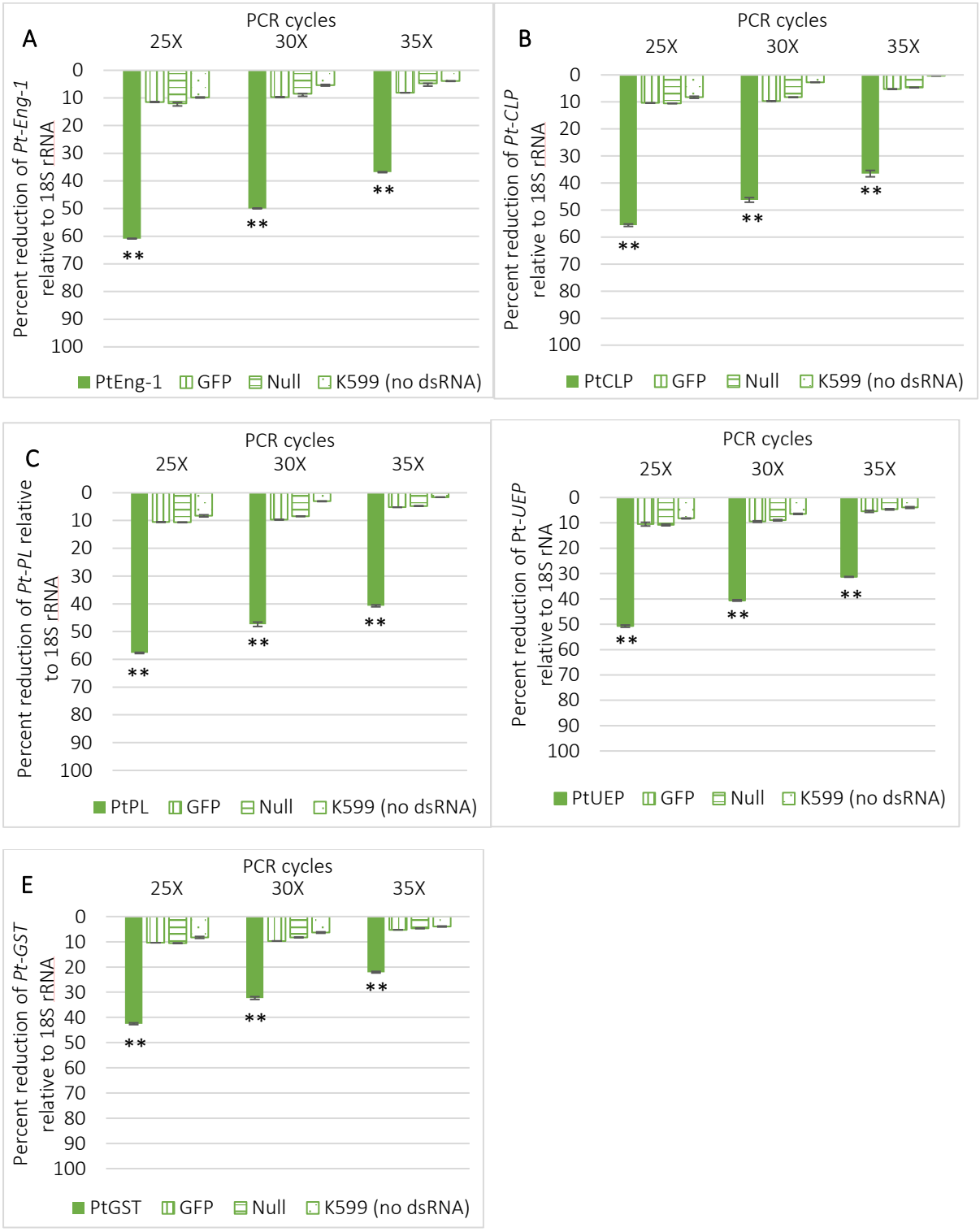

Figure 5.12. Percentage of reduction of *P. thornei* target genes relative to 18S rRNA after feeding on hairy root events of axenic soybean for 21 days. Data represents ± standard errors of mean with $n = 3$ per group. Significance with respect to each control at an experimental time-point (PCR cycle) is denoted by asterisks. ** indicates $p < 0.001$.

5.3.4.2 Effects on target gene expression for *P. thornei* after feeding on axenic alfalfa hairy root events

The housekeeping gene, 18S rRNA gene was amplified from cDNA of nematodes that fed on alfalfa controls: untransformed K599, *GFP*, null, and the five target genes (5.13A). Similarly, transcripts of all five target genes were amplified from nematodes that fed on alfalfa expressing untransformed K599, *GFP*, null and target genes (5.13B). The results show a clear reduction in the amplification of each target gene in nematodes that fed on alfalfa expressing the target genes compared to the three controls, which were visually brighter and consistent. For all target genes, the target transcript accumulation at each PCR cycle tested appeared lower than in their three respective controls. Amongst these genes, the reduction in transcript accumulation of *Pt-Eng-1*, *Pt-CLP* and *Pt-PL* was most evident at all PCR cycles. For *Pt-GST* and *Pt-UEP*, there was a visible reduction in transcript accumulation especially at 25 and 30 PCR cycles.

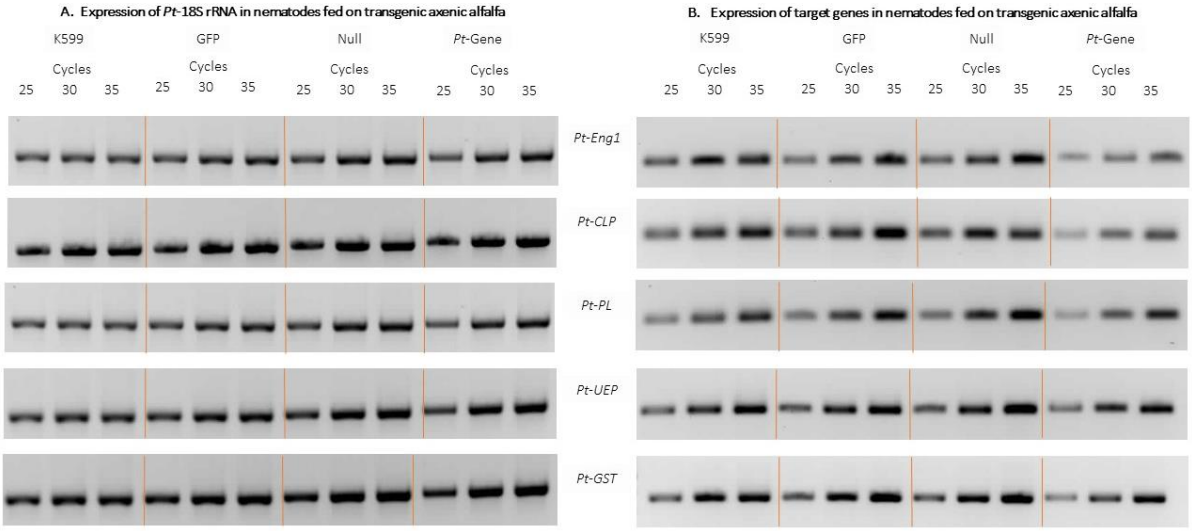

Figure 5.13. Inverse images of agarose gels for semi-quantitative PCRs of cDNA of *P. thornei* fed on transgenic hairy roots of axenic alfalfa for 21 days.

The reductions in transcript accumulation relative to 18S rRNA at 25, 30 and 35 PCR cycles were as follows. The greatest reduction was seen for *Pt-CLP*: 61.0%, 52.1% and 39.3% (Figure 5.14B) followed by *Pt-Eng-1*, which had a reduction of 59.6%, 49.9% and 36.8% (Figure 5.14A), after which was *Pt-PL* with a reduction of 55.5%, 47.7% and 41.2% (Figure 5.14C); while for *Pt-GST* it was found to be 35.1%, 28.3% and 14.2% (Figure 5.14E); finally, for *Pt-UEP,* it was 32.4%, 20.7% and 10.2% which was also the lowest as compared to other target genes indicating a lesser knockdown compared to the others (Figure 5.14D).

These data complemented the visual appearances in that that the lowest band intensity was for *Pt-Eng-1* which did not appear to differ from the intensity of *Pt-CLP* transcript. The densitometric analysis also indicated similar reductions for these two genes. Visually, intensities of *Pt-PL, Pt-GST* and *Pt-UEP* exhibited similar brightness, followed with the latter two seeming only very subtly different. Again, this complemented the reduction data indicating less knockdown in *Pt-GST* followed by *Pt-UEP.*

5.3.4.3 Effects on target gene expression of *P. thornei* after feeding on non-axenic soybean hairy root events.

The housekeeping gene, 18S rRNA gene was successfully amplified from cDNA of nematodes that fed on soybean controls: untransformed K599, *GFP*, null, and the five target genes (5.15A). Similarly, transcripts of *Pt-Eng-1* and *Pt-CP* were amplified in nematodes that fed on soybean expressing untransformed K599, *GFP*, null and the two target genes (5.15B). However, there was an obvious reduction in the amplification of both target genes in nematodes that fed on soybean expressing them compared to their three respective controls which were visually brighter and consistent. For all target genes, the target transcript accumulation at each PCR cycle tested was lower than in their three respective controls. Between the two genes, there were subtle differences in the reduction of transcript accumulation; *Pt-Eng-1* appeared slightly less than *Pt-PL* at all PCR cycles. The reductions in transcript accumulation relative to 18S rRNA at 25, 30 and 35 PCR cycles for *Pt-Eng-1* were 61.2%, 51.2% and 40.1% (Figure 5.16A), and this was greater than *Pt-PL*, which had a reduction of 55.2%, 47.1% and 40.8% (Figure 5.16B) suggesting a greater knockdown in *Pt-Eng-1* than *Pt-PL*. This data complemented the visual observation of the intensity of transcript accumulation as seen on the agarose gels

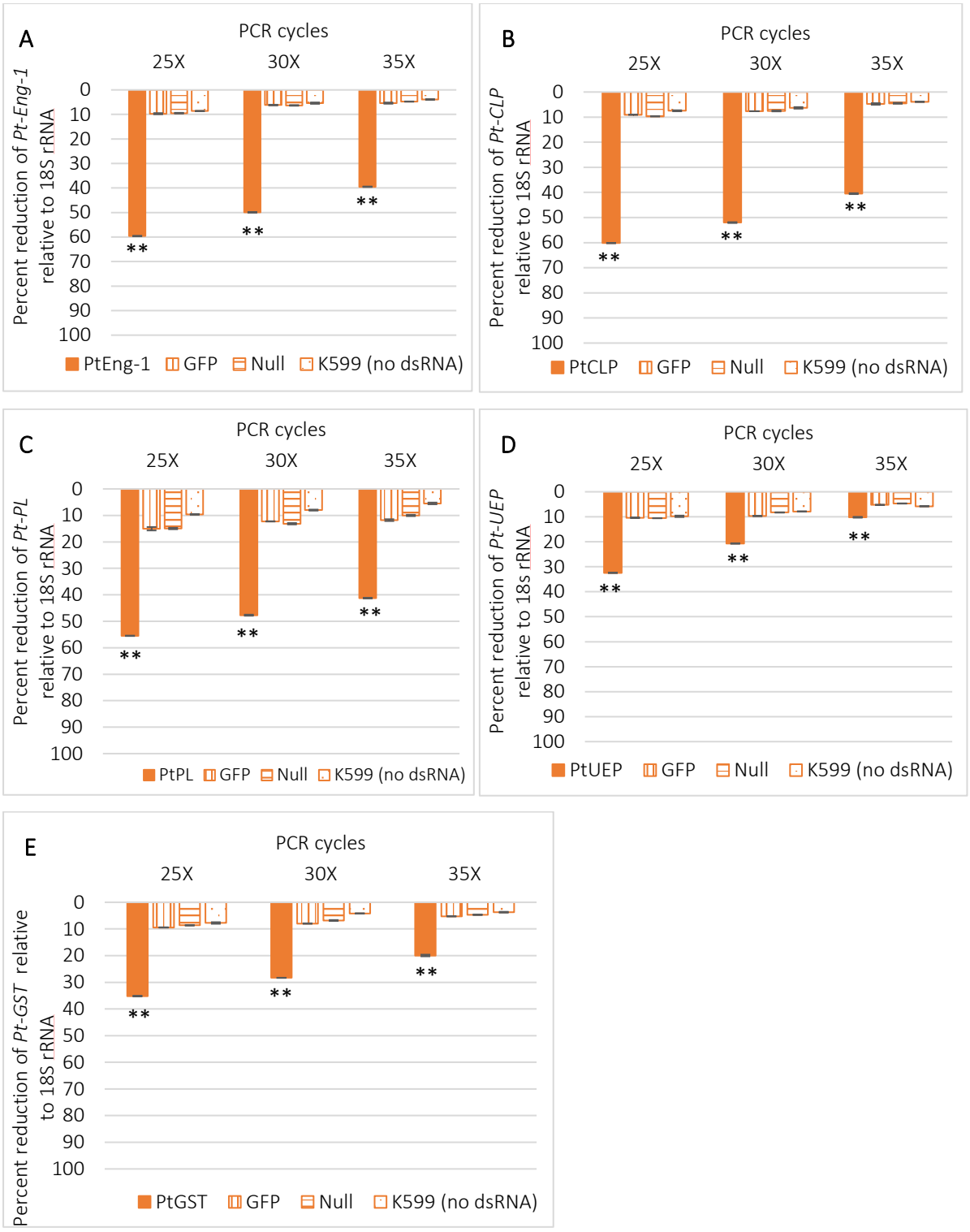

Figure 5.14. Percentage of reduction of *P. thornei* target genes relative to 18S rRNA after feeding on hairy root events of axenic alfalfa for 21 days. Data represents ± standard errors of mean with $n = 3$ per group. Significance with respect to each control at an experimental time-point (PCR cycle) is denoted by asterisks. ** indicates $p < 0.001$.

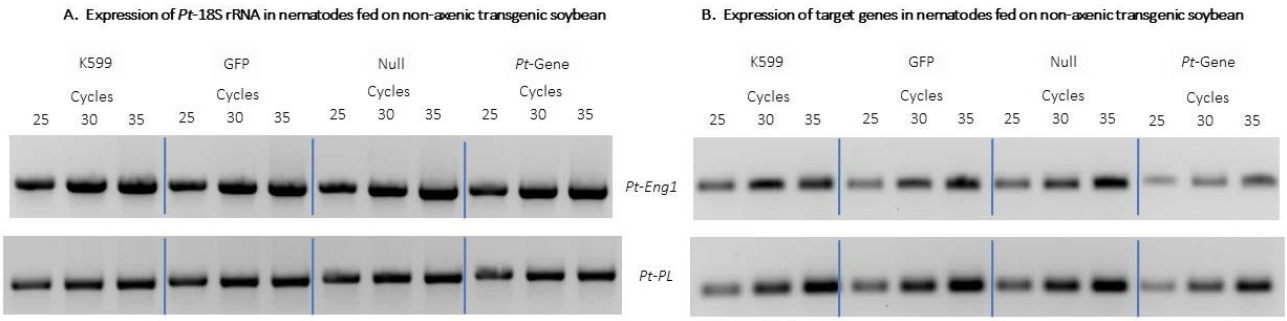

Figure 5.15. Inverse images of agarose gels from semi-quantitative PCRs of cDNAs of *P. thornei* fed on transgenic hairy roots of non-axenic soybean for 21 days.

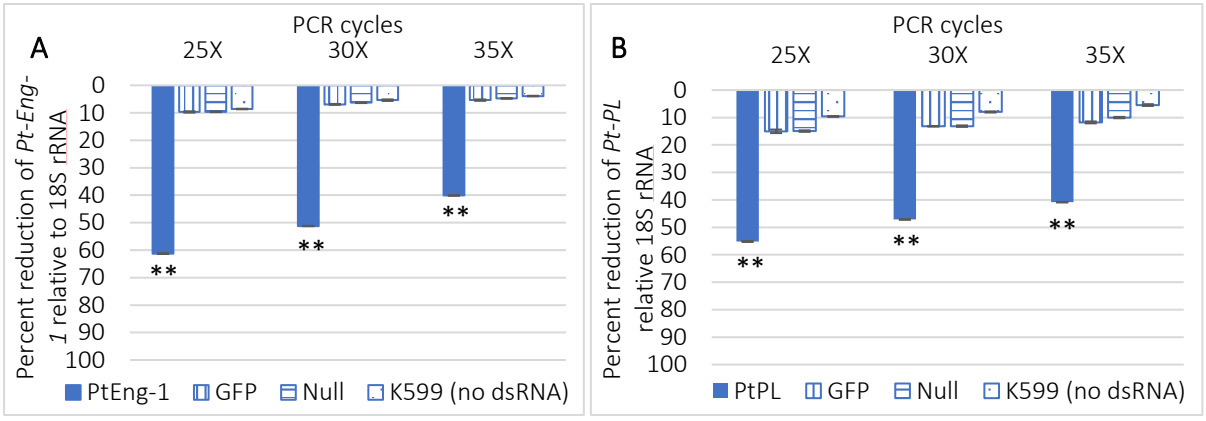

Figure 5.16. Percentage of reduction of *P. thornei* two target genes relative to 18S rRNA after feeding on hairy root events of non-axenic soybean for 21 days. Data represents ± standard errors of mean with n = 3 per group. Significance with respect to each control at an experimental time-point (PCR cycle) is denoted by asterisks. ** indicates $p < 0.001$.

For the above densitometry data, one-way ANOVA to test the hypothesis of equal reduction in target transcript accumulation relative to 18S rRNA was done at each time-point (PCR cycle). At each PCR cycle, statistically significant differences in reduction of transcript accumulation were found between target genes and controls (hairy roots from untransformed K599 *i.e* no dsRNA, *GFP* and null), and are denoted by asterisks (Tukey's HSD, $p < 0.001$) in Figures 5.12, 5.14 and 5.16. Similarly, in these data, no significant differences in reduction of target transcript accumulation were observed among the three controls ($p < 0.05$).

5.4 Discussion

The results of the work in this Chapter demonstrate that host-delivered target dsRNA homologous to parasitism effector gene sequences in *P. thornei* can cause a reduction in transcript accumulation and also reduced nematode numbers on subsequent culture.

The method of generating axenic hairy roots of soybean was achieved successfully, with one modification that involved transferring cotyledons to Petri dishes lined with layers of sterile Whatman™ filter paper soaked in liquid MXB media containing antibiotics. This provided a favourable environment which promoted callus and hairy root formation, and significantly reduced the time (8 to 10 days) required for callusing and hairy root induction, compared to 10 to 14 days using previous methods. In the process of inoculating alfalfa with *A. rhizogenes* caution is needed during the inoculation procedure which should resemble 'touch-and-go' ensuring no pressure is applied to the seedling while gently tapping on the inoculum paste. Applying more than the required amount of inoculum results in a need for several passages on media plates to remove *Agrobacterium* contamination that can grow on the plate, thereby causing experimental delays.

Agrobacterium-mediated hairy root transformation of soybean yielded more roots than for alfalfa, however, the hairy root induction in alfalfa was faster than for soybean. This may be caused for example by genotypic differences or the age of seedling with regard to totipotent cells at the stage of plant growth and development used. The efficiency of hairy root formation varies with genotypes of a plant species, including soybean genotypes (Cao et al., 2009; Cho et al., 2000; Mazarei et al., 1998; Savka et al., 1990; Weber et al., 2011). While there are no similar reports for alfalfa (*M. sativa*), there are genotypes with differing responsiveness in culture, and such responsiveness may extend to hairy root induction. There was not time to study different genotypes for such responsiveness. The age of seedlings is indirectly linked to the presence of proliferating cells in the pericycle and the surrounding cell layers that give rise to adventitious roots induced by *A. rhizogenes*, as reported in squash (Ilina et al., 2012). Moreover, the seedlings need to be old enough to survive wounding and subsequent infections with *Agrobacterium,* and yet young enough to have cells that progress through the cell cycle in the pericycle and adjacent cells. There are observations in *Agrobacterium*-mediated transformation of squash where cells of the tissue around the site of inoculation probably do

not divide, and this affects hairy root formation (Katavić et al., 1991). Another possibility may be host-specificity on *Agrobacterium* strains, which can also have affect transformation efficiency, root initiation and development (Baranski et al., 2006; Smarrelli et al., 1986). From this work, if time is a major constraint, then using alfalfa can provide a quicker option to deliver hairy root events.

For both, soybean and alfalfa, the mean number of hairy root events that emerged from untransformed and transformed K599 were not significantly different, suggesting that K599 transformed with GFP or target genes did not affect hairy root emergence. However, some statistically non-significant differences were seen in root length of the three controls (untransformed K599, null and GFP) versus the target genes for both hosts. This was also reflected in the rate of root elongation, and could be due to some stress that these genes may have caused, the site of insertion or copy number. More studies, such as Southern blots to estimate number of copies inserted, need to be done to test these hypotheses. In any case, essentially the same number of hairy roots emerged from K599 transformed with target genes as from controls: both were greatest at 15dpi.

An interesting observation in stained samples of hairy roots of both soybean and alfalfa was that some nematodes appeared coiled in the root cells. These may reflect a resting phase – there is a similar observation on the invasion and endoparasitic behaviour of *P. penetrans* in wild-type tobacco roots using transmission electron microscopy (Zunke, 1990b). In that study, feeding and migration was frequently interrupted by resting phases lasting for several hours, during which the nematodes exhibited characteristic coiling inside host root cells (Zunke, 1990b).

Overall, the semi-quantitative PCR results indicated that there was a significant reduction in transcript accumulation and nematode numbers for all five target genes after *in planta* RNAi of *P. thornei* inoculated to transgenic soybean and alfalfa hairy roots. The assessment of transcript accumulation was based on visual observations and densitometry analysis relative to the housekeeping gene 18S rRNA, and is discussed with respect to effects on reproduction of *P. thornei* for each gene.

Amongst the axenic hairy root events of soybean, on average, in comparison to untransformed K599, the nematode numbers were 41%, 44%, 50%, 60% and 62% in events of *Pt-Eng-1*, *Pt-PL*, *Pt-CLP*, *Pt-GST* and *Pt-UEP*, respectively. This result correlated with the target transcript accumulation as determined by semi quantitative PCR at 25 PCR cycles for all five genes (in the same order as above) relative to 18S rRNA, suggesting that the levels of knockdown correlated with the reduced nematode numbers found on soybean transgenic roots. In other words, greater knockdown correlates with reduced nematode numbers.

However, this pattern was slightly different for axenic hairy root events of alfalfa. On average, compared to untransformed K599, the nematode numbers were 25%, 26%, 29%, 36% and 39% in events of *Pt-Eng-1*, *Pt-PL*, *Pt-CLP*, *Pt-GST* and *Pt-UEP*, respectively. Only two of the genes tested showed a direct correlation between reduced number of nematodes and the percentage reduction in target transcript accumulation found at all PCR cycles. The percent reduction in transcript accumulation of *Pt-Eng-1* and *Pt-PL* relative to 18S rRNA at 25 PCR cycles, for example, was 60% and 56%, respectively. This also correlated with the lowest numbers of nematodes of 25% and 26% found in alfalfa transgenic roots expressing *Pt-Eng-1* and *Pt-PL* respectively, in comparison to untransformed K599. However, this was not true for the three other genes, *Pt-CLP*, *Pt-GST* and *Pt-UEP.* This could be perhaps because the effect of knockdown was transient or the nematodes may have compensated for the effects of knockdown to a certain level, or there may have been differential host responses to nematode infection. As *P. thornei* is a migratory nematode that can enter and exit transgenic roots, and may not feed continuously, this behaviour may give these nematodes a chance to recover. Interestingly, in alfalfa transgenic events, even though the reduction in target transcript accumulation was not inversely proportional to nematode numbers as was the case for soybean, it nevertheless showed the same trend of *P. thornei* numbers, where fewest nematodes were found on events of *Pt-Eng-1* followed by *Pt-PL*, *Pt-CLP*, *Pt-GST* and *Pt-UEP* in the same order.

Another observation for *in planta* RNAi studies of axenic soybean and alfalfa which paralleled results for *in vitro* RNAi, was that for all the target genes there were 16% to 24% fewer nematodes compared to respective controls on transgenic alfalfa than on soybean, indicating that there were consistent host specific differences which affected nematode reproduction.

For example, alfalfa expressing *Pt-Eng-1* supported 16% fewer nematodes than soybean expressing *Pt-Eng-1*. In soybean and alfalfa, nematode numbers were reduced by 59% and 75% respectively, in comparison to untransformed K599-only controls. Thus, reproduction of *P. thornei* on ds*Pt-Eng-1* fed nematodes was reduced more on alfalfa than on soybean. Similarly, in alfalfa expressing- *Pt-PL*, *Pt-CLP*, *Pt-UEP* and *PT-GST* there was 74%, 71%, 60% and 63.5% reduction in nematode numbers respectively, as compared to untransformed K599-only controls. In soybean expressing *Pt-PL*, *Pt-CLP*, *Pt-UEP* and *PT-GST* there were 56%, 50%, 38% and 40% reduction in nematodes respectively, than on untransformed K599-only controls. These results may again reflect differences in genotype and variety, host preference, host defence responses or root exudates attracting nematodes that eventually affect nematode reproduction.

There was also a positive correlation in nematode numbers from non-axenic composite soybean transgenic roots and the percentage reduction in target transcript accumulation at 25 PCR cycles for *Pt-Eng-1* and *Pt-PL*. There was 53.4% reduction in nematode numbers from soybean roots expressing *Pt-Eng-1* as compared to untransformed K599 control and this correlated with the 61% reduction of *Pt-Eng-1* relative to 18S rRNA. Similarly, there was 48.5% reduction in nematode numbers in soybean roots expressing *Pt-PL* as compared to the controls. This suggests that greater knockdown of *Pt-Eng-1* transcripts resulted in greater reduction in nematode numbers than *Pt-PL*. This trend of positive correlation of greater knockdown resulting in lower nematode numbers was also evident when long-term effects on *P. thornei* were studied after *in vitro* RNAi of *Pt-Eng-1, Pt-PL* and *Pt-CLP,* when wild-type soybean and alfalfa were infected with target dsRNA-soaked nematodes.

Compared to control values (no dsRNA), the nematode numbers found on the two hosts were the lowest for *Pt-Eng-1* followed by *Pt-PL* and *Pt-CLP*. Overall, based on the results of *in vitro* RNAi, *in planta* RNAi *i.e.* axenic soybean and alfalfa, and non-axenic soybean transgenic hairy root studies, it can be said that, for the two genes, *Pt-Eng-1* and *Pt-PL*, the results suggest that the level of knockdown correlated with the reduction in nematode numbers.

Additionally, the percent reduction in nematode numbers found on axenic and non-axenic soybean hairy root events expressing *Pt-Eng-1* and *Pt-PL* compared to their controls also

showed the same trend and range (59% and 75% for soybean- and alfalfa-expressing *Pt-Eng-1*, respectively; 56% and 74% for soybean- and alfalfa- expressing *Pt-PL*, respectively) indicating a similarity in target gene performance. The slightly lower reduction in nematode numbers observed in non-axenic soybean can be perhaps because of normal nematode reproduction on non-transformed hairy roots, or intermittent/insufficient feeding on transformed roots thus yielding lesser RNAi silencing. Unfortunately, these are only speculations which need further investigation to qualify as solid reasons.

In this study, irrespective of the transgenic host and method (axenic/non-axenic) used to obtain them, *Pt-Eng-1, Pt-PL*, *Pt-CLP* and *Pt-UEP* transcript levels were significantly reduced after dsRNA treatments compared to the controls. There was also a significant reduction in nematode numbers on transgenic soybean and alfalfa roots, indicating that silencing these genes affected *P. thornei* reproduction. These results are in line with similar studies done for the same genes in other PPNs using RNAi constructs expressed in transgenic plants. For example, transgenic tobacco expressing *Mj-Eng-3* of *M. javanica* showed a significant decrease in gene expression, and number of nematodes and galls (Hu et al., 2013). Similar results of reduction in transcript levels, egg numbers and subsequent nematode infectivity have been found in tobacco expressing cysteine protease cathepsin L type, *Mi-Cpl-1* (de Souza Júnior et al., 2013). When soybean (cv. Williams 82) expressing pectate lyase, *Hg-pel-6* was challenged with *H. glycines*, there was a significant reduction in transcript levels, accompanied by a 30% to 45% reduction in nematode numbers compared to dsGFP controls (Peng et al., 2016). Similarly, potato expressing a ubiquitin carboxyl extension protein with a signal peptide, *GrUBCEP12* of *G. rostochiensis* was challenged with nematodes, resulted in 2.5 to 5.5-fold reduction in target mRNA expression and was accompanied by significant reduction in average number of females per root system (Chronis et al., 2013).

On the overall success of the five target genes from *in vitro* and axenic *in planta* RNAi studies, *in planta* RNAi using composite plants was studied. The advantage of the latter approach was that such plants could be generated relatively quickly, and more importantly, it mimicked natural host infections. This system has not so far been used to study the effects of *in planta* RNAi on *Pratylenchus* spp., and so it is the first report of composite plant-based RNAi for *P. thornei.* While it would have been desirable to test all five target genes, growth chamber space

and time limited extending the study. Hence, only the two most effective target genes, *Pt-Eng-1* and *Pt-PL,* from the *in vitro* and *in planta* RNAi studies, were tested using composite plants. In addition, soybean was chosen as test plant since the hairy root system was the most convenient for *Agrobacterium* inoculation. Soybean, and specifically cv. Williams 82, has also used to develop composite plants in other studies (Ibrahim et al., 2011; Klink et al., 2009; Li et al., 2010a; Li et al., 2010b; Niu et al., 2012; Peng et al., 2016).

One aspect of the composite soybean plants with wild-type shoots and transgenic hairy roots was that not all the roots at the inoculation sites were transgenic. However, the transformation frequency was checked, and was 93% for *Pt-Eng-1* roots and 92% in *Pt-PL* roots. Nevertheless, this issue could be overcome by using a reporter or selectable marker gene such as GFP and antibiotic resistance genes in the binary vector so that only transformed roots can be selected. For example, the *gfp* marker gene has been used to identify green fluorescence of transformed roots under blue light, so non-transformed roots could be removed before replanting (Ibrahim et al., 2011). In the current study, while the *npt II* resistance gene was present in the binary vector, a step to select transformed roots *in solium* by spraying kanamycin could have been included, but remaining kanamycin in the soil could affect the survival and activity of *P. thornei*, and so this form of selection was not done. If the 7-8% of non-transgenic roots had been identified and excised the percent reduction in nematode numbers would have been greater, since non-transgenic roots would have allowed normal reproduction. Nevertheless, the system used clearly showed that RNAi significantly reduce nematode numbers.

For other host-pathogen studies, composite soybean seedlings with transgenic roots have been used to study the effects of silencing the reproductive and fitness by RNAi of genes, *Cpn-1*, *Y25* and *Prp-17* of *H. glycines,* resulting in reduced eggs per gram of root by 95%, 81% and 79% respectively, together with knockdown of transcript levels (Li et al., 2010a). Similarly, composite soybean with transgenic roots were used to study *in planta* RNAi silencing of *Rpn7* of *M. incognita*. In addition to a significant reduction in gene expression, there was a reduction in egg masses per gram of root tissue and number of eggs per gram of root tissue of 34% and 51%, respectively (Niu et al., 2012). Similar plants of soybean cv. Williams 82 with roots expressing tyrosine phosphate and mitochondrial stress-70 protein precursor genes also

reduced size of *M. incognita* adults, with reduced target gene expression and reduced gall formation by 92% and 95% respectively (Ibrahim et al., 2011).

In considering the use of composite plants, apart from the issue described above that not all roots are transgenic at the inoculation site, it may not be possible to generate transgenic soybean plants from hairy roots: if that were the case then seed could not be produced and useful resistance could not be transferred by crossing. However, there has been success of generating transgenic plants from hairy roots, and perhaps the same would be possible in soybean (Giri and Narasu, 2000; Hu and Du, 2006). It is more likely that target sequences effective in reducing nematodes identified in this way would be introduced into elite crop lines as an additional transgenic trait

CHAPTER 06

General Discussion

PPNs are one of the four major groups of plant pathogens and their control is vital to help ensure globally sustainable agriculture and food security. Estimates of PPN-associated crop losses now exceed $100 billion worldwide, and PPNs pose a serious threat to the global food supply on which the growing human population relies. Indeed, PPNs may be responsible for more crop losses than that caused by insect pests (Nicol et al., 2011). These crop losses point towards limitations of currently used approaches to PPN control, which have relied primarily on chemicals and non-host rotations. Many chemical nematicides have been banned or their use restricted because they can persist in soil and be detrimental to the environment and can also affect non-target organisms. There are also limited sources of genetic resistance against many PPNs. Although the use of integrated pest management, which includes nematode-free planting material, crop rotations, use of bio-control agents and planting crop varieties with some resistance to PPNs are frequently deployed, in many cases their success is limited, especially in broad scale agricultures, and this is the case for RLNs. Therefore, additional forms of nematode control are needed, such as an RNAi-based crop protection strategy, in the form of synthetic resistance genes. The success of a host-mediated crop protection requires the selection of suitable target genes that play a crucial role in the life-cycle of the organism, and which on silencing, result in an altered behaviour and negative effects on growth, development and reproduction, thereby conferring host resistance.

RNAi is a well-conserved phenomenon in the eukaryotes (Fire et al., 1998). Since then, RNAi has been found in many organisms including PPNs, in which it is has been used to understand gene function and their potential for nematode control (Fosu-Nyarko and Jones, 2015). After the first demonstration of *in planta* RNAi in RKNs and CNs, other studies have followed including those on *Pratylenchus* species, thus illustrating the potential of host-derived RNAi as a strategy of crop protection (Adam et al., 2008; Bakhetia et al., 2005a; Chen et al., 2005b; Dubreuil et al., 2011; Fairbairn et al., 2007; Fanelli et al., 2005; Fanelli et al., 2014; Fosu-Nyarko and Jones, 2015; Fosu-Nyarko and Jones, 2016; Fosu-Nyarko et al., 2009; Haegeman et al., 2009; Huang, 2006; Jones and Goto, 2011; Jones and Fosu-Nyarko, 2014; Joseph et al., 2012; Lilley et al., 2012; Nsengimana et al., 2013; Papolu et al., 2013; Park et al., 2008; Sindhu et al., 2009; Tan et al., 2013; Urwin et al., 2002; Vieira et al., 2015; Yadav et al., 2006). Although these RNAi studies include *Pratylenchus* species, those on *P. thornei* are limited, providing an opportunity to investigate the efficacy of silencing target genes by *in planta* RNAi. The only

record of host-mediated RNAi-based on *P. thornei* are by Tan (2015), with limited additional data on silencing parasitism effector genes in *P. vulnus* and *P. thornei* (Fanelli et al., 2014; Tan, 2015). Taken together, this provided an opportunity to study target parasitism effector genes of *P. thornei* using *in vitro* and *in planta* RNAi.

Regarding the initial goals of the project, these were to identify and silencing target effector genes of *P. thornei* by soaking in dsRNA and by delivery *via* transgenic hairy roots. Specific aims included *in silico* identification and molecular characterisation of putative parasitism effector genes and to study the effects of *in vitro* and *in planta* RNAi silencing on phenotype, gene expression and reproduction. Most of these initial aims were achieved successfully, providing valuable new information on the potential of these target genes for developing a host resistance trait against *P. thornei,* and perhaps other RLNs. Major findings, advantages and limitations, and future research prospects are discussed here.

6.1 Target gene sequences

The key to successful RNAi is the selection of effective target genes. The first step was selection of target putative parasitism effector gene sequences of *P. thornei*. At the time this research was initiated, a comprehensive literature review was undertaken which generated a list of 88 published characterised effector genes of sedentary nematodes were used as queries in BLAST suites to identify orthologues in the TSA of *P. thornei.* Gene identification would have been improved using characterised full-length sequence information of other *Pratylenchus* species, but unfortunately these were not available. In the absence of *P. thornei* genomic sequence, the availability of its TSA was used to identify transcripts that may be expressed in different life stages. The selection of five putative target effector gene sequences was based on satisfying one or more of the following criteria: 1. A direct role in host invasion, migration and feeding, plant cell wall degradation, intracellular digestion, plant defence suppression, detoxification of ROS, protein degradation and avirulence. 2. Have been identified *in silico* in either or all of the three available transcriptomes of *Pratylenchus* species, (*P. thornei*, *P. coffeae* and *P. zeae*). 3. The corresponding TSA contig sequence matched the query sequence within its cds, the sequence had the lowest e-value, and highest alignment and bit scores in a TBLASTX. This resulted in the identification of five target effector genes that are directly involved in

parasitism: *Pt-Eng-1* (β-1,4-endoglucanase), *Pt-PL* (pectate lyase), *Pt-CLP* (cathepsin L-protease), *Pt-UEP* (ubiquitin extension protein) and *Pt-GST* (glutathione S-transferase).

The *P. thornei* sequence that matched the *Tpx* gene encoding peroxiredoxin of *G. rostochiensis* was not advanced as was discussed in section 3.4. Further work on this target needed designing and testing additional primer sets or reducing the nematode collection time and post-processing to obtain samples that were still within the window of the infection process, thereby increasing the chances of amplifying *Tpx* transcripts.

6.2 RACE PCRs

Four partial sequences of *Pt-Eng-1, Pt-PL, Pt-CLP* and *Pt-GST* were obtained using RACE PCRs. For *Pt-Eng-1,* using sequence analysis of 5' and 3' RACE data allowed the identification of the overlapping sequence thus generating a single sequence. This *Pt-Eng-1* sequence exhibited conserved cellulase and CBM-2 domains separated by a linker region, and the presence of signature sequences within them, which provided convincing evidence of the identity of *Pt-Eng-1.* The sequence was similar to endoglucanases of *P. vulnus*, *P. penetrans* and *M. incognita.* This sequence conservation suggests that *these Eng-1* genes probably originated from a common ancestor via horizontal gene transfer (Haegeman et al., 2011a; Jones et al., 2005). Importantly, this study provides the first *Pt-Eng-1* translation start codon and the full coding sequence. Evidence was also found for a cleavage site, a signal peptide at the N-terminus, classically secreted protein, with no predicted transmembrane helices.

The partial sequence of *Pt-PL* obtained from 5'RACE indicated that it was a class III pectate lyase, since it contained the conserved BF domain and seven residues that are known to be active and calcium binding sites, a hallmark of the class III pectate lyases (Hatada et al., 2000; Huang et al., 2005; Kudla et al., 2007). A translation start codon was also obtained for *Pt-PL.* A consensus sequence based on the top five BLASTP and BLASTX hits that matched *Pt-PL* was used to modify the two missing residues near the methionine at the N-terminal end, thus providing important information on the signal peptide and cleavage sites, which suggests a non-classical secretory pathway. The phylogenetic analysis of *Pt-PL* showed a relationship with the pectate lyases of bacterial origin, particularly Gram-positive. Again, this can be explained

by lateral gene transfer events of cell wall modifying enzymes like cellulases, pectate lyases, polygalacturonase, xylanase, chorismite mutase, and expansins of bacterial and fungal origins to be transferred to PPNs (Haegeman et al., 2011a; Jones et al., 2005). Genes like the cell wall modifying, *Pt-Eng-1* and *Pt-PL* in this study belong to multigene families and may have resulted from multiple horizontal gene transfer and or alternatively of gene duplication events (Haegeman et al., 2011a).

The *Pt-CLP* sequence information from 3'RACE PCRs provided evidence of two of the three residues, histidine (H) and asparagine (N) that make the catalytic triad together with a cysteine (C) residue, which was not identified in the signature conserved site that is normally present in all cysteine cathepsins, due to incomplete sequence information (Guha and Padh, 2008). Similarly, the *Pt-GST* sequence information from 3'RACE PCRs provided evidence of the seven essential and conserved residues for dimer interface, substrate binding pocket (having an H site), and an N-terminal domain interface in addition to two more glutathione binding moieties (Marchler-Bauer et al., 2006; Marchler-Bauer et al., 2014; Marchler-Bauer et al., 2010). However, the two tyrosine (Y) residues associated with the stabilisation of GSH known to be conserved in the N-terminal end closer to the start codon (Y4 and Y8) (Torres-Rivera and Landa, 2008) were not found because of missing 5'RACE sequence information. With the limited information available on GSTs of PPNs, it was not surprising to find only two GSTs of *M. incognita* and *R. similis* that clustered with *Pt-GST:* most GSTs belong to free-living and APNs and have been the most investigated (Brophy et al., 1995a; Brophy et al., 1995b; Campbell et al., 2001; van Rossum et al., 2001b; Xie et al., 2015; Zhan et al., 2010).

In all of the gene sequences generated by RACE PCRs, it would have been desirable to have found the few missing nucleotide sequences needed to generate a complete sequence, however, this would have require designing and trying more primer sets for RACE and long-distance PCRs, which was a limitation. Nevertheless, the evidence provided here clearly validated the identities of these genes.

The aim was to use RACE PCRs to generate full-length gene sequences for RNAi studies. Factors which prevented fully achieving this aim included the time and resources needed for Sanger sequencing, primer design, the cost of RACE kits, and the lack of *Pratylenchus* genome data,

which would have been useful. Another factor was that the in-house RNAi vector, pDoubler, used for *in vitro* transcription, had a limited choice for restriction enzyme sites.

6.3 Root lesion nematode effectors

In Table 6.1 of characterised or putative effectors of PPNs, there appears to be two classes of effectors (Fosu-Nyarko and Jones, 2016; Jones and Fosu-Nyarko, 2014). First a common subset of related effectors present in most PPNs appears to be involved in host penetration, migration and suppression of host defences. This subset is present in all PPNs species, including RLNs such as *P. thornei*. The second subset is more diverse and includes a much wider array of species-specific effectors which are required for feeding-cell formation by endoparasites, with major differences in this set of effectors for example between RKNs and CNs.

In CNs there is a general motif in the promoter of some effectors, termed a 'DOG' box, which is common to and associated with expression of dorsal gland cell-generated effectors (Eves-van den Akker et al., 2016): it would be interesting to know if such a motif is present in RLN promoters. A genomic sequence for *P. thornei* is needed to look for such motifs, and it would also be useful to know how the genome size compares to the only known RLN genome of *P. coffeae* which is 19.67 Mb (Burke et al., 2015).

6 .4 *In vitro* RNAi

In vitro RNAi silencing of *P. thornei* in dsRNA homologous to the target gene sequence is an effective method for initial screening of candidate target genes, understanding their function and evaluating their suitability for host-derived RNAi for nematode control (Fosu-Nyarko et al., 2011; Fosu-Nyarko and Jones, 2015). Of course, RNAi can be applied to any vital nematode gene, not just effectors, but the focus here was on effectors since they are of comparative interest between different PPN species. The results obtained here for five target putative effector genes both reduced transcript accumulation and also reduced nematode reproduction in long-term assays, suggesting their suitability for *in planta* RNAi.

Table. 6.1. Secreted proteins and effectors of parasitism genes of sedentary and migratory endoparasitic nematodes. Source: Jones and Fosu-Nyarko, 2014.

Nematode Parasitism Gene	Nematode Genus Known to Secrete Protein/Effector			Putative or Known Function
	Meloidogyne	*Heterodera*/ *Globodera*	*Pratylenchus*	
CAZymes (cell wall modifying enzymes)				
Endoglucanases and precursor	+	+	+++	Hydrolysis of beta 1,4-glucan
Pectate lyase	+	+	+++	Hydrolysis of alpha 1,4-linkages in pectate
Xylanase	+	Unknown	++	Hydrolysis of xylan
Expansin-like proteins	+	+	++	Cell wall softening or extension
Endo-1,3-β-glucanase	Unknown	Unknown	++	Hydrolysis of beta 1,3-glucan
Polygalacturonase	+	Unknown	++	Hydrolysis of alpha 1,4-D-galactosiduronic linkages
Arabinogalactan galactosidase/arabinase	+	+	++	Hydrolysis of pectin
Cellulose binding proteins	+	+	+++	Promote hydrolysis of crystalline cellulose
Protection from host defences				
Thioredoxin	+	Unknown	+	Detoxification of ROS
Peroxiredoxin	+	+	+++	Detoxification of ROS
Superoxide dismutase	+	+	++	Detoxification of ROS
Glutathione-S-transferase	+	+	+++	Detoxification of ROS
Glatathione synthetase	+	+	+	Detoxification of ROS
Glutathione peroxidase	+	+	+++	Detoxification of ROS
SPRYSEC-RBP-1	+	+	+++	Suppression of host defences
Sec-2/FAR	+	+	+++	Reduction in host defence response
Targeting regulation and signalling pathways				
Annexin	+	+	++	Protection of plant cells against stress
RANBP	Not present	+	+++	No known specific function in nematodes
14-3-3 and 14-3-3b	+	+	+++	No known specific function in nematodes
SKP-1	+	+	++	Involved in ubiquitination, signal transduction
Ubiquitin extension protein	+	+	+++	Involved in ubiquitination
Calnexin/Calreticulin	+	+	+++	Calcium spiking
Beta-galactoside binding lectin (Galectin)	Unknown	+	++	No functional data available for nematodes
Feeding				
Aminopeptidase	+	+	+	Protein digestion/degradation
Proteases (e.g. serine, cysteine)	+	+	++	Protein digestion/degradation
Initiation and maintenance of feeding site				
Auxin	+	+	Not identified	Involved in giant cell formation
Cytokinin	+	+	Not identified	Activation of cell cycle
Peptide hormones (CEPs)	+	Absent	Not identified	Involved in giant cell formation
CLE peptides	+	+	Not identified	Mimic plant CLEs, functional evidence available
16D10 CLE related peptide	+	Unknown	Not identified	Promotion of giant cell induction
Chorismate mutase	+	+	Unclear	Plant defence suppression, targeting SA pathway
NodL-like	+	Unknown	Not identified	No functional data available for nematodes
19C07	Unknown	+	Not identified	Modification of auxin influx in syncytium
10A06	Unclear	+	Not identified	Indirect induction of antioxidant genes in syncytium
7E12	Unknown	+	Not identified	No functional data available
Potential effectors with unknown functions				
Transthyretin-like proteins	+	+	+++	Unknown function
Vap-1	+	+	++	Unknown function
SXP-RAL2	+	+	++	Unknown function

+++, the gene has been identified in *Pratylenchus thornei*, *Pratylenchus coffeae* and *Pratylenchus zeae* transcriptomes; ++, the gene has been identified in transcriptomes of two of the three species: *Pratylenchus thornei*, *Pratylenchus coffeae* and *Pratylenchus zeae*; +, the gene has been identified in at least one species of the genus; unknown, the gene has not yet been identified in any species of the genus; unclear, the identity of the gene reported to known sequences is very low; CEPs, C-terminally encoded peptides; CLE, CLAVATA (CLV)-like element.

Expression of *Pt-Eng-1* and *Pt-PL* was detected in by semi-quantitative RT-PCRs, whereas, the other three genes, *Pt- CLP, Pt-UEP* and *Pt-GST* were not detected in eggs. The presence of transcripts for both β-1,4-endoglucanases is consistent with findings in *H. glycines*, *M.*

incognita, *M. javanica* and *R. similis* (Gao et al., 2004; Haegeman et al., 2008; Hu et al., 2013; Ledger et al., 2006; Rosso et al., 1999). Similarly, the presence of pectate lyase in eggs of *P. thornei agrees* with observations in *M. incognita*, G. *rostochiensis,* and *H. glycines* (Ali et al., 2015; Huang et al., 2005; Peng et al., 2016). Expression of these two genes probably reflects the presence of non-hatched J2s in some eggs such that the pre-parasitic J2s are primed for plant invasion and migration (Gao et al., 2004; Haegeman et al., 2008; Huang et al., 2005; Long et al., 2013; Roman and Hirschmann, 1969b). Conversely, failure to detect expression of the genes *Pt- CLP, Pt-UEP* and *Pt-GST* in eggs of *P. thornei* suggests that their expression is activated post egg emergence in the parasitic stages of the nematode, with involvement in detoxification of ROS, ubiquitination and protein digestion during plant invasion and feeding.

Soaking *P. thornei* in dsRNA of *Pt-Eng-1* significantly reduced transcript accumulation: this agrees with results for *G. rostochiensis*, *P. coffeae*, and *P. vulnus* (Chen et al., 2005b; Fanelli et al., 2014; Joseph et al., 2012). There were also long-term effects of soaking resulting in only 39% and 30% nematodes in soybean and alfalfa respectively, as compared to untreated controls. This reduction in reproduction was lesser than that reported in ds*Pv-eng-1* treated *P. vulnus* where a greater reduction in reproduction was found (Fanelli et al., 2014). However, it is important to note that there are a few major differences in the current study and the study on *P. vulnus* which may explain the difference in percentage of reproduction. These differences are: the infection was monitored on carrot discs in sterile/controlled environment versus soybean and alfalfa grown in pasteurised soil and maintained in a growth chamber; even though the two organisms belong to *Pratylenchus* genera, they are different species; although the concentration of dsRNA used was the same for the two studies, the sequence of endoglucanase itself that was targeted; finally, the number of days allowed for infection were higher in comparison to the current study.

Similar observations of transcript reduction accompanied by lesser reproduction in ds*Pt-CLP* treated *P. thornei* was seen in this study and this was in line with the observations on ds*Mi-cpl-1* treated *M. incognita* which, however, showed greater reduction in reproduction on roots of adzuki beans grown in growth pouches (Shingles et al., 2007). Interestingly, even though the number of nematodes and infection period was the same in the two studies, 50 nematodes/plant and 21 dpi, there was a difference in number of nematodes observed. This

can be perhaps due to difference in the concentrations of dsRNA used; 2 to 5 mg/mL of dsRNA corresponding to *Mi-cpl-1* whereas 1 mg/mL ds*Pt-CLP* used in the current study. Also, the species of PPN tested were different; sedentary versus migratory nematode used in this study which are known to move in and out of their host freely accounting for difference in nematode numbers. Additionally, the gene sequences itself in the two studies were different, and finally, the soil used in the current study allowed for the nematodes to mimic natural migration as opposed to being in a restricted area of a growth pouch used in the *M. incognita* study. It is possible that sedentary PPNs are more sensitive to down-regulation of vital gene because they are not limited to feed on a few cells.

In this study, *in vitro* RNAi of *Pt-UEP* resulted in lower transcript accumulation accompanied by 60% and 44% reproduction in soybean and alfalfa, respectively, as compared to controls. There is only one report of *in planta* RNAi of the ubiquitin carboxyl extension protein in *G. rostochiensis*, *GrUBCEP12,* in potato, which reduced nematode parasitism (Chronis et al., 2013). *In vitro* RNAi of *Pt-UEP* resulted in lower transcript accumulation and a reduction in reproduction of 60% and 44% in soybean and alfalfa, respectively. Interestingly the knockdown of *Pt-UEP* was not great as for *Pt-Eng-1*, *Pt-PL* and *Pt-CLP*, this was possibly a result of lower transcript abundance. One suggested role of UEP is to modulate plant immunity (Chronis et al., 2013; Jaouannet et al., 2013a; Postma et al., 2012). The lower transcript abundance *P. thornei* could be related to its migratory nature and intermittent feeding behaviour. However, more investigation into temporal and developmental expression of this gene in RKNs or CNs versus RLNs is required.

The transcript reduction of *Pt-PL* and *Pt-GST* was also accompanied by a reduction in nematode numbers 21 dpi in soybean and alfalfa. These observations were similar to those in ds*Hs-pel-6* treated *H. glycines,* ds*Hs-pel-2* treated *H. schachtii* and ds*Mi-gsts-1* treated *M. incognita* where nematode numbers were found to be reduced on soybean, *A. thaliana* and tomato roots, respectively (Dubreuil et al., 2007; Peng et al., 2016; Vanholme et al., 2007), although the reduction in nematode numbers was not as great.

An overall feature of the results found for *P. thornei* it appears that that nematode numbers at 21 dpi, compared to those in other studies for orthologues in other PPNs, is that reduction in

reproduction is less marked. As discussed this probably reflects differing hosts: carrot, soybean, adzuki beans or *A. thaliana* versus soybean and alfalfa used in this study.

6.5 *In planta* RNAi

In planta RNAi silencing of all five putative target effector genes resulted in a reduction of transcript accumulation and nematode numbers on axenic and non-axenic transgenic soybean and alfalfa hairy roots. For some genes, there was also a positive correlation between reduction in transcript and nematode numbers suggesting that higher levels of knockdown have a greater impact on nematode numbers. This study is, so far, the first study to demonstrate host-mediated RNAi of *P. thornei* putative parasitism effector genes in two plant species and has resulted in reduced nematode numbers. Through this study, it was possible to show that both hosts, soybean and alfalfa, can be successfully transformed to express *P. thornei* genes. The cultivars of both host species (wild-type or untransformed) were found to be susceptible to *P. thornei* infections in preliminary studies and chosen for RNAi studies. The successful reduction in nematode numbers on transformed soybean and alfalfa that were susceptible to *P. thornei* infections provides an opportunity to transform partially-resistant cultivars thus enhancing their resistance.

Remarkably, amongst the five genes assessed, axenically produced transgenic hairy roots of soybean and alfalfa, and non-axenically produced soybean hairy roots expressing *Pt-Eng-1*, *Pt-PL* and *Pt-CLP* showed the lowest nematode numbers as compared to controls indicating their suitability as targets in future transgenic crop development. The success of *Pt-Eng*-1, *Pt-PL* and *Pt-CLP* in host-mediated RNAi found in this study was similar to that reported in transgenic tobacco expressing *Mj-Eng-3* of *M. javanica* and *Mi-Cpl-1* of *M. incognita*, and soybean expressing *Hg-pel-6* of *H. glycines* where reduction in transcript levels was accompanied by reduced nematode numbers and egg or galls (de Souza Júnior et al., 2013; Hu et al., 2013; Peng et al., 2016). As there was limited information available on GSTs as *in planta* RNAi targets for PPNs, it is difficult to compare or contrast the results obtained in this study. Finally, for *Pt-UEP*, even though the reduction in transcript accumulation or reproduction was not as high as *Pt-Eng-1*, *Pt-PL* and *Pt-CLP*, reduction in target mRNA expression and average number of

females per root system has been reported in potato expressing *GrUBCEP12* of *G. rostochiensis* (Chronis et al., 2013).

Composite plants provided a good opportunity to test target genes under more natural infection conditions, this is the first application of this system to study the effects of *in planta* RNAi on *Pratylenchus* species. The concern of including non-transformed chimeric hairy roots in quantitative assessments, can be overcome using selectable marker or reporter genes, ensuring only transformed roots are studied (Ibrahim et al., 2011). The composite soybean system will also enable research to study long-distance silencing in the future. The spread of gene silencing triggers from root to shoot using reporter genes or synthetic hairpin RNA transgenes has been demonstrated in tobacco and Arabidopsis, where root stock-to-scion a GFP silencing system was used (Brosnan et al., 2007; Kalantidis et al., 2008; Mlotshwa et al., 2002). It may be possible to transform hairy roots with a construct containing nematode and insect resistance genes to assess the spread of the silencing signals from roots to the shoots, and this would enable a new opportunity to protect plants from two pests at the same time.

Based on the densitometric analysis of semi-quantitative PCR results and reduction in nematode population due to *in vitro* and *in planta* RNAi silencing of five target *P. thornei* effector genes is summarised below (Table 6.2).

6.6 Benefits of RNAi-based resistance

The advantages of using RNAi for PPN include: 1) choice of target gene specificity to only the pathogen/pest, with minimal or no sequence homology to plants, animals, beneficial insects, as well as free-living nematodes; 2) the possibility of silencing gene families with a single sequence by use of conserved region within the targets (Gupta et al., 2013; Sinha and Rajam, 2013); 3) In comparison to traditional plant breeding and multigenic resistance transfer, the RNAi-based strategy could be used to create a single resistant locus which would be relatively easy to transfer across cultivars. 4) RNAi may also offer resistance that is stable and durable based on the target gene, its sequence and its homology among PPN populations.

Table 6.2. An overview of performance of *P. thornei* target effector genes based on percent reduction of target transcripts and reduction in nematode numbers after *in vitro* and *in planta* RNAi.

Gene name	*In vitro* RNAi			*In planta* RNAi					
	Percent transcript reduction†	Percent reduction in *P. thornei* numbers		Soybean*		Alfalfa*		Soybean**	
		Soybean	Alfalfa	Percent transcript reduction†	Percent reduction in *P. thornei* numbers	Percent transcript reduction†	Percent reduction in *P. thornei* numbers	Percent transcript reduction†	Percent reduction in *P. thornei* numbers
Pt-Eng-1	66.4	61.1	70.0	60.8	59.0	59.6	75.0	61.2	53.4
Pt-PL	59.2	58.1	67.3	57.7	56.0	55.5	74.0	55.2	48.5
Pt-CLP	55.2	51.0	63.0	55.6	50.0	61.0	71.0		
Pt-UEP	53.4	40.3	55.5	50.7	38.0	35.1	60.0		
Pt-GST	35.4	45.0	56.4	42.5	40.0	32.4	63.5		

* and ** indicate axenic and non-axenic (composite) hosts whereas, † indicates percentage of reduction of *P. thornei* target genes relative to 18s rRNA at 25 PCR cycles.

5) In RNAi tolerance developed in the pest population, the RNAi locus can be engineered to target multiple genes (Li et al., 2011a; Tamilarasan, 2013); 6) The use of a wound inducible promoter could allow activation of RNAi at the specific points of PPN entry into plant, to limit the effects of RNAi to the root-invading PPNs (Xue et al., 2012); 7) RNAi does not encode a functional protein, unlike the highly specific transgenic β-endotoxins of *Bacillus thuringiensis* (Bt) which, may have non-target effect during its use for controlling many insect pests in a number of crops (Bakhetia et al., 2005b).

The classical approach of breeding for resistance against insect pests and pathogens is time-consuming with an increase in complexity for addition of extra traits (Younis et al., 2014). The most widely used biotechnological approach hasly relied on the use of a Bt-toxin gene: transgene encoded RNAi s to control invading pests can be chieved faster and may be equally efficient. The use of Bt genes is limited to certain pests and crops, and there is also evidence for resistance development, whereas RNAi offers a robust and a more selective pathway in the battle against various destructive pests and diseases (Rajamohan et al., 1998; Vaughn et al., 2005; Younis et al., 2014).

6.7 Translation of RNAi technology to practice

The application of RNAi technology in crop protection shows great promise, but its full potential compared to conventional breeding remains to be realised. Nevertheless, there are some excellent examples that demonstrate the success of RNAi-based crop protection. For example, RNAi can impart disease resistance against fungal and bacterial diseases: examples include the overexpression of a fatty acid metabolism gene AtFAAH in Arabidopsis alters phytohormone signalling and is involved in the plant defence pathway against certain flagellate bacterial pathogens (Kang et al., 2008). Similarly, RNAi knockdown of the OsSSI2 gene involved in fatty acid desaturase activity increases resistance against the bacterial pathogen of leaf blight (*Xanthomonas oryzae* pv. *Oryzae*) and the blast fungus (*Magnaporthe grisea*) (Jiang et al., 2009). RNAi silencing of *A. tumefaciens ipt* and *iaam* genes for cytokinin and auxin synthesis using their respective

inverted repeats is effective against the crown gall disease in *Arabidopsis*, Nicotiana and *Lycopersicon* species. Host-induced gene silencing of the wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) fungal pathogen, *Blumeria graminis*, through a transient gene expression resistant to RNAi due to silent point mutations limits the disease potential of the pathogen (Escobar et al., 2001). RNAi has also been used successfully to control Plum pox virus (PPV) of woody perennials by introducing the PPV coat protein gene in the host plants (Ding, 2010; Ding and Voinnet, 2007; Scorza et al., 2001), similarly, many viral plant diseases, such as the cassava brown streak disease in cassava and the bean golden mosaic virus in bean have been controlled using an anti-sense or hairpin RNAi constructs (Sudarshana et al., 2007). RNAi silencing of CYP6AE14 cotton bollworm (*Helicoverpa armigera*) reduces the insect population, as is alos the case for RNAi of the glutathione S-transferase (GST1) gene in the herbivorous insects (Mao et al., 2007). Similarly, western corn rootworm, pea aphid (*Acyrthosiphon pisum*), green peach aphid (*Myzus persicae*) and many PPNs have been shown to be controlled using host-mediated and *in vitro* RNAi approaches (Mutti et al., 2006; Pitino et al., 2011).

In addition to offering resistance to pests and associated diseases, RNAi has been used for crop quality and trait improvement (Scheurer and Sonnewald, 2009; Tang et al., 2007;) such as enhanced nutrient content in wheat (lysine) and rice (lysine and lower glutelin) (Kusaba et al., 2003), tomato (lycopene, carotene) (Davuluri et al., 2005), cotton seed (high-stearic, high-oleic contents and low gossypol) (Liu et al., 2002; Sunilkumar et al., 2006), rapeseed (carotene, zeaxanthin, violaxanthin and lutein) (Yu et al., 2008), wheat (Regina et al., 2006), corn (Houmard et al., 2007) and sweet potato (amylose) (Shimada et al., 2006). It has also been used to reduce the alkaloid content in coffee (decaffeination) (Ogita et al., 2003), morphine to non-narcotic reticuline in opium poppy (Allen et al., 2004), key carcinogens in tobacco (Lewis et al., 2008), allergenicity in tomato (Le et al., 2006), apple (Gilissen et al., 2005), peanuts and ryegrass (Ali et al., 2010; Younis et al., 2014). Similarly, RNAi has also been used to increase ethylene sensitivity in tomato for longer shelf-life (Xiong et al., 2005) and delayed ripening (Meli et al., 2010) for introducing male sterility in tobacco and tomato in hybrid seed industry; drought stress tolerance;

salt stress tolerance; cold and heat stress tolerance; UV-B radiation stress tolerance; mechanical stress tolerance (Ali et al., 2010; Younis et al., 2014).

It is quite clear from the above examples that RNAi is a powerful approach that can be applied in various fields of plant agricultural research. Additionally, its commercial application in some crops is an indication of growing acceptance. The earliest record of a commercialised RNAi-based crop was in 1994 and was the introduction of the Flavr Savr® tomato that was designed for increased storage life through the suppression of the tomato polygalacturonase gene. Another example is the transgenic papaya expressing the papaya ring spot coat protein gene that was first commercialised in Hawaii, USA, in 1998. More recently, the United States Department of Agriculture (USDA) approved non-browning Arctic®Golden and Arctic®Granny apples developed by Okanagan Specialty Fruits Inc., Canada, for the consumer market in 2015. Similarly, Monsanto's SmartStax Pro corn encoding DvSnf7 dsRNA against the western corn rootworm and Simplot's generation 1 and 2 Innate® non-bruising potatoes with improved starch and colour quality in addition to reduced acrylamide were also approved for consumption in 2016-17. In Australia, the Commonwealth Scientific and Industrial Research (CSIRO) has demonstrated successful examples of the application of RNAi for improved wheat breeding, development of cereals resistant to barley yellow dwarf virus, trans fatty-acid-free cottonseed oil, high oleic acid safflower seed oil for industry purposes and also in aquaculture research. Currently, the CSIRO's licensees for RNAi technology are developing rice resistant to rice stripe virus in China and new corn varieties with increased resistance to western corn rootworm.

There are also several global examples of successful applications of RNAi in medicine thus making it a powerful tool beyond the field of plant agriculture. CSIRO, for example, is working towards improving antibodies to treat human diseases such as Non-Hodgkin's lymphoma and inflammatory arthritic conditions, and silencing the reproductive ability of the Hepatitis B virus. There are also reports of successful clinical trials using RNAi in other parts of the world, in the United States, for example, pharmaceutical companies like Alnylam, Arrowhead, Dicerna and Arbutus Biopharma Corporation (previously known as Tekmira Pharmaceuticals) are developing RNAi-based drugs for

liver diseases including hepatitis, haemophilia, cancer, blood cholesterols, Ebola virus diseases. Similarly, in Israel, SilenSeed Ltd. is developing treatment for RNAi-based cancer drugs and delivery systems to effectively penetrate and treat malignant solid tumours to treat pancreatic, prostate and brain cancers. Such examples of RNAi-based approaches to treat human diseases, improve crops for human consumption and making agriculture sustainable provide a very promising future for improving the quality of food and health of humankind.

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Appendix 1. Comprehensive list of plant parasitic effector genes and proteins from PPNs used for comparative bioinformatics in this research.

No.	Known or putative effector	NCBI Accession number	Description	Reference
1	10A06	ACU12489	Heterodera schachtii 10A06 effector protein isoform 1	Haegeman 2011
2	14-3-3b	AAL40719	Meloidogyne incognita 14-3-3 product	
3	16D10	Q06JG6	Meloidogyne javanica Clavat3/ESR (CLE)-related protein 16D10	
4	19C07	AAO85458	Heterodera glycines putative gland protein G19C07	
5	7E12	AAQ10021	Meloidogyne incognita putative oesophagus gland cell secretory protein 7 (msp7)	
6*	Acid phosphatase	AAN08587	Meloidogyne incognita putative oesophagus gland cell secretory protein 21	
7	Annexin	AAN32888	Heterodera glycines annexin 4C10	
8	Calreticulin	AAL40720	Meloidogyne incognita calreticulin	
9*	Chitinase	AAN14978	Heterodera glycines chitinase	
10*	Chorismate mutase	ABB02655	Meloidogyne arenaria chorismate mutase 1	
11	CLE peptide	AAO33474	Heterodera glycines gland-specific protein g4g12	
12	ERp99	AAG21337	Heterodera glycines hypothetical oesophagus gland cell secretory protein 8 (hsp8)	

13	Galectin	AAB61596	Globodera rostochiensis galectin	
14*	Glutathione peroxidase	CAD38523	Globodera rostochiensis secreted glutathione peroxidase (Gpx1 gene)	
15*	Glutathione S-transferase	ABN64198	Meloidogyne incognita glutathione S-transferase-1	
16	Map-1	CAC27774	Meloidogyne incognita putative avirulence protein	
17*	Peroxiredoxin	CAB48391	Globodera rostochiensis peroxiredoxin (Tpx)	
18	SPRYSEC RBP-1	AM491356	Globodera pallida RBP-1 protein (Rbp-1)	
19	RING-H2 zinc finger protein	AAP30834	Heterodera glycines putative gland protein G10A06	
20	SEC-2	CAA70477	Globodera pallida mRNA for putative SEC-2 protein	
21	SKP1-like protein	AAP30763	Heterodera glycines putative gland protein G8H07	
22	SXP/RAL-2	CAB75701	Globodera rostochiensis putative hypodermis secreted protein (Sxp1 gene)	
23	Transthyretin-like protein	CAM84510	Radopholus similis mRNA for transthyretin-like protein 1 (Ttl-1 gene)	
24*	Ubiquitin extension protein	AAO33478	Heterodera glycines ubiquitin extension protein	
25	Venom allergen protein	AAD01511	Meloidogyne incognita secreted protein MSP-1	

26	SXP/RAL	CAB75701	Globodera rostochiensis putative hypodermis secreted protein (Sxp1 gene)	Abad, 2008
27	SXP/RAL	CAB66341	Globodera rostochiensis putative amphid protein (Ams1)	
28	Expansin	CAC83611	Globodera rostochiensis EXPB1 protein	
29	VAP-1	AAK60209	Heterodera glycines VAP1 protein (Vap-1)	
30	VAP	AAK55116	Heterodera glycines secreted venom allergen-like protein (Vap-2)	
31	14-3-3b	AAR85527	Meloidogyne incognita 14-3-3b protein	
32*	Cysteine proteases	CAD89795	Meloidogyne incognita putative cathepsin L protease (Cpl-1 gene)	
33	RAN-BP-like	CAC21849	Globodera rostochiensis hypothetical partial protein	
34	RAN-BP-like	CAC21848	Globodera rostochiensis hypothetical protein	
35*	RAN-BP-like	AAV34698	Globodera pallida IC5	
36	Ubiquitin extension proteins	AAP30081	Heterodera schachtii ubiquitin extension protein	
37*	Chrosimate mutase	AAO19577	Heterodera glycines chorismate mutase (Cm-1 gene)	
38*	Chrosimate mutase	AAD42163	Meloidogyne javanica chorismate mutase (NC30 gene)	
39*	Chrosimate mutase	CAD29887	Globodera pallida putative chorismate mutase (Cm-1 gene)	

40	CLE	AAG21331	Heterodera glycines hypothetical oesophagus gland cell secretory protein 1 (Hsp1 gene)	
41	NodL-like	AW570655	Meloidogyne nodulation protein (EST sequence)	
42	Transthyretin-like protein precursor	CB374976	Heterodera glycines transthyretin-like protein precursor (EST sequence)	Nicol, 2012
43*	GHF5 beta-1,4-endoglucanase	Q9UA57	Meloidogyne incognita beta-1,4-endoglucanase (eng-1)	Haegeman 2013
44*	GHF45 beta-1,4-endoglucanase	AFG30029	Bursaphelenchus xylophilus GHF45 family protein	
45*	Beta-1,3-endoglucanase	BAE48357	Bursaphelenchus xylophilus Bx-13g-1 mRNA for beta-1,3-endoglucanase,	
46	Cellulose binding protein	A2VBB1	Meloidogyne arenaria cellulose binding protein precursor (Cbp-1 gene)	
47*	Pectate lyase	AAQ09004	Meloidogyne incognita pectate lyase	
48*	Arabinogalactan endo-1,4-beat-galactosidase	ACY02855	Heterodera schachtii ararbinogalactan endo-1,4-beta-galactosidase	
49*	Polygalacturonase	AAM28240	Meloidogyne incognita polygalacturonase	
50*	Xylanase	AAF37276	Meloidogyne incognita xylanase (Xyl-1 gene)	
51	Expansin	ADX36366	Meloidogyne javanica expansin B (EXPB gene)	
52*	30C02	AAP30836	Heterodera glycines putative gland protein G30C02	

53	VAP (venom allergen proteins)	CAD60978	Globodera rostochiensis VAP1 protein	
54	Mj-NULG1a	AFB73917	Meloidogyne javanica secretory protein	
55	C-type lectin	AAM18623	Heterodera glycines C-type lectin domain protein (Ctl-1 gene)	
56	2E07	AAQ10015	Meloidogyne incognita putative oesophagus gland cell secretory protein 1 (msp1)	
57	2G02	AAQ10016	Meloidogyne incognita putative oesophagus gland cell secretory protein 2 (msp2)	
58	2G10	AAN15807	Meloidogyne incognita putative oesophagus gland cell secretory protein 27 (msp27)	
59	4D01	AAQ10017	Meloidogyne incognita putative oesophagus gland cell secretory protein 3 (msp3)	
60	4D03	AAN15808	Meloidogyne incognita putative oesophagus gland cell secretory protein 28 (msp28)	
61	5G05	AAN15806	Meloidogyne incognita putative oesophagus gland cell secretory protein 26 (msp26)	
62	6F06	AAQ10018	Meloidogyne incognita putative oesophagus gland cell secretory protein 4 (msp4)	

63	6G07	AAQ10019	Meloidogyne incognita putative oesophagus gland cell secretory protein 5 (msp5)	
64	7A01	AAQ10020	Meloidogyne incognita putative oesophagus gland cell secretory protein 6 (msp6)	
65	7H08	AAQ1002	Meloidogyne incognita putative oesophagus gland cell secretory protein 8 (msp8)	
66	8D05	AAQ10024	Meloidogyne incognita putative oesophagus gland cell secretory protein 9 (msp9)	
67	8H11	AAQ10025	Meloidogyne incognita putative oesophagus gland cell secretory protein 10 (msp10)	
68	9H10	AAQ10022	Meloidogyne incognita putative oesophagus gland cell secretory protein 11 (msp11)	
69	10A08	AAN52091	Meloidogyne incognita putative oesophagus gland cell secretory protein 34 (msp34)	
70	10G02	AAN15809	Meloidogyne incognita putative oesophagus gland cell secretory protein 29 (msp29)	
71	11A01	AAN08578	Meloidogyne incognita putative oesophagus gland cell secretory protein 12 (msp12)	

72	12H03	AAN08579	Meloidogyne incognita putative oesophagus gland cell secretory protein 13 (msp13)	
73	13A12	AAN08580	Meloidogyne incognita putative oesophagus gland cell secretory protein 14 (msp14)	
74	14E07	AAN08581	Meloidogyne incognita putative oesophagus gland cell secretory protein 15 (msp15)	
75	16E05	AAN08583	Meloidogyne incognita putative oesophagus gland cell secretory protein 17 (msp17)	
76	17H02	AAN08584	Meloidogyne incognita putative oesophagus gland cell secretory protein 18 (msp18)	
77	19F07	AAN52090	Meloidogyne incognita putative oesophagus gland cell secretory protein 32 (msp32)	
78	21E02	AAN08585	Meloidogyne incognita putative oesophagus gland cell secretory protein 19 (msp19)	
79	25B10	AAN52092	Meloidogyne incognita putative oesophagus gland cell secretory protein 33 (msp33)	
80	28B04	AAN52093	Meloidogyne incognita putative oesophagus gland cell secretory protein 35 (msp35)	

81	30G11	AAN08587	Meloidogyne incognita putative oesophagus gland cell secretory protein 21 (msp21)	
82	30H07	AAN08586	Meloidogyne incognita putative oesophagus gland cell secretory protein 20 (msp20)	
83	31H06	AAN08588	Meloidogyne incognita putative oesophagus gland cell secretory protein 22 (msp22)	
84	34D01	AAN08589	Meloidogyne incognita putative oesophagus gland cell secretory protein 23 (msp23)	
85	34F06	AAN08590	Meloidogyne incognita putative oesophagus gland cell secretory protein 24 (msp24)	
86	35A02	AAN08591	Meloidogyne incognita putative oesophagus gland cell secretory protein 25 (msp25)	
87	35E04	AAN52095	Meloidogyne incognita putative oesophagus gland cell secretory protein 31 (msp31)	
88	35F03	AAN52094	Meloidogyne incognita putative oesophagus gland cell secretory protein 30 (msp30)	

Appendix 2. List of virtual amino acid sequences of *Pt-Eng-1*, *Pt-CLP*, *Pt-PL* and *Pt-GST*, and back-translated sequence of *Pt-Eng-1*.

> Pt-Eng-1 amino acid sequence based on 5' and 3' RACE PCRs (465 aa)

SSSSSHPLPFTQFNFNDTSTTMAAPQLLALFXFISLQFAXSVFGADXPYGALKVSGTQVVGSGGQAVVLRGMS
LFWXXFXEGSPFYNAETVKALKCXXNXXVXRAXMGVEEGSGYLSNSGTQMALXEAVIQAAIDQGIYVIVDWH
DHNGQNHQSXXIEFFKKIATKYGSYPHIIYETFNEPLQVGWSGVKSYHQAVVAAIRAIDPDNLIIMGTTTWSQD
VDTASQDKVSGSNLCYTXHYYAASHKQELRNKAQTALNNGACVFVTEYGTXDASGGGGVDTTSSNEWWTW
LESKKISYVNWAVDAKSEGSAALVPGTGSSQVGSDSVLTASGKLXXXXLKSKNNGVSCSGSSATTTTTARTTTRA
GQTTTTTKAGQTTTTTKASSSGGGGVSVSASAQVXSTWNGGGQFKLVVKNNGSKPVCKVTVRINTASGQTIT
XIXNASGSXGQYELASWLNIAPGASFQDTGFXVSGSTSTPSVSVVSASAC

>*Pt-Eng-1* nucleotide sequence

TCATCATCATCATCACATCCACTTCCATTCACACAATTCAATTTCAATGATACATCAACAACAATGGCTGCT
CCACAACTTCTTGCTCTTTTCNNNTTCATTTCACTTCAATTCGCTNNNTCAGTTTTCGGAGCTGATNNNCC
ATATGGAGCTCTTAAAGTTTCAGGAACACAAGTTGTTGGATCAGGAGGACAAGCTGTTGTTCTTAGAGGA
ATGTCACTTTTCTGGNNNNNNTTCNNNGAAGGATCACCATTCTATAATGCTGAAACAGTTAAAGCTCTTA
AATGTNNNNNNAATNNNNNNGTTNNNAGAGCTNNNATGGGAGTTGAAGAAGGATCAGGATATCTTTC
AAATTCAGGAACACAAATGGCTCTTNNNGAAGCTGTTATTCAAGCTGCTATTGATCAAGGAATTTATGTT
ATTGTTGATTGGCATGATCATAATGGACAAAATCATCAATCANNNNNNATTGAATTCTTCAAAAAAATTG
CTACAAAATATGGATCATATCCACATATTATTTATGAAACATTCAATGAACCACTTCAAGTTGGATGGTCA
GGAGTTAAATCATATCATCAAGCTGTTGTTGCTGCTATTAGAGCTATTGATCCAGATAATCTTATTATTATG
GGAACAACAACATGGTCACAAGATGTTGATACAGCTTCACAAGATAAAGTTTCAGGATCAAATCTTTGTTA
TACANNNCATTATTATGCTGCTTCACATAAACAAGAACTTAGAAATAAAGCTCAAACAGCTCTTAATAATG
GAGCTTGTGTTTTCGTTACAGAATATGGAACANNNGATGCTTCAGGAGGAGGAGGAGTTGATACAACAT
CATCAAATGAATGGTGGACATGGCTTGAATCAAAAAAAATTTCATATGTTAATTGGGCTGTTGATGCTAAA
TCAGAAGGATCAGCTGCTCTTGTTCCAGGAACAGGATCATCACAAGTTGGATCAGATTCAGTTCTTACAG
CTTCAGGAAAACTTNNNNNNNNNNNNCTTAAATCAAAAAATAATGGAGTTTCATGTTCAGGATCATCAG
CTACAACAACAACAACAGCTAGAACA

>3'Pt-CLP (189 aa)

XXXXXXXXXNGXPAIGLXXXTKPDGLXXXXXNDGCEGGLMXXXXKYVKDNKGIDTEKSYPYKAEDSKKCYFKRK
NVGATDTGYVDIPQGDEEKLKLAVATXGPISVXIDXXHDSFQSYQSGVYYEEECSAEELDHGVLVVGYGTXXXX
GDYWIVKNSWSKSWGMDXXXLMSRNKDNNCXIASAASYPLA

> 5'Pt-PL (177 aa) (yellow highlight indicates two unknowns, X, replaced by L and I, respectively, based on the consensus of top five BLASTP hits).

MLHSNLATTNCIVPVLLFISLLQLDVVRCEFWPKSKGADITVDVSITVNASQTFDCNYTRYIPNPDKLGDGSQAE
RQKAVFILLDGATLKNCIIGAKAGAAGSADGVHCKGAGCTVKNVWFENVGEDAVTFYXLSSDSITYTVDGGGA

RNSEDKIFQFDGKGTAYIKNFWADTFARFARSCGNCKNQYERHLVLSNVTALNGASGQFIAGINTNYGDSATL
TGIKLGGSTAKKVNXXKXFIGVTSGESXXXXXXADGVYCIYKSSEIXXXXXXXXXX

>3’Pt-GST (179 aa)
XXXXXXXXXXHXXEXWXXKXXSSSTXXXXEXXXKQLXQXXXXCQYLGKKFGLAGKDDWEEAKASEVADFLKDM
GNQLFPYIAVKRGFRQGDAEKLRTEEFLPGVDKHFPIAEKLLADSGSGFMLPSGLSYVDFMFIQFLLMIKSADA
DVFAKHTKLVDYIDRVXGLPQLKEYVKSSPLA