Characterising putative parasitism genes for root lesion nematodes and their use in RNA interference studies

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University 2015
Declaration

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

Jo-Anne Chiew Hwa, TAN
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Abstract

Root lesion nematodes (RLNs, *Pratylenchus* spp.) are economically important migratory endoparasitic pests of crop plants. The overall aim of the work in this thesis was to undertake molecular studies on *Pratylenchus thornei* to characterise genes potentially involved in plant parasitism, and to determine if they are amenable to gene silencing (RNA interference). The recent availability of the transcriptomes of three RLN species, *Pratylenchus coffeae*, *P. thornei* and *P. zeae* significantly expanded the resources available for this study. Amongst the transcriptome data, putative parasitism genes (PPGs) were identified. A common assembly platform was also used to analyse transcriptome data to determine whether differences between PPGs identified here and those reported previously were consistent when the same assembly was used. Bioinformatic analysis was also undertaken to compare PPGs between different *Pratylenchus* spp. The results showed that there were some differences in the PPGs identified in *P. coffeae*, *P. thornei* and *P. zeae*. From the transcriptome of *P. thornei*, genes encoding potential protein or peptide effectors were identified and three aspects of plant parasitism were studied – cell wall modifying proteins (CWMPs) needed to overcome the physical barrier of cell walls in host plant roots, proteins required for detoxification of reactive oxygen species produced as a host defence response, and those thought to have other roles in parasitism. There was no information available on whether RLNs would take up dsRNA by ‘soaking’, and so conditions for optimisation of dsRNA uptake were established using *P. thornei* and *P. zeae*. Using these conditions, down-regulation of target genes in *P. thornei* by RNAi was studied. Mixed stage populations of nematodes of *P. thornei* and *P. zeae* were induced to ingest dsRNA when incubated in a basic soaking solution (M9 buffer, 0.05% gelatine, 3 mM spermidine) in the presence of the 10-50 mM neurostimulant octopamine, 0.1-1.0 mg/mL FITC, and 0.5-6 mM spermidine for 16h. Under these conditions, nematodes vitality was not affected. Knockdown of *pat-10* and *unc-87* genes of *P. thornei* and *P. zeae* resulted in aberrant movement in both species, more so in *P. thornei*. A greater reduction of transcript expression of both genes in *P. thornei* also indicates that it may be more susceptible to RNAi than *P. zeae*. *P. thornei* treated with dsRNA of *pat-10* and *unc-87* have reduced replication in mini carrot discs, showing an 81% and 77% decrease in numbers of nematodes harvested after 5 weeks culture following RNAi. Hairy roots generated with constructs containing *Ptpat-10* also resulted in significant decrease in *P. thornei* reproduction. Additionally, dsRNA constructs of either nematode species elicited RNAi
effects in both species indicating a possible cross-species control of nematodes via RNAi. After soaking mixed stages of *P. thornei* in dsRNA for 16 hr and transfer to carrot mini-discs for 5 weeks, down-regulation of genes encoding CWMPs significantly decreased nematode reproduction by between 86-95%, respectively, showing that secretion of cellulase, pectate lyase and polygalacturonase are required for *P. thornei* to infect host plants effectively. Similarly, reproduction of *P. thornei* was also reduced when RNAi was initiated against cathepsins, fatty acid and retinol binding protein, peroxiredoxin, transthretin-like protein and venom allergen-like protein (46-88%, respectively). However, RNAi did not reduce replication of *P. thornei* when RNAi was directed against galectin and thioredoxin. These results show that RLNs are amenable to RNAi, and indicate that the efficiency of RNAi-based gene silencing can differ depending on the target gene chosen, whether it is a member of a multi-gene family, the specific sequence chosen and also details of experimental treatments. This research described here also contributes to describing genes needed for entry into and migration in host roots and evading host defences. However, genes encoding effectors required by sedentary endoparasitic nematodes for feeding site formation were not found in RLNs.
## Abbreviations

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<th>Abbreviation</th>
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<td>APN</td>
<td>Animal parasitic nematode</td>
</tr>
<tr>
<td>bp</td>
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<td>Cyst nematodes</td>
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Chapter 1 : Literature Review
1.0 Introduction

Plant parasitic nematodes (PPNs) are acknowledged as one of the greatest threats to crops worldwide, having been found to attack almost every part of the plant including roots, stems, fruits and seeds. To be such successful pests they have to be able to find host tissues, migrate within them, suppress and evade host defences, and for sedentary endoparasites, modify development of host cells to form specific feeding sites. It is now well established that secretions produced by the glands of these pests are responsible for the dramatic modification of specific feeding cells in the host plants (Haegeman et al., 2012, Jones et al., 2013, Mitchum et al., 2013). Elucidation of the transcriptomes of these pests with the aim of isolating nematode effectors synthesised in gland cells is a promising avenue to understanding nematode parasitism and its evolutionary origins, in addition to developing nematode control strategies.

Traditional approaches such as cultural practices, applications of nematicides and natural resistance genes have been deployed to control these pests but none of these approaches is fully effective. RNA interference (RNAi) has shown to be an apt biotechnological strategy to confer host resistance to phytonematodes. The initial application of RNAi concentrated on control of sedentary endoparasitic nematodes, and study of the migratory nematodes i.e. Pratylenchus spp. were neglected. Hence, this review focuses on nematode effectors which have been or could be secreted by Pratylenchus spp. and the potential of RNAi as a control strategy against these genes of nematode pests.

1.1 Nematodes

Nematodes are unsegmented roundworms that range from 80 µm to 10 m in length (Maggenti, 1981). They constitute one of the most ubiquitous phyla of organisms, having successfully adapted to nearly every niche in terrestrial, aquatic and marine environments (Bird and Koltai, 2000). The number of species in the phylum Nematoda has been estimated to vary from 40, 000 to 100 million (Lambshead, 1993, Dorris et al., 1999). Although they possess very similar body design, the genetic diversity among nematodes is high, which is displayed in their physiological flexibility. Most nematode diversity is represented by species that are free-living such as fungivores, omnivores, microvores and predators, while a comparatively small fraction constitutes parasites of animals and plants.

Although presence of nematodes can influence humans both economically and in relation to health, they are not widely understood. Animal parasitic nematodes can infest livestock and companion
animals, and cause exotic human diseases such as ascariasis and elephantiasis (Baird et al., 1986). In addition, PPNs cause substantial crop losses, estimated at $157 billion per annum globally (Abad et al., 2008). In Australia, losses from phytonematodes amount to at least AUD$600 million per annum (Hodda and Cook, 2009, Murray and Brennan, 2009).

### 1.1.1 Plant parasitic nematodes

Nematodes have been found in fossilised plant material from as early as 400 million years ago. Molecular and paleontology studies have shown that terrestrial plants originated between 425 and 490 million years ago, this implies that plant parasitism occurred quite early in plant evolution (Poinar et al., 2008). However, based on genomic sequence data, it now seems that plant parasitism by nematodes evolved at least three times independently: twice in the Enoplea class (orders Triplonchida and Dorylaimida) and once in the Chromadorea class (order Rhabditida) (Blaxter et al., 1998, De Ley and Blaxter, 2002) (Fig.1.1).

Plant nematodes have evolved morphologically to facilitate parasitism. All phytonematodes have a needle-like protrusible stylet through which they can withdraw nutrients from plant cell cytoplasm and also secrete proteins into host tissues. Stylet types differ for the independently evolved nematode groups (Triplonchida = onchiostylet; Dorylaimida = odontostylet; Tylenchida and Aphelenchida = stomatostylet). Other morphological adjustments to plant parasitism include the pharynx structure and pharyngeal gland cells, dorsal and subventral. These gland cells are essential for the production of the proteins and other molecules which are secreted through the stylet (Vanholme et al., 2004, Haegeman et al., 2012).
Figure 1.1 Phylogenetic relationships between nematode species, modified from Mitreva et al. (2005). Dark blue: animal parasitic nematodes; turquoise: free living nematodes; light blue: plant parasitic nematodes. Note: tree not drawn to scale.

Phytomonematodes can feed from cells of all parts of the plant including roots, stems, leaves, flowers, tubers and seeds. They specialise either in migratory ectoparasitism, burrowing endoparasitism or various types of independently evolved sedentary ecto- and endoparasitism and a full range of intermediates (Baldwin et al., 2004). Ectoparasites remain outside the plant and use their stylet to perforate plant cells. Some e.g. Belonolaimus and Longidorus spp. can have extremely long stylets, which aid them in feeding deep within the plant root on nutrient-rich cells (Cohn, 1970, Huang and Becker, 1997).

On the other hand, migratory endoparasites move through root tissues and massive tissue apoptosis can occur as a result of their feeding and migration. In contrast, obligate sedentary parasites can transform plant cells into discrete feeding cells (e.g. giant cells or syncytia) from which they feed throughout their life cycle (Bird, 1961, Jones and Northcote, 1972, Jones, 1981).

Nematodes that feed as semi-endoparasites such as Rotylenchus reniformis are able to penetrate the plant partially and develop feeding cells at some stage of their life cycle. Usually the
anterior part of the nematode penetrates the root, while the posterior part remains in the soil. In this project, the nematodes of interest are the root lesion nematodes (RLNs) *Pratylenchus thornei* and *Pratylenchus zeae*, which are migratory endoparasites. These are economically important migratory endoparasites that attack wheat and sugarcane respectively in Australia.

### 1.1.2 Root lesion nematodes (RLNs)

*Pratylenchus* spp. are regarded as the third most agriculturally important PPNs, after root-knot nematodes (RKNs) and cyst nematodes (CNs) (Jatala and Bridge, 1990, Jones et al., 2013). Like RKNs and CNs, *Pratylenchus* spp. is polyphagous. They can reduce the quality and quantity of marketable crops such as wheat, barley, sugarcane and many dicotyledons such as chickpeas and carrots. RLNs are migratory endoparasites of subterranean plant parts (roots, tubers, pods), in contrast to the more highly studied RKNs and CNs which are sedentary. However, *Pratylenchus* spp. can also reproduce and develop in the soil when the host plants are ageing, sick or stressed, or when their host plants have been harvested and the soil tilled up after harvest (ectoparasitic) (Zunke, 1990a). *Pratylenchus* spp. are widespread and endemic in tropical and temperate environments worldwide, encompassing 45-89 nominal species (Siddiqi, 2000, Ryss, 2002, Castillo and Vovlas, 2007). *Pratylenchus thornei* together with *P. zeae*, *P. vulnus*, *P. neglectus*, *P. coffeae* and *P. penetrans* are considered major species of *Pratylenchus* genus on the basis of their worldwide geographical distribution and very large host range (Jones and Fosu-Nyarko, 2014).

#### 1.1.2.1 *Pratylenchus thornei* and *Pratylenchus zeae*

*P. thornei* was first described in California and Utah associated with a range of plants which included grasses, wheat and sugar beet (Sher and Allen, 1953, Thorne, 1961). Infestation of *P. thornei* has also been reported in the Mediterranean region, America and the Indian subcontinent associated with cereals and legumes (Sharma et al., 1992, Di Vito et al., 1994, Castillo et al., 1996, Nicol et al., 1999, Nicol and Ortiz-Monasterio, 2004, Smiley et al., 2005). In Australia, *P. thornei* infest cereals, clovers and carrots (Colbran and McCulloch, 1965, Grandison and Wallace, 1974, Riley and Wouts, 2001, Riley and Kelly, 2002, Hay and Pethybridge, 2005). *P. thornei* (and the related species *P. neglectus* and *P. penetrans*) infestation of wheat is reported to cause losses of at least $AU46M in Australia annually (Thyer, 2008).
*P. zeae* was first found in North Carolina and Georgia in the 1940s associated with corn, rice, sugarcane, sorghum and tobacco. It is commonly distributed throughout tropical parts of the world (Khan, 1959, Allow and Katcho, 1967, Olowe and Corbett, 1976). In Australia, it has been found in sugarcane, cereals, rice, apple, white clover and peach (Colbran and McCulloch, 1965, Mathur and McLeod, 1977, Riley and Kelly, 2002).

### 1.1.2.2 Life cycle of *P. thornei* and *P. zeae*

*P. thornei* and *P. zeae* conform to the common format of nematode life cycles in which including the egg stage, they go through six life stages and four moults. These start with embryogenesis inside the egg followed by a first moult in the egg to the second juvenile (or larval) stage (J2). On hatching the motile, infective J2s are attracted to and then penetrate the zone of elongation behind the root tips and at the junctions of lateral roots, preferably at root hairs (Freckman and Chapman, 1972, Townshend, 1978, Jones and Fosu-Nyarko, 2014) (Fig. 1.2). The initial invasion track is often followed by other nematodes, possibly attracted to the contents of the damaged cells (Baxter and Blake, 1968). Following invasion of the host roots, the nematodes migrate between parenchyma cells to the cortex by rupturing consecutive cell walls using a series of persistent thrusting by the stylet and probably secretion of cell wall degrading enzymes (CWMPs) through the stylet (Castillo and Vovlas, 2007). Although not sedentary, feeding and reproduction are restricted almost entirely to the cortical region in the root. Reproduction is usually parthenogenetic and eggs are laid singly and deposited in groups or in rows mainly in the root cortex. *P. thornei* and *P. zeae* continue their developmental stages in the cortex, proceeding to further moults to third and fourth stage juveniles before the final moult to the adult stage. Some nematodes will leave the lesion, enter the soil, and move to other parts of the root or different roots to start a new infection.

It is evident that all mobile life stages of *Pratylenchus* spp. are able to penetrate host roots (Townshend and Wolynetz, 1991, Castillo et al., 1996) (Fig. 1.2). There may be two to three generations within the plant host per year. For *P. zeae*, eggs take 15-20 days to hatch and juveniles take 35-40 days to reach maturity. The number of *P. zeae* increases when soil temperature increases from 16-21°C to 27-32°C (Graham, 1951). The time required for *P. thornei* to complete its life cycle varies from 40-65 days, depending on environmental conditions such as host susceptibility, adequate temperature and moisture. The optimal soil temperature for reproduction is between 20°C and 25°C (Smiley et al., 2005).
The available evidence shows that even when unfavourable conditions such as drought, high soil temperature and low humidity occurred, the number of *P. thornei* do not decline drastically, suggesting that *P. thornei* survives the adverse conditions in an anhydrobiotic state and is rehydrated when rain falls (Orion et al., 1979).

![Infection cycle of Pratylenchus spp.](image)

**Figure 1.2** Infection cycle of *Pratylenchus* spp.

### 1.1.2.3 Signs and symptoms of *Pratylenchus* infection

The impact of *P. thornei* and *P. zeae* infestation on roots of host plants is very characteristic, causing root distortions and localised brown discolouration which expands with further feeding longitudinally along the root, accompanied by a reduction in the number and size of lateral roots and root hairs. Above ground, several symptoms can be observed. For wheat, shrinkage of grains and tillers, chlorosis and reduction in size and number of ears and head weight occurs (Thorne, 1961, van Gundy et al., 1974). However, death of plants rarely occurs with *Pratylenchus* infestation. The optimal conditions for most prominent disease symptoms are when rainfall is insufficient, unusually high temperatures occur early in the growing season, soil fertility is low and pH, and/or root-rot organisms attack the plants (Glazer and Orion, 1983, Smiley et al., 2005). However, very often, the damage inflicted by *Pratylenchus* is not clear: they are often mistaken for nutrient deficiency and thus the extent of damage caused by *Pratylenchus* is often underestimated.
At the cellular level, RLN migration and feeding leads to organelle degeneration and membrane disintegration, vacuolation and build-up of tannin (Sijmons et al., 1994). In addition, cortical cells adjacent to cells penetrated by Pratylenchus styles have enlarged nucleus and nucleolus, thickened walls and dense granular cytoplasm (Green et al., 1983, Zunke, 1990b, Castillo et al., 1998).

1.1.2.4 Associations with other pathogens/ Disease complex involving Pratylenchus spp.

P. thornei, P. zeae and other Pratylenchus spp. interact with other soil organisms, acting either as a vector for bacterial or fungal pathogen spores, or otherwise increasing plant susceptibility to other pathogens (e.g. wilt or root-rot fungi). An example of this is the severe attack by smut on oats and maize infested by P. thornei (Thorne, 1961). Another example is the increased susceptibility of wheat roots to soil fungi, mainly by Fusarium solani after P. thornei attack hence creating a disease complex in wheat (van Gundy et al., 1974). Similarly for chickpeas, Castillo and co-workers (1998) found that P. thornei infestation resulted in many more propagules of Fusarium oxysporum f. sp. ciceris in roots of Fusarium-susceptible cultivars. They concluded that damage to the cortical cells of chickpea caused by P. thornei facilitated more extensive colonisation by F. oxysporum f. sp. ciceris (Castillo et al., 1998).

For P. zeae, Khan (1959) showed that presence of Phytophthora spp. in sugarcane correlated with more P. zeae, and that this combination significantly increased yield losses in sugarcane. Similarly, the interaction of P. zeae together with P. brachyurus and the root-rot fungus, Fusarium moniliforme leads to more severe reduction in growth of maize (Jordaan et al., 1987). Because of direct and indirect losses caused by these nematodes, it is important to know how they parasitise the host in order to find a solution to combat them effectively.

1.2 Nematode artillery versus the plant cell wall

Before parasitising a plant, nematodes need to overcome the barrier of the cell walls that protect the plant protoplast. The fundamental structure of the cell wall in all terrestrial plants (monocots and dicots) appears to be similar. It is composed of various polysaccharides held together mainly by non-covalent bonds (Fig. 1.3). Amongst the polysaccharides, cellulose constitutes the most
abundant component (30-45%) and functions as a framework for the binding of other wall components (Carpita and McCann, 2000). The cellulose microfibrils of the wall are embedded in a cross-linked matrix of noncellulosic molecules which include hemicelluloses and pectins, and is reinforced by structural proteins such as glycoproteins and aromatic components (McCann and Roberts, 1991, Carpita and Gibeaut, 1993) (Fig. 1.3). The most common hemicelluloses are glucuronorabinoxylans, galactogluco- and glucomannans, xylglucans (XyG) and mixed-linkage glucans while the most abundant pectin is usually rhamnogalacturonan I and II, xylogalacturonan and homogalacturonan (O’Neill et al., 1990).

Figure 1.3 General structure of a plant cell wall.

Although all plant species contain the same sets of polysaccharides, their relative amounts and layout within the cell wall differs depending on the plant species, cell type and position and phase of growth and differentiation (Carpita and McCann, 2000). Hence differences in plant cell wall compositions have to be matched by nematodes with their inventory of cell wall modifying enzymes (CWMPs) to enable their entry into different plant species in a stealthy manner.

One of the first genes identified from PPNs which counters the plant cell wall is β-1,4 – endoglucanases which hydrolyses polysaccharides possessing β-1,4-glucan backbones such as cellulose and the hemicellulose XyG in the host cell walls (Smant et al., 1998, Yan et al., 1998). These cellulases have since been isolated from the genera Heterodera, Meloidogyne, Pratylenchus, Ditylenchus, Bursaphelenchus, Rotylenchus and Xiphinema (de Meutter et al., 1998, Gao et al., 2002, Goellner et al., 2000, Kikuchi et al., 2004, Kyndt et al., 2008, Rosso et al., 1999, Uehara et al., 2001, Yan et al., 2001, Wubben et al., 2010). The numbers and types of cellulases produced were found to vary for different species. Thus far, four cellulases were reported to be produced by G. rostochiensis, 12 by M. incognita,
two by *P. penetrans* and six by *H. glycines* (Abad et al., 2008, Gao et al., 2004b, Huang et al., 2004, Ledger et al., 2006, Rosso et al., 1999, Uehara et al., 2001). These cellulases, except for those found in *B. xylophilus* and *X. index*, belong to the glycosyl hydrolase family 5 (GHF5) of β-1,4-endoglucanases (E.C. number 3.2.1.4) and comprise two main categories: catalytic domain with and without a cellulose binding domain.

The PPNs repertoire of CWMPs also consists of endo-1,4-β-xylanase (EC 3.2.1.8) which was identified in *M. incognita* and the burrowing nematode *Radopholus similis* (Mitreva-Dautova et al., 2006, Haegeman et al., 2009). Endo-1,4-β-xylanase degrades the xylans of the hemicellulose layer of the cell wall. An arabinogalactan endo-1,4-β-galactosidase has also been isolated from *H. schachtii* and *H. glycines* which hydrolyses the 1,4-β-galactosidic bonds of the arabinogalactans of the hemicellulose layer of host cell walls (Vanholme et al., 2009).

Besides cellulose and hemicellulose modifying enzymes, PPNs also possess a combination of pectin degrading enzymes (PDEs) to depolymerise the middle lamella during intracellular migration. PDEs identified in PPNs include pectate lyases and polygalacturonase. PPNs such as *G. rostochiensis*, *H. glycines*, *M. javanica* and *P. penetrans* have been observed to produce pectate lyases (EC 4.2.2.2) which are known to catalyse random cleavage of internal α-1,4-linkages of pectate by β-elimination (Doyle and Lambert, 2002, Huang et al., 2003, Myers, 1965, Popeijus et al., 2000). In contrast, polygalacturonase (EC 2.1.1.5) was found to cleave the α-1,4-glycosidic linkages in pectate by hydrolysis (Barras et al., 1994). Thus far, PPNs reported to produce polygalacturonase are *Meloidogyne*, *Ditylenchus dipsaci*, *D. destructor* and *P. zeae* (Jaubert et al., 2002, Krusberg, 1960, 1964).

PPNs also produce cell wall modifying proteins with more subtle roles in parasitism such as cellulose binding protein which were only characterised in *H. glycines* (HG-CBP-1) and *M. incognita* (MI-CBP-1) (Ding et al., 1998, Gao et al., 2004c). Although lacking enzymatic activities, these proteins may have an indirect involvement in cell wall dissolution by activating a plant pectin methylesterase (Hewezi et al., 2008).

Another class of effectors that lack hydrolytic activity are the β-expansins, identified from *G. rostochiensis* (GR-EXPB1), *B. xylophilus* and *M. incognita* (Abad et al., 2008, Kikuchi et al., 2009, Kudla et al., 2005, Qin et al., 2004). β-expansins cause the loosening of hydrogen bonds between cellulose and hemicellulose polymers therefore initiating plant cell wall extension (McQueen-Mason and Cosgrove, 1995). Extension of cell walls by expansins is thought to increase the accessibility of plant cell wall polysaccharides to enzymatic attack and hence facilitating the invasion of plants by PPNs (Cosgrove, 2000).
In addition to the plant cell wall, PPNs also have to contend with the defence mechanism e.g. production of reactive oxygen species (ROS) elicited by the host in response to nematode invasion. Some of the genes which are potentially used by PPNs for detoxification of ROS have been identified, namely glutathione peroxidase, glutathione –S-transferase, glutathione synthetase, superoxide dismutase, thioredoxin and peroxiredoxin (Table 1.1). Although these antioxidants have been found in all PPNs, only glutathione peroxidase in *G. rostochiensis* (Jones et al., 2004a), peroxiredoxin in *G. rostochiensis* and *M. incognita* (Robertson et al., 2000, Dubreuil et al., 2011) and glutathione-S-transferase (Dubreuil et al., 2007) have been functionally analysed.

Table 1.1 Some of the nematode genes with known or putative functions in plant parasitism

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Group identified</th>
<th>Putative or known function</th>
<th>Gene isolation method</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell wall modifying enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-1,3-β-glucanase</td>
<td>? ? +</td>
<td>Hydrolysis of beta 1,3-glucan</td>
<td>4</td>
<td>Nicol et al. (2012)</td>
</tr>
<tr>
<td>Expansin-like proteins</td>
<td>+ + +</td>
<td>Cell wall extension</td>
<td>5</td>
<td>Qin et al. (2004)</td>
</tr>
<tr>
<td>Polygalacturonase</td>
<td>+ ? +</td>
<td>Hydrolysis of alpha 1,4 D-galactosiduronic linkages</td>
<td>3</td>
<td>Jaubert et al. (2002)</td>
</tr>
<tr>
<td><strong>Protection from host defences</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione-S-transferase</td>
<td>+ + +</td>
<td>Detoxification of ROS</td>
<td>11</td>
<td>Dubreuil et al. (2007)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>+ + +</td>
<td>Detoxification of ROS</td>
<td>4</td>
<td>Abad et al. (2008) Haegeman et al. (2011)</td>
</tr>
<tr>
<td>Protein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Action</td>
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<td>---------</td>
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<tr>
<td>synthetase</td>
<td></td>
<td></td>
<td></td>
<td>Possible interference with host defence response</td>
</tr>
<tr>
<td>Ichorismatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Detoxification of ROS</td>
</tr>
<tr>
<td>SPRYSEC-RBP-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Suppression of host defences</td>
</tr>
</tbody>
</table>

**Targeting regulation and signalling pathways**

<table>
<thead>
<tr>
<th>Protein</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calnexin/Calreticulin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Calcium spiking</td>
<td>Jaubert et al. (2002) Nicol et al. (2012)</td>
</tr>
</tbody>
</table>

**Feeding**

<table>
<thead>
<tr>
<th>Protein</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteases (e.g. serine, cysteine)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Protein degradation/digestion</td>
<td>Neveu et al. (2003b) Bellafiore et al. (2008)</td>
</tr>
</tbody>
</table>

**Initiation and maintenance of feeding site**

<table>
<thead>
<tr>
<th>Protein</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>7E12</td>
<td>?</td>
<td>+</td>
<td>-</td>
<td>No functional data available</td>
<td>Souza et al. (2011)</td>
</tr>
<tr>
<td>16D10 CLE related peptide</td>
<td>+</td>
<td>?</td>
<td>-</td>
<td>Promotion of giant cell induction</td>
<td>Huang et al. (2006b)</td>
</tr>
</tbody>
</table>
Some PPNs are also equipped with genes encoding effectors which aid in the suppression of host responses e.g., fatty acid and retinol binding protein (far/sec-2), chorismate mutase (cm) and venom allergen-like protein (vap) (Lambert et al., 1999, Jones et al., 2003, Iberkleid et al., 2013, Lozano-Torres et al., 2014). FAR represses host response by inhibiting the lipoxygenase-mediated metabolism of plant linoleic and linolenic acids, which are the precursors of the plant defence compound, jasmonic acid (Prior et al., 2001, Curtis, 2007, Haegeman et al. 2012, Iberkleid et al., 2013). In contrast, CM manipulates the hosts’ endogenous shikimate pathway by depleting the cytoplasmic chorismate of host cells, thereby reducing the synthesis of cellular salicylic acid (SA), an important host defense-related secondary metabolite (Doyle and Lambert, 2003). Another enzyme involved in the SA pathway in plants, isochorismatase (ICM), was also identified in plant parasitic nematodes, namely M. hapla and H. oryzae (Opperman et al., 2008, Burke et al., 2015). ICM converts isochorismate to 2,3-dihydroxybenzoate and pyruvate, thereby depleting the pool of isochorismate and reducing SA synthesis. VAPs of a phytoparasitic nematode have been reported to have potential roles as an inhibitor of the extracellular cysteine protease receptor which operates as a signalling node in innate immunity of tomato plants (Lozano-Torres et al., 2014). Far, vap and cm were found in both sedentary and migratory nematodes (Table 1.1). Other effectors also secreted by sedentary nematodes included those required for the induction and maintenance of permanent feeding sites, feeding and those required for signalling pathway (Table 1.1) (Jones and Fosu-Nyarko, 2014).

Effectors encoded by these PPGs are produced in the nuclear region of three large, unicellular pharyngeal or oesophageal gland cells. Two of the gland cells are located subventrally while the other is positioned dorsally in the nematode body (Fig. 1.4). Each gland is a large single secretory cell with a wider basal region and a long narrow extension at the apical region, which ends in an ampulla (Seinhorst, 1971). The valve of the single dorsal gland (DG) is located near the base of the stylet whereas the two subventral glands (SvG) empty into the oesophagus just posterior to the metacorporal pump.
chamber (Hussey and Mims, 1991). The products of oesophageal gland cells can be released either into the external environment through the hollow stylet or passed down the alimentary tract. The volume, shape and metabolic activity of the gland cells change during nematode development, particularly for sedentary PPNs.

For RKNs and CNs, it has been shown by immunolocalisation and cytologically that the SvG is highly metabolically active during migration through host tissue (Wang et al., 1999, Tytgat et al., 2002). There appears to be reduced production of secretions by the SvG and increase in DG activity at the initiation of feeding sites, which has led to speculation that triggers for feeding site induction by sedentary PPNs are believed to originate mainly in the DG (Bird, 1983, Endo, 1987, Tytgat et al., 2002). It will be interesting to see if SvG and DG activities of RLNs act like that of its sedentary counterparts since it do not establish long term feeding sites in the host tissues. Hence there is a need to identify and characterise secretory products of RLNs to understand their role in plant parasitism.
1.3 Identification and analysis of putative parasitism genes of plant parasitic nematode

1.3.1 Analysis of putative parasitism genes

Genes encoding proteins secreted by PPNs have been identified using several approaches. The most widely used strategy is the ‘candidate gene strategy’. Degenerate oligonucleotides designed from conserved regions have been used to isolate candidate genes via PCR amplification (Smant et al., 1998, Robertson et al., 2000, Gheysen and Jones, 2006). Genes identified from this strategy include cysteine and serine proteases from *G. pallida* and *H. glycines* (Lilley et al., 1996a, 1997) and β-1,4-endoglucanase from *P. penetrans* (Uehara et al., 2001). However the most common method used to discover candidate genes has been via expressed sequence tags (ESTs). Many sequences of PPNs are now available and accessible in public databases. As of October 2014, the Parasitic Nematode Sequencing Project (http://nematode.net/NN3) had already generated over 426,000 ESTs from forty different parasitic nematode species, of which nearly 132,000 ESTs originated from PPNs. Mining of EST datasets has not only resulted in the identification of numerous putative phylum- and family-specific genes but also given insight in the potential for horizontal gene transfer, for example in *Meloidogyne* (McCarter et al., 2003).

However, there are drawbacks in using ESTs as research tools. For example, since ESTs reflect the abundance of mRNAs present in the starting material that is used to make a cDNA library, it is difficult to identify rare transcripts that may include putative parasitism related sequences (McCarter et al., 2000). In addition, most ESTs only represent fragments of transcripts, hence different ESTs that derive from the same mRNA sequence may have different 5’ and 3’ ends as the beginning and end of the sequence may be prone to error. Low quality sequences, especially those with insertions and deletions of bases that cause frameshifts could compromise the predictions of open reading frames (Nagaraj et al., 2007). This could make annotation of sequences difficult.

To a certain extent, construction of contigs could reduce some of the problems encountered when analysing individual ESTs (McCarter et al., 2000). Recent advances in cDNA sequencing, using next generation sequencing platforms (NGS) offer almost limitless insights on the genome and transcriptome of numerous PPNs. By assembling transcripts, redundancy within a library can be estimated and reduced (Abad et al., 2008, Opperman et al., 2008, Haegeman et al., 2011, Nicol et al., 2012). Assembly of ESTs into contigs also enables the identification of both abundant transcripts and also rare ones (McCarter et al., 2003). Further analysis of the assembled ESTs to identify PPGs can then be done by using a series of programs such as BLAST, followed by validation of the site of expression using in situ hybridisation.
A commonly used method to analyse ESTs derived from libraries is the screening for short, N-terminal, leader peptide sequences known as signal peptides. Most secreted proteins in PPNs are exported through classical endoplasmic reticulum and Golgi secretory pathway via such signal peptides. SignalP is a precise program which allocates signal peptide prediction scores and cleavage sites to amino acid sequences (Nielsen et al., 1999, Menne et al., 2000). Using this program, it is possible to identify proteins containing putative signal peptides from ESTs regardless of the presence of a similar sequence in the database. Some EST projects have employed such predictions to identify putative secreted products in plant pathogens, i.e. in the plant pathogenic oomycete *Phytophthora infestans* and the PPN *Heterodera schachtii* (Torto et al., 2003, Vanholme et al., 2006). The set of ESTs with such characteristics can then be subjected to comparative EST analysis to assess if they are nematode-specific, plant parasitic-specific, or family-specific. An additional filter is to use the TMHMM (Prediction of transmembrane helices in proteins) server which identifies membrane localisation domains. In general, secreted proteins should lack such domains. However, many secretory proteins lacking the amino-terminal signal peptides are not predicted by this method (Nickel, 2003). Whilst programs such as SecretomeP has been used to predict non-classical secretory proteins in most organisms, it has been fairly erratic in its prediction of secretory proteins for parasitic nematodes, as demonstrated in the study of *B. malayi* (Bendtsen et al., 2004). Therefore the next best approach to analyse the secreted proteins is at the protein level.

1.3.2 Analysis of the putative parasitism effectors

Generally, methods such as two-dimensional gel electrophoresis (2-DE) or mass spectrometry were used to analyse the nematode secretions. Acquisition of secreted proteins has been done using micro-aspiration and induced secretion by soaking nematodes in serotonin (5-methoxy-N, N-dimethyltryptamine) and resorcinol (McClure and von Mende, 1987, Gao et al., 2001). The most abundant proteins can then be identified by microsequencing (De Meutter et al., 2001, Jaubert et al., 2002). Although 2-DE is an efficient method for the separation of complex mixtures of proteins, this procedure has a number of drawbacks. This technique is time consuming and laborious, requires skill to interpret data and yields poor reproducibility between gels. It is also difficult to detect proteins at low concentrations and hydrophobic membrane proteins, possess a low linear range of visualisation and cannot be readily automated (Fenyo and Beavis, 2002).

In contrast to the cumbersome 2-DE, Bellafiore and colleagues (2008) developed a high-throughput method to analyse the secretome of *M. incognita*. Using liquid chromatography, nano-
electrospray ionisation and tandem mass spectrometry (nano LC ESI MS/MS) to identify secreted proteins: 486 secreted proteins which could play a role in root-knot formation were found. Some of these lacked signal peptides (Bellafiore et al., 2008). This technique has since been employed to study the secretome of another plant parasite B. xylophilus (Shinya et al., 2013). Although at present, proteomic analysis represents the most successful approach to identify most if not all peptides present in the secretome, it is not without its drawbacks. The foremost difficulty is the collection of enough secretions and to concentrate them to enable proteomic analyses.

1.4 Molecular approach to functional genomics studies

Since many secreted products may be associated with parasitism, uncovering the identity of parasitism genes is very important and understanding the actual function of products of parasitism genes in the host-parasite interaction is essential to understand the molecular basis of the interaction, and will inform new methods to control and/or prevent the destructive effect of PPNs on hosts. However this is hindered by the multiplicity of functions ascribed to similar genes in other eukaryotes, the absence of homologous genes in databases, or the lack of known function for homologous genes. There are many approaches to determine the actual function of a gene. This includes overexpression in a host, or knock out of expression using other approaches such as antisense sequences, aptamers (nucleic acid macromolecules that binds tightly to a specific molecular molecule), DNAzymes and ribozymes (Rutz and Scheffold, 2004, Thakur, 2003). Increasing numbers of completely sequenced genomes from a broad array of species including C. elegans, M. incognita, M. hapla, Drosophila and humans has presented an opportunity to develop techniques that allow more systematic and rapid investigations not only of gene function in specific organisms but also to obtain crucial insights into the functional regulation of cellular pathways based on the conserved regions between species (Consortium, 1998, Consortium, 2001, Kamath et al., 2003, Abad et al., 2008, Opperman et al., 2008). RNA interference (RNAi) has emerged as one of the most powerful approaches to study gene function and this may be done on a high throughput scale.
1.4.1 RNA Interference

1.4.1.1 History of RNA interference (RNAi)

RNAi was initially believed to be a component of an ancient surveillance system of cells to combat the deleterious effects of transposons and infection by RNA viruses (Waterhouse et al., 1998, Voinnet, 2001). When the transposon-specific or viral messengers copy themselves, they may replicate dsRNA in intermediate, which is detected and degraded by the host cell (Waterhouse et al., 1998, Schepers and Kolter, 2001). Now, it is generally believed that RNAi not only plays a role in self-defense against such viruses but is also widely involved in regulation of gene expression. Similarly, it has long been known that infection of a plant with a mild strain of a virus protected the plant from infection by a more virulent strain. This was thought to be caused by preventing the uncoating of introduced virus particles by the presence of excess coat protein in cells. This gave rise to experiments in which virus coat protein genes were overexpressed in transgenic plants, and these plants did indeed exhibit resistance to the virus. However, it is now accepted that the mechanism is post-transcriptional gene silencing rather than coat protein mediated.

The first case of RNAi ‘phenomenon’ was documented by Napoli and colleagues in 1990. When a gene encoding a key pigment pathway step in the anthocyanin biosynthesis (chalcone synthase) was introduced, a deep violet pigmentation in petunia flowers was expected. Instead, transformed plants exhibited white and/or variegated flowers. The homology-dependent silencing of an endogenous locus by the introduced transgene was coined ‘cosuppression’ although the underlying mechanism was not known yet at that time (Napoli et al., 1990, van der Krol et al., 1990). Since then, RNA-mediated gene silencing has been reported in organisms from different kingdoms (Table 1.2).
Table 1.2 Example of RNA-mediated silencing in different organisms

<table>
<thead>
<tr>
<th>Species</th>
<th>Phylum</th>
<th>Stage/ type of organism tested</th>
<th>Reference (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>Nematode</td>
<td>Larval and adult stage</td>
<td>Fire et al. (1998b)</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Fruit-fly</td>
<td>Cell lines, adult, embryo</td>
<td>Bernstein et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kennerdell and Carthew (2000)</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Plants</td>
<td>Plant cells</td>
<td>Akashi et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kennerdell and Carthew (2000)</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>Fungus</td>
<td>Filamentous fungi</td>
<td>Romano and Macino (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pandit and Russo (1992)</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>Protozoan</td>
<td>Procyclic forms</td>
<td>Ngô et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wang et al. (2000)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Bacterium</td>
<td>-</td>
<td>Tchurikov et al. (2000)</td>
</tr>
<tr>
<td><em>Danio rerio</em></td>
<td>Zebradish</td>
<td>Embryo</td>
<td>Wargelius et al. (1999)</td>
</tr>
<tr>
<td><em>Hydra magnipapillate</em></td>
<td>Cnidarian</td>
<td>Adult</td>
<td>Lohmann et al. (1999)</td>
</tr>
<tr>
<td><em>Scmidtea mediterrania</em></td>
<td>Pl Animals</td>
<td>Adult</td>
<td>Alvarado and Newmark (1999)</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>Amphibian</td>
<td>Embryo</td>
<td>Dirks et al. (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Li and Rohrer (2006)</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Mammal</td>
<td>Human cell lines</td>
<td>Elbashir et al. (2001)</td>
</tr>
<tr>
<td><em>Acyrthosiphon pisum</em></td>
<td>Insect</td>
<td>Third instar</td>
<td>Jaubert-Possamai et al., (2007)</td>
</tr>
</tbody>
</table>

The first evidence that dsRNA could lead to silencing in animals was reported by Guo and Kemphues (1995), who noted that the *par-1* message in *C. elegans* was degraded when either sense or antisense RNA to *par-1* mRNA was introduced. This was unexpected because only dsRNA formed from the hybridization of antisense RNA with endogenous mRNA was anticipated to be targeted. The misconception that sense RNA was also targeted for destruction was rectified by Fire and colleagues (1998b), also working with *C. elegans*. They extensively purified their sense and antisense RNAs and directly compared their effects to dsRNA on the *unc-22* gene, which encodes a nonessential myofilament protein. Downregulation of *unc-22* activity generates a twitching phenotype while complete loss of function results in further structural muscle impairments and defective motility. By simultaneously injecting both the sense and antisense RNA in *C. elegans*, a 10-fold increase in silencing potency was revealed compared to injections of the sense RNA or antisense RNA alone (Fire et al., 1998). Similar results were obtained with four other genes: (i) *unc-54* which encodes an isoform of myosin required for full muscle contraction (ii) *fem-1* which encodes an ankyrin-repeat-containing protein required in hermaphrodites for sperm production (iii) *hlh-1* which encodes a *C. elegans* homologue of myoD- family proteins that is required for proper body shape and motility and (iv) *mex-3* found in gonad cells (Fire et al., 1998). These findings represented a major step forward in the field of gene regulation.
1.4.1.2 General mechanism of RNA interference

RNAi is a process through which exposure to dsRNA leads to the silencing of homologous genes (Fire et al., 1998b). The general mechanism of RNAi is conserved among diverse organisms (Agrawal et al., 2003) and can be mediated either by small interfering RNAs (siRNAs) or non-coding RNA molecules known as microRNAs (miRNAs) (Fig. 1.5). The attributes of siRNA and miRNA pathways are compared in Table 1.3.

**Figure 1.5** RNA interference mechanism via siRNA and miRNA pathways in nematodes.

**(I) siRNA pathway:** (A) exogenous dsRNA is cleaved into a series of 21-23 nt siRNAs by a Dicer. (B) siRNAs produced are unwound by a DEAD-box helicase associated with RNA-induced silencing complex (RISC) to generate two strands. (C) The antisense strand of siRNAs is conveyed into RISC, where it acts as a guide to identify and base-pair with target mRNA with complementary sequence, triggering mRNA cleavage. (D) Cleavage of target mRNA leads to silencing of the target gene and prevents the production of the encoded protein.

**(II) miRNA pathway:** (A) Transcription of primary miRNA (pri-miRNA) by RNA polymerase II in the nucleus forms a 70 nt long hairpin which enables binding by Pasha and subsequent cleavage by Drosha, yielding miRNA precursor (pre-miRNA). Pre-miRNA is exported to the cytoplasm via, Exportin 5. (B) Pre-miRNA processed by Dicer to form miRNA-miRNA duplex. (C) The duplex is unwound to form mature miRNA and incorporated into a complex similar to the RISC complex. (D) Mature miRNA pairs with target mRNA, leading to translation inhibition (Agrawal et al., 2003, Dykxhoorn et al., 2003).
Table 1.3 A comparison between siRNA and miRNA-mediated RNAi mechanisms

<table>
<thead>
<tr>
<th>Attributes</th>
<th>siRNA</th>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor</td>
<td>Long dsRNA</td>
<td>70 nt stem-loop RNA (shRNA)</td>
</tr>
<tr>
<td>Hairpin structure</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Length of siRNA/miRNA</td>
<td>21-23 bases</td>
<td>19-24 bases</td>
</tr>
<tr>
<td>Enzymes</td>
<td>RNA-dependent RNA polymerase, Dicer</td>
<td>RNA polymerase II; Drosha; Dicer</td>
</tr>
<tr>
<td>Complementarity to target</td>
<td>100% match</td>
<td>Imperfect match</td>
</tr>
</tbody>
</table>

An additional step in the siRNA mechanism of RNAi which is present in some organisms such as nematodes and some plants involves the amplification of the silencing signal through the action of an RNA-dependent RNA polymerase (RdRP) (Sijen et al., 2001). siRNA is used as a primer by RdRP to convert the cognate mRNA present into dsRNA (Lipardi et al., 2001, Sijen et al., 2001, Alder et al., 2003). The newly generated dsRNA serves as a substrate for Dicer cleavage activity, generating secondary siRNAs. The RdRP chain reaction is perpetuated until no target mRNAs remain (Lipardi et al., 2001). The amplification step was postulated to be the reason for the spread of dsRNA signal throughout the organism (Fire, 1999).

In *C. elegans*, gene silencing is observed even in distal regions from the initiation site implying the presence of some sort of transport mechanism that allows systemic RNAi. The *sid-1* and *sid-2* (systemic RNAi deficient) genes encode a membrane spanning protein in *C. elegans* that are involved in the uptake of dsRNA from the environment (Feinberg and Hunter, 2003, Winston et al., 2007). Besides the SID proteins, three *rsd* (RNAi spreading defective) mutants, *rsd-2, rsd-3* and *rsd-6*, appear to have normal ability in the initial uptake of dsRNA from the gut to the somatic tissues but are defective in the distribution of the RNAi effect to the germline. However, only *rsd-3* has homologous genes in RKNs. No homologues of *sid-1, sid-2, rsd-2* and *rsd-6*, which are genes involved in systemic RNAi and dsRNA spreading to surrounding cells were found in RKNs or in the root lesion nematode *P. coffeae* (Abad et al., 2008, Opperman et al., 2008, Burke et al., 2015). These genes are also absent from animal parasitic nematodes, *Brugia malayi and Haemonchus contortus*, suggesting that systemic RNAi may spread through the action of novel or poorly conserved factors in parasitic nematodes (Abad et al., 2008).

The RNAi effect is a non-stoichiometric reaction in that concentrations of only a few siRNA molecules per cell can trigger a prominent silencing effect, implying that the effect involves a catalytic stage (Fire et al., 1998b, Tijsterman et al., 2002). Usually, even though levels of target mRNA are drastically reduced, minimal amounts can still be detected. Thus, the term ‘knockdown’ rather than ‘knockout’ is preferred for RNAi-mediated silencing of a particular gene.
1.4.1.3 Establishing RNAi for phytonematodes

In *C. elegans*, introduced dsRNA has been used to successfully used to induce gene knockdown and this led many researchers to believe that the same could also be applied to other nematode species. However for phytonematodes, delivery of dsRNA is not as straightforward as for *C. elegans* because (i) infective stages of PPNs are small in size making microinjection technically challenging and (ii) pre-parasitic nematodes are non-feeding and do not normally ingest fluid before infestation of plant roots.

Urwin and co-workers (2002) overcame this problem by treating J2s of *G. pallida* and *H. glycines* with the neurotransmitter octopamine, which stimulated oral uptake of external solutions. Resorcinol and serotonin, both which increase pharyngeal pumping in nematodes (McClure and von Mende, 1987) have also been used to induce uptake of external solution containing dsRNA in PPNs i.e. J2s of *M. incognita* (Rosso et al., 2005b). This protocol was further modified by Chen and co-workers (2005) who supplemented the soaking buffer with spermidine and extended the incubation time to increase the penetrance of RNAi into *G. rostochiensis*. Gelatin (0.05%) was also added to improve penetrance of dsRNA into CNs (Maeda et al., 2001, Urwin et al., 2002). A dsRNA solution also containing fluorescein isothiocyanate (FITC) as a visual tracer for uptake efficiency and for selection of desired individuals was used for subsequent experiments. Alternatively, fluorescently labelled dsRNA/siRNA has been used instead of FITC in the soaking medium (Arguel et al., 2012). Soaking approaches utilising octopamine have since been employed to elicit RNAi responses in various stages of PPNs including eggs (Table 1.4).
Table 1.4 Examples of methods of delivery of dsRNAs/siRNAs in plant parasitic nematodes and genes targeted by RNAi

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Target</th>
<th>Site of gene expression</th>
<th>Incubation time (h)</th>
<th>dsRNA/ siRNA concentration</th>
<th>RNAi effect (s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. artiellia</em></td>
<td>Eggs</td>
<td>Chitin synthase</td>
<td>Egg</td>
<td>24-72</td>
<td>1 µg/ µl</td>
<td>Delayed egg hatch</td>
<td>(Fanelli et al., 2005)</td>
</tr>
<tr>
<td><em>M. incognita</em></td>
<td>J2</td>
<td>Dual oxidase</td>
<td>Extracellular matrix</td>
<td>4</td>
<td>2 µg/ µl</td>
<td>Decreased number of established nematodes</td>
<td>(Bakhetia et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca reticulin</td>
<td>Subventral gland</td>
<td>4</td>
<td>4 mg/ml</td>
<td>Depleted transcript</td>
<td>(Rosso et al., 2005a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polygalacturonase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathepsin L cysteine proteinase</td>
<td>Intestine</td>
<td>4</td>
<td>2-5 mg/ml</td>
<td>Reduction in number of nematodes and females</td>
<td>(Shingles et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutathione-S-transferase</td>
<td>Subventral gland</td>
<td>1</td>
<td>2 mg/ml</td>
<td>Decrease in number of egg masses</td>
<td>(Dubreuil et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dual oxidase (Miduox)</td>
<td>Extracellular matrix</td>
<td>24</td>
<td>5 µg/50 µl</td>
<td>Reduction in nematode numbers and retardation of female development</td>
<td>(Charlton et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subunit of signal peptidase</td>
<td>Endoplasmic reticulum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>genes (MispC3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J2 and eggs</td>
<td></td>
<td>Drosha</td>
<td>Unknown</td>
<td>24</td>
<td>0.05 mg/ml siRNA</td>
<td>Decrease in transcripts and delayed normal embryonic development and aberrant phenotype</td>
<td>(Dalzell et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pasha</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNAseIII enzyme dicer</td>
<td></td>
<td></td>
<td></td>
<td>Insignificant decrease in dicer transcripts</td>
<td></td>
</tr>
<tr>
<td>J2</td>
<td></td>
<td>Posterior segregation gene,</td>
<td>Probably in gonads</td>
<td>4</td>
<td>1 µg/ µl</td>
<td>Reduced hatching</td>
<td>(Matsunaga et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pos-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. glycines</em></td>
<td>J2</td>
<td>Cysteine proteases</td>
<td>Intestine</td>
<td>4</td>
<td>2-5 mg/ml</td>
<td>Increased male: female ratio</td>
<td>(Urwin et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-type lectin</td>
<td>Adult female</td>
<td></td>
<td></td>
<td>Decreased number of nematodes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Major sperm protein</td>
<td>Sperm</td>
<td></td>
<td></td>
<td>Reduction in transcript abundance</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aminopeptidase</td>
<td>Female reproductive system</td>
<td>4</td>
<td>2-5 mg/ml</td>
<td>Decreased number of established nematodes</td>
<td>(Lilley et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-1, 4- endoglucanase</td>
<td>Subventral gland</td>
<td>16</td>
<td>2-5 mg/ml</td>
<td>Decreased number of established nematodes</td>
<td>(Bakhetia et al., 2007)</td>
</tr>
<tr>
<td>Protein Family</td>
<td>Source</td>
<td>Stage</td>
<td>Gland</td>
<td>Concentration</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>---------------</td>
<td>--------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Pectate lyase</td>
<td>G. pallida</td>
<td>J2</td>
<td>Oesophageal gland</td>
<td>Increased male: female ratio</td>
<td>2.5 or 5 mg/ml</td>
<td>Depleted transcript abundance (Sukno et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Chorismate mutase</td>
<td>G. pallida</td>
<td>J2</td>
<td>Dorsal gland</td>
<td>Increased male: female ratio</td>
<td>2.5 or 5 mg/ml</td>
<td>Depleted transcript abundance (Sukno et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Secreted peptide SYV46</td>
<td>G. pallida</td>
<td>J2</td>
<td>Dorsal gland</td>
<td>Decreased In number of established nematodes</td>
<td>2.5 or 5 mg/ml</td>
<td>Depleted transcript abundance (Sukno et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Unknown gland protein</td>
<td>G. pallida</td>
<td>J2</td>
<td>Subventral gland</td>
<td>Increased male: female ratio</td>
<td>2.5 or 5 mg/ml</td>
<td>Depleted transcript abundance (Sukno et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Pectate lyase</td>
<td>G. rostochiensis</td>
<td>J2</td>
<td>Subventral gland</td>
<td>Decreased in number of established nematodes</td>
<td>2.5 mg/ml</td>
<td>Impaired motility (Chen et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Cysteine proteinase</td>
<td>G. rostochiensis</td>
<td>J2</td>
<td>Intestine</td>
<td>Increased male: female ratio</td>
<td>2-5 mg/ml</td>
<td>Impaired motility (Urwin et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>FMRF amide like peptides (flip-1, -6, -12, -14, -18)</td>
<td>G. rostochiensis</td>
<td>J2</td>
<td>Nervous system</td>
<td>Increased male: female ratio</td>
<td>24 h to 7 days</td>
<td>Impaired motility (Kimber et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Flip-12</td>
<td>G. rostochiensis</td>
<td>J2</td>
<td>Nervous system</td>
<td>Increased male: female ratio</td>
<td>24</td>
<td>Impaired motility (Dalzell et al., 2010a)</td>
<td></td>
</tr>
<tr>
<td>β-1, 4-endoglucanase</td>
<td>P. coffeae</td>
<td>Mixed</td>
<td>Subventral gland</td>
<td>Decreased in number of established nematodes</td>
<td>2-5 mg/ml</td>
<td>Impaired motility (Chen et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Secreted amphid protein</td>
<td>P. coffeae</td>
<td>Mixed</td>
<td>Amphids</td>
<td>Decreased ability to locate and parasitise host</td>
<td>2-5 mg/ml</td>
<td>Impaired motility (Chen et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Pat-10, Unc-87</td>
<td>P. thornei</td>
<td>Mixed</td>
<td>Muscle</td>
<td>Decreased motility</td>
<td>1 µg/µl</td>
<td>Decreased reproduction (Tan et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Pat-10, Unc-87</td>
<td>P. thornei</td>
<td>Mixed</td>
<td>Muscle</td>
<td>Decreased motility</td>
<td>1-2 mg/ml</td>
<td>Decreased reproduction (Tan et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Pat-10, Unc-87</td>
<td>P. zeae</td>
<td>Mixed</td>
<td>Muscle</td>
<td>Decreased motility</td>
<td>1-2 mg/ml</td>
<td>Decreased reproduction (Tan et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>β-1,4-endoglucanase</td>
<td>P. vulnus</td>
<td>Female</td>
<td>Subventral gland</td>
<td>Decreased reproduction rate</td>
<td>12 and 24</td>
<td>Decreased reproduction rate (Fanelli et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Myo-3 Tmy-1</td>
<td>B. xylophilus</td>
<td>L2-L3</td>
<td>Muscle</td>
<td>Abnormal locomotion</td>
<td>1 day</td>
<td>Decreased reproduction rate (Fanelli et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Hsp-1</td>
<td>B. xylophilus</td>
<td>L2-L3</td>
<td>Unknown</td>
<td>Abnormal locomotion</td>
<td>1 day</td>
<td>Decreased reproduction rate (Fanelli et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>β-1,4-endoglucanase</td>
<td>B. xylophilus</td>
<td>Mixed</td>
<td>Subventral gland</td>
<td>Inhibit dispersal ability</td>
<td>200 uL</td>
<td>Decreased reproduction rate (Fanelli et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Arginine kinase gene</td>
<td>B. xylophilus</td>
<td>Mixed</td>
<td>Unknown</td>
<td>Defective motility and morphology</td>
<td>2 µg/µl</td>
<td>Decreased reproduction rate (Fanelli et al., 2014)</td>
<td></td>
</tr>
</tbody>
</table>
The effectiveness of RNAi depends on many factors e.g. incubation time (Table 1.4) as well as design, length and concentration of dsRNA/siRNA (Sukno et al., 2007, Arguel et al., 2012). Different studies have also shown considerable variation in the effectiveness of silencing using different target genes, some being more amenable to the approach than others (Fraser et al., 2000, Cutter et al., 2003, Zawadzki et al., 2006, Sukno et al., 2007). Further studies are required to understand the basis of these differences in nematode responses to RNAi.

1.4.1.4 In vivo RNAi experiments

One of the most important goals of studies involving plant pests is to develop control methods against them. Whilst in vitro experiments are useful for understanding gene function in PPNs, it is not appropriate for real-life applications because the effect may be transient. Traditional methods of PPNs control such as cultural practices and application of chemical nematicides and the host plants ‘natural resistance’ have been deployed but none are ideal for controlling PPNs in most crop plants.

A viable strategy which could be employed is the delivery of compounds via plants that disrupt the different aspects of nematode life cycle i.e. entry into and migration in roots, evasion of host defense mechanisms and formation or functioning of feeding cells of endoparasites (Jones and Fosu-Nyarko, 2014). Successful use of transgenic proteinase inhibitors (phytocystatins) to confer a level of resistance to RKNs (M. incognita), CNs (G. pallida, H. glycines and H. schachtii), P. penetrans and R. reniformis was one of the earliest indications that such approaches may be effective in conferring host resistance to PPNs (Urwin et al., 1995, Atkinson et al., 1996, 1997b, Vain et al., 1998, 2000, Samac and Smigocki, 2003). However, in the case of RLNs, the degree of resistance conferred to P. penetrans was significantly lower (only 29-32% reduction in population) compared to that in other nematodes found in plant expressing the different isoforms of oryzacystatin (Urwin et al. 1995; 1997; 2000; Atkinson et al. 1996; Vain et al. 1998; Samac and Smigocki, 2003) and therefore alternative control strategies against Pratylenchus spp. were required. The effectiveness of in vitro RNAi silencing and in planta delivery suggests that a potential solution was to use plants to deliver dsRNA/siRNAs derived from the nematodes’ own genes to confer host resistance against these pests.
Yadav and colleagues (2006) were the first to report RNAi based resistance to *M. incognita* by *in planta* delivery of dsRNA in tobacco plants against *M. incognita*. The plants were engineered with an RNAi vector containing target genes made to be transcribed to mRNA that formed a hairpin containing sense and antisense sequences of a target nematode gene. This was then processed by the plant RNAi machinery to produce silencing triggers such as dsRNA and/or siRNAs. When these triggers were ingested by nematodes during feeding, the expression of the homologous target genes was reported to be strongly downregulated. Plants expressing hairpin constructs for the targeted nematode splicing factor and integrase gene not only demonstrated a near complete depletion of target mRNA but also provide the hosts effective resistance against *M. incognita* (Yadav et al., 2006). There are some criticisms of the data presented by Yadav et al. (2006) in terms of the apparently clear cut results presented. Nevertheless, this finding was followed closely by data from Huang and co-workers (2006) who found that broad resistance against different RKN species can also be obtained with *in planta* RNAi, depending on the gene targeted. Since then, *in planta* RNAi has been developed against different genes of root-knot and cyst nematodes with considerable success (Table 1.5).
### Table 1.5: A summary of host-derived RNAi against plant parasitic nematodes

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Host Plant</th>
<th>Target Gene</th>
<th>Target mRNA depletion</th>
<th>Observable phenotypes</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. incognita</em></td>
<td>J2</td>
<td>Tobacco</td>
<td>Splicing factor</td>
<td>Yes</td>
<td>Almost complete resistance to <em>M. incognita</em></td>
<td>(Yadav et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Integrase</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td></td>
<td></td>
<td>Tyrosine phosphatase</td>
<td>Yes</td>
<td>Reduced gall number</td>
<td>(Ibrahim et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mitochondrial stress-70 precursor protein</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>Rpn7</td>
<td>Tobacco</td>
<td>Cathepsin L cysteine protease</td>
<td>Yes</td>
<td>Reduced egg production and hatching</td>
<td>(de Souza Junior et al., 2013)</td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td></td>
<td>Cathepsin D aspartic protease</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td></td>
<td>Chymotrypsin-like serine protease</td>
<td>Yes</td>
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<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td></td>
<td>FMRF amide like peptide genes, <em>flip-14</em> and <em>flip-18</em></td>
<td>Yes</td>
<td>Reduced galling, number of females and fecundity</td>
<td>(Papolu et al., 2013b)</td>
</tr>
<tr>
<td>A. thaliana</td>
<td></td>
<td>Calreticulin</td>
<td>Parasitism gene <em>BD05</em></td>
<td>Not analysed</td>
<td>Reduction in gall number</td>
<td>(Xue et al., 2013)</td>
</tr>
<tr>
<td>M. javanica</td>
<td>Eggs</td>
<td>Tobacco</td>
<td>Putative transcription factor, MjTis11</td>
<td>Not analysed</td>
<td>-</td>
<td>(Fairbairn et al., 2007)</td>
</tr>
<tr>
<td><em>M. arenaria</em></td>
<td>J2</td>
<td>A. thaliana</td>
<td>16D10</td>
<td>Not analysed</td>
<td>Reduced gall number and egg production</td>
<td>(Huang et al., 2006a)</td>
</tr>
<tr>
<td><em>M. hapla</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. incognita</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. javanica</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. glycines</td>
<td>J2</td>
<td>Soybean</td>
<td>Major sperm protein</td>
<td>Not analysed</td>
<td>Reduced egg production</td>
<td>(Steeves et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced reproductive rate of progeny</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td></td>
<td>Cpn 1</td>
<td></td>
<td>Yes</td>
<td>Reduction in reproduction and development</td>
<td>(Li et al., 2010a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y25</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Prp 17</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>H. glycines</td>
<td>J2</td>
<td>Chimeric soybean</td>
<td><em>Fib-1</em></td>
<td>Yes</td>
<td>Suppression of nematode reproduction and development</td>
<td>(Li et al., 2010b)</td>
</tr>
<tr>
<td>Species</td>
<td>Stage</td>
<td>Description</td>
<td>Detection</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------</td>
<td>---------------------------------------------</td>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td>H. schachtii</td>
<td>J2</td>
<td>Protein similar to plant SCF complex involved in polyubiquitination, 8H07</td>
<td>Yes</td>
<td>Decreased number of developing females</td>
<td>(Sindhu et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein similar to ubiquitin but has unique C-terminal amino acid extension peptide, 4G06</td>
<td>Yes</td>
<td>Decreased number of females</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Region covering the ring H2 zinc finger domain, 10A06</td>
<td>Not analysed</td>
<td>Decreased number of developing females</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellulose-binding protein, 3B05</td>
<td>Yes</td>
<td>Decreased number of females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. thaliana</td>
<td></td>
<td>Annexin-like effector, Hs4F01</td>
<td>Yes</td>
<td>Reduction in number of females</td>
<td>(Patel et al., 2010)</td>
<td></td>
</tr>
</tbody>
</table>
This approach therefore shows potential for commercial application to control sedentary endoparasites. However, when this project was first started, there had been no published studies on RNAi to RLNs. Since then, from this study and from similar work at the lab of Professor Godelieve Gheysen in Ghent University, the data indicates that some Pratylenchus spp. (P. coffeae, P. thornei, P. zeae) are amenable to RNAi (Joseph et al. 2012; Tan et al. 2013). These results strongly suggest that in planta RNAi could be used confer resistance against Pratylenchus spp. Another indication that the in vivo RNAi strategy would work for Pratylenchus spp. is the absence of a feeding tube in Pratylenchus spp. which may limit the size and amount of dsRNA taken up from host cells when present, as is formed when cyst or RK nematodes feed from syncytia or giant cells respectively. Without the added filter of a feeding tube, Pratylenchus spp. may well be more amenable to transgenic control using an in vivo RNAi strategy than the sedentary endoparasites.

1.5 Outline of the thesis and aims

Based on the work on gene silencing described for sedentary endoparasitic plant nematodes, the overall aim of the work reported here was to find out how amenable Pratylenchus species might be to RNAi, and to optimise methods required to study gene silencing in Pratylenchus spp. Because molecular studies for Pratylenchus spp. were limited, there was no information on whether RNAi could in fact be used to study gene expression in Pratylenchus spp. There was therefore a need to establish and optimise protocols for both in vitro and in vivo RNAi for these root lesion nematodes: with success in these areas functional analysis of putative effectors and other genes could then be undertaken to identify gene targets as potential candidates for their control.

Specific aims of the thesis were as follows:

- To optimise parameters such as incubation time, incubation medium and concentration of dsRNA for feeding to RLNs to find the best conditions for effective in vitro RNAi: the work undertaken on these aspects is provided in Chapter 3. Effect of preliminary in vivo RNAi studies using hairy roots expressing a P. thornei specific gene will be observed.

- To identify genes comprising the secretome/parasitome of P. thornei. To achieve this aim, an efficient pipeline of bioinformatics was used to mine the available Pratylenchus transcriptome data for secretory proteins. Candidate genes were then identified via PCR and validated further.
using *in situ* hybridisation to test whether the corresponding genes were expressed in organs associated with secretion of molecules at the host-parasite interface. To further characterise the genes, *in vitro* RNAi was employed: the work undertaken on these aspects is described in **Chapter 4**

- To study the genes expressed in the penetration phase of the nematode-plant interaction. The focus was to be on cell wall degrading enzymes of *P. thornei*. The work undertaken on these aspects is provided in **Chapter 5**. The focus was on characterising cellulase, pectate lyase and polygalacturonase. An optimised *in vitro* RNAi soaking protocol on *P. thornei* was used to determine whether candidate genes identified in Chapter 3 were involved in parasitism.

- To study and characterise cysteine proteases and venom-allergen proteins of *P. thornei*: the work undertaken on this aspects is described in **Chapter 6**

- To study some of the genes used by *P. thornei* encoding fatty acid and retinoid binding protein and antioxidants that might be involved in host immunosuppression. The work undertaken on these aspects is described in **Chapter 7**

- The aim of the work described in **Chapter 8** was to study additional genes, namely transthyretin-like protein and galectin, which may have putative roles in nematode parasitism but have yet to be fully characterised.

**The overall results and the context of the work in this thesis are discussed in Chapter 9.**
Chapter 2 : General materials and methods
2.1 Carrot callus culture

Either ‘Mojo’ or ‘Stefano’ cultivars of carrots (*Daucus carota*) (Mercer Mooney, Canning Vale, Australia) were cultured *in vitro* to propagate *P. thornei* and *P. zeae* and for *in vitro* RNAi experiments. The carrots were first surface-sterilised with sodium hypochlorite (2% of available chlorine) and 3 drops of Tween 20 for 30 min on a shaker at 150 rpm. The carrots were removed and then soaked in sterile water before the bleached skins were removed with a sterile peeler. The peeled carrots were then cut into discs, soaked in sterile water and flamed before being placed in autoclaved polypropylene containers. Surface-sterilised carrots discs were transferred to an incubation oven maintained at 21°C in the dark. After 14-20 days, the carrots discs will normally have sufficient callus for nematode infection.

To establish a system to measure reproduction rates of *P. thornei* and *P. zeae* after soaking experiments, mini discs of 10 mm thickness were cut from carrots prepared as above using a 10 mm diameter cork borer. Each disc were placed in one well of a 24-well plate (Greiner Bio-One, Germany) containing 2.2 g/L MS basal solution with Gamborg’s vitamins (Sigma Aldrich, Australia) solidified with 0.7 % agar. The plates were covered with a sterile lid, sealed with parafilm and incubated at 25°C for 2 weeks to ensure sterility and to initiate callusing. When callus started to form, discs were inoculated with 50 sterile nematodes of either *P. thornei* or *P. zeae* and incubated in the dark at 25°C. For each nematode species, one plate with 24 mini discs was infected and three replicates were analysed for numbers of nematodes present at weekly intervals over a period of five weeks.

2.2 Nematode propagation and collection

*P. thornei vic33* used in this study was obtained from stock cultures maintained on carrot discs at the Department of Agriculture and Food Western Australia (DAFWA) (Perth, Western Australia, Australia). The identity of the nematodes has been previously verified by Dr. Jackie Knobbs (South Australian Research and Development Institute, Australia). Dr. Jennifer Cobon (The Department of Agriculture, Fisheries and Forestry, Queensland) provided the starting culture for *P. zeae*. Carrots infested with nematodes were collected using the mist chamber: cut discs were placed onto coffee filters on a 5 cm sieve with 0.1 cm aperture which were sprayed with a fine mist of water for 10 seconds every 10 minutes for 24 h. The sieve was placed on a funnel attached to a 0.25 cm wide rubber tubing (Fig. 2.1). Nematodes moved down the tube and accumulated in a U-shaped section of tubing sealed
with a clip until collected. Nematodes were collected every two days with a 15 mL Falcon tube and placed in a 15°C incubation oven overnight to let them settle. Excess water was removed the following day and the nematodes were surface-sterilised with 1 % chlorhexidine gluconate (hibitane) for 20 min, followed by 1 % streptomycin sulphate for 5 min and five washes with sterile water. Washing involved suspending nematodes in water in 1.5 mL or 15 mL tubes, inverting the tube 3-5 times and centrifuging at 1,000 g for 3 min. This was repeated 4-5 times. The nematodes were then observed using a light microscope to check for vitality before use. Approximately 1000 live nematodes were used for infecting fresh carrot callus. Infected carrots were then placed in 21°C for 6-8 weeks.

![Figure 2.1 Carrot mini disc culture and extraction system for root lesion nematodes. (A). Carrot mini discs in a 24-well plate. (B). Mist chamber set-up in a glasshouse for extraction of mixed stages of root lesion nematodes.](image)

2.3 Gene amplification and analysis of gene expression

2.3.1 RNA extraction and DNase I treatment

RNA was extracted from either eggs, mixed stages of *P. thornei* and *P. zea* or from dissected section of *P. thornei*. The amount of nematodes used depended on the purpose of the experiment. Briefly, samples were snap-frozen in liquid nitrogen and crushed using three 5 mm stainless steel beads in a tissue lyser at 2.5 rpm/s for 5 min (Qiagen, Hilden, Germany). Macerated samples were then extracted using TRIzol® Reagent (Invitrogen Corporation, California, USA) and chloroform: isoamyl alcohol (24:1). Extracted RNA was ethanol-precipitated overnight and resuspended in RNase-free water. DNase I treatment was then carried out to remove genomic DNA from RNA using RNase-free DNase set according to RNeasy Mini Handbook page 71 (09/2010) (Qiagen, Hilden, Germany). After clean-up with chloroform and ethanol precipitation, purified RNA was quantified with NanoDrop™ 1000
Spectrophotometer (Thermo Scientific, Wilmington, USA) which enables accurate UV/Vis analyses with 1 μL of samples.

### 2.3.2 Reverse transcription

Reverse transcriptase (obtained as MultiScribe™ Reverse Transcriptase (Applied Biosystem, Foster City, California, USA) was frequently used to transcribe 100 ng of RNAs to complementary DNA (cDNAs) according to manufacturer’s protocol. The mixture was incubated at 25°C for 10 min to allow annealing of hexameric primers, followed by 37°C for 120 min for reverse transcription and finally 85°C for 5 sec for denaturation of the reverse transcriptase. The cDNA produced were used in PCR reactions.

### 2.3.3 Polymerase chain reaction (PCR)

PCR was normally performed in a reaction volume of 20 μL containing variable amounts of DNA (10-200 ng), 0.2-0.4 U of MyTaq DNA polymerase, 5X Taq polymerase PCR buffer (Bioline Inc., London, UK) 0.5 mM of each forward and reverse primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) and 6 μL of cresol red (1% cresol red, 17 g sucrose) (Sigma Aldrich Corporation, Castle Hill, Australia). Cycling conditions were a single initial denaturation step of 94°C for 3 min, followed by 25 to 40 cycles of denaturation at 94°C for 30 sec, extension at 72°C for 30 sec and a final incubation step at 72°C for 7 to 10 min to ensure complete extension. The primer annealing temperature varied with primer pairs. PCR reactions were carried out in Perkin Elmer GeneAmp® DNA Thermal Cycler model 2400 or Applied Biosystems Thermal Cycler model 7200.

Touchdown PCR (TD-PCR) was also performed to increase the specificity of PCR especially for primer pairs with large differences in melting temperature (Tm). Condition of the TD-PCR was similar to normal PCR except that the initial annealing temperature used was above the Tm of the primers which then progressively decreased by 1°C per cycle until an annealing temperature that is equal or 2-5°C below the Tm of the primers was reached.

Products of PCR amplification were ran on 0.8% to 1.5% TAE agarose gel in a horizontal gel apparatus (Bio-Rad Laboratories, Hercules, California, USA) at 65-90 V for 30 min to 2 h. For visualisation of DNA fragments, agarose gel was pre-stained with 5 μL of SYBR Safe™ DNA gel stain (Invitrogen
Corporation, California, USA) per 50 mL of 1x TAE buffer which was diluted from 50x TAE (242 g Tris, 100 mL 0.5M EDTA pH 8.0, 57.1 mL glacial acetic acid) as described by Sambrook et al. (1989). Molecular weight standard markers used included 100 bp DNA marker (New England Biolabs Inc., Massachusetts) and 1kb DNA marker (Axygen Scientific Inc., Union City, USA). Gels were visualised and captured using bio-vision imaging system (Vilber Lourmat, Germany).

2.3.4 Quantitative PCR (qPCR)

Analyses of gene expression in nematodes were done with the GoTaq® qPCR Master Mix (Promega Corporation, Australia) in a Corbett RotorGene Quantitative Thermal Cycler (Qiagen Pty Ltd, Australia). Briefly, 1 µl of cDNA, converted from 200 ng of RNA of experimental and control samples, was added to 1X GoTaq qPCR master mix, 10 µM each of the gene specific primer pair and sterile deionised water to make the reaction mixture to 20 µL. All PCRs were done in triplicate and the mean of Ct values determined. Expression of respective genes was quantified with primer pairs stated in Table 2.1. PCRs were done at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 55°C for 60 sec. Gene expression was normalised with 18S ribosomal RNA (rRNA) genes using primer pairs q18SrRNA-F and q18SrRNA-R which amplified 147 bp in P. thornei (Table 2.1). Relative gene expression was determined using the ΔΔCt method as described in the ABI PRISM 7700 Sequence Detection System Bulletin 2 (Applied Biosystems, USA).

2.3.5 Primer design

All primers used in the study were designed manually from sequences obtained from analysis of the transcriptomes of three RLNs and checked for melting temperatures and formation of secondary structures using IDT DNA OligoAnalyzer. Primer sequences are listed in Table 2.1.

Table 2.1: Primers used for amplification of DNA templates for cloning, generation of dsRNA template and for quantitative PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
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<td>GAATGGCTACATCATGGC</td>
<td>1</td>
</tr>
<tr>
<td>PpPat10-R</td>
<td>AACAATCTTCGTTGACAGTC</td>
<td>1</td>
</tr>
<tr>
<td>Ppunc87-F</td>
<td>GTGACTCCAGAAGTTGATGAC</td>
<td>1</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Ppunc87-R</td>
<td>TTGTTTGTACCAGACTGGAG</td>
<td></td>
</tr>
<tr>
<td>PthTTL-4F</td>
<td>CAGCAAACACAGTCTCCAGT</td>
<td></td>
</tr>
<tr>
<td>PthTTL-4R</td>
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<td></td>
</tr>
<tr>
<td>PtAminoPep-Xbal.Xhol</td>
<td>TCTAGACTCGAGTTCGCCGACCAACAACA</td>
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<tr>
<td>PtAminoPep-BamHI.KpnI</td>
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<tr>
<td>Ptβ-1,4-endoglucanase-Xbal.Xhol</td>
<td>TCTAGACTCGAGTGTTGATACTGCTCTT</td>
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<td>Ptβ-1,4-endoglucanase-BamHI.KpnI</td>
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<tr>
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<td>qPtGh5-R</td>
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<tr>
<td>qPtGalectin-R</td>
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<td>qPtGlured-F</td>
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</tr>
<tr>
<td>qPtGlured-R</td>
<td>CCTCTCCACAGCATTACGAC</td>
<td></td>
</tr>
<tr>
<td>qPtGST-F</td>
<td>GCAAGGTGAGATGAGGAGAAV</td>
<td></td>
</tr>
</tbody>
</table>
### 2.4 Restriction digestion

Restriction digestion was performed to linearise vectors and to excise desired DNA inserts from vectors for downstream experiments e.g. to generate template for dsRNA synthesis and in situ hybridisation and for ligation to other digested fragments. Digestion was done at 37°C for 3-8 h with either a single enzyme or two enzymes simultaneously. A typical digestion mixture contained 20 U/μL enzyme (s) (NEB, USA), 10x buffer, 100x BSA and 1-10 μg template DNA.

### 2.5 DNA transformation

#### 2.5.1 Ligation of PCR products to cloning vector

After PCR and gel extraction, purified PCR products were ligated to pGEM-T Easy cloning vector (Promega Corporation, Annandale, NSW, Australia) or pDoubler (Fosu-Nyarko et al., 2015). Ligations were done according to manufacturer’s protocol (Promega Corporation Technical Manual; pGEM-T and pGEM-T Easy Vector Systems, 2005).
2.5.2 Transformation of competent *Escherichia coli* cells

Ligated products were added to *E. coli* strain JM109 competent cells prepared using a modified rubidium chloride method (Promega Subcloning Notebook) and transformed according to Promega *E. coli* Competent Cells Technical Bulletin #TB095 with some modifications. Four hundred μL of Luria-Bertani (LB) broth (10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl) was added to the cells transformed with ligation mixtures instead of 900 μL of SOC medium and incubated for 1.5 h at 37 °C with shaking at 225 rpm. pGEM-T Easy vector transformants were seeded on LB plates containing 100 μg/mL ampicillin with 40 µg/mL X-Gal and 0.5 mM IPTG while products ligated to pDoubler were spread on LB plates containing 25 μg/mL kanamycin plates without X-Gal and IPTG.

2.5.3 Analysis of transformants by PCR and bacterial growth

Individual white colonies were picked with sterile pipette tips and resuspended in 20 μL of sterile water of which 5 μL was used as PCR template. T7/SP6 primers were used to screen pGEM-T Easy transformants whereas products ligated to pDoubler were screened with M13 forward and reverse primers. Amplicon size of pGEM-T Easy vector transformants containing insert from PCR with T7/SP6 primers was 140 bp plus expected insert size whereas PCR with M13 primers on pDoubler transformants containing insert generated amplicons of 200 bp plus expected insert size. After positive screening by PCR, single isolated colonies were inoculated to 5-10 mL of LB broth with 100 μg/ mL ampicillin or 25 μg/mL kanamycin in 20 mL McCartney bottles and incubated on a shaker at 225 rpm for 16-18 h. Bacterial cultures were then purified to yield plasmids for downstream applications.

2.6 DNA purification

PCR products and digested product from plasmid DNA were normally purified from agarose gels using Promega Wizard® SV Gel and PCR Clean-Up System according to manufacturer’s protocol (Promega Corporation, Madison, USA).

Plasmid DNA from cell cultures were purified using Wizard® *Plus* Minipreps DNA DNA Purification Kit (Promega Corporation, Madison, USA) according to Promega’s protocol presented in
Technical Bulletin #225 (2007). Purified DNA was quantified with NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA) which enables accurate UV/Vis analyses with 1 μL of samples.

2.7 DNA sequencing

DNA was sequenced using the Big Dye Terminator version 3.1 Cycle Sequencing Kit from Applied Biosystems Industries (California, USA). Sequencing of plasmid DNA was performed using 10 μL 1/16 reactions, each containing 0.5 μL dye terminator (ABI), 1.75 μL of 5x sequencing buffer, 3.2 pmol of primers and 150-300 ng of plasmid DNA. Thermocycling reactions were performed at: 96°C (2 min) followed by 25 cycles of denaturation at 96°C (10 sec), annealing at 50°C (5 sec), extension at 60°C (4 min) and a final 14°C hold in a Perkin Elmer GeneAmp PCR System 7200 thermocycler.

Unincorporated dye from the PCR product was removed by the addition of 1 μL of 125 mM EDTA, 1 μL of 3 M sodium acetate pH 5.2 and 25 μL 100% ethanol. This was mixed and left to stand at room temperature for at least 15 min to precipitate the extension products. The mixture was then spun at 3000 g for 30 min in Beckman Avanti™ centrifuge. The resulting supernatant was removed by inverting the plate gently without disturbing the precipitates and spun up to 185 g. The DNA pellet was then washed by adding 35 μL of 70% v/v ethanol and centrifuged at 1650 g for 15 min. The supernatant was removed as described above. The reaction was processed by Ms. Frances Brigg, Western Australia State Agricultural Biotechnology Center (Murdoch University, Australia) with an ABI 3730 48 capillary machine (Perkin Elmer Inc., California, USA). DNA sequence chromatograms were viewed and edited with FinchTV (Geospiza Inc.) and identities of genes confirmed using BLAST programs.

2.8 In vitro RNA interference

2.8.1 Generation of double-stranded RNA (dsRNA)

Double stranded RNA corresponding to putative RLN parasitism genes were used in in vitro RNAi ‘soaking’ experiments to test the effect of RNAi on P. thornei and P. zeae. To generate templates for dsRNA, approximately 10 μg of pDoubler containing the genes of interest were digested with 1 μL of EcoRI (20 U/μL) at 37 °C for 8h of which 1 μg of desired inserts were used as templates for dsRNA production. A 500 nucleotide long dsRNA corresponding to the gfp gene of Aequorea victoria was used.
as control. Alternatively, dsRNA was also generated by using 1 μg of template DNA generated from PCR with gene specific primers which have T7 promoter sequence upstream appended (Table 2.1).

Double stranded RNA (dsRNA) was produced using MEGAscript®T7 Kit (Life Technologies, USA) and HiScribe T7 In vitro transcription according to the manufacturers’ protocols (NEB, USA). For HiScribe-produced dsRNA, DNase I treatment was done according to RNeasy Mini Handbook page 71 (09/2010) (Qiagen, Hilden, Germany). After clean-up with chloroform and ethanol precipitation, purified RNA was quantified with NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). DsRNA integrities were then checked on a 1.5 % non-denaturing agarose gel prepared with 1x TBE (diluted from 10x TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.3).

### 2.8.2 Nematode ‘soaking’ experiments

For most soaking experiments nematodes were incubated in a basic soaking solution consisting of M9 buffer (22 mM KH₂PO₄, 42.3 mM Na₂HPO₄, 85.6 mM NaCl, 1 mM MgSO₄), 50 mM octopamine, 1.0 mg/mL fluorescein isothiocyanate (FITC) (Sigma Aldrich, 46952), 0.05 % gelatine and 3 mM spermidine. When dsRNA was added to the soaking solution, a final concentration of 2.0 mg/mL was used. Approximately 2000 nematodes were treated in 50 μL of soaking solution in replicates, covered and incubated in the dark at 21-23°C for 16h. After incubation nematodes were transferred to a 15 mL Falcon tube and washed three times with sterile water and centrifugation at 1, 200 g for 2 min each time to remove the soaking solution. Nematodes were suspended in 100 μL of sterile water and assessed for vitality and RNAi phenotypes using an inverted light microscope (Olympus LH50A) while fluorescence from nematodes resulting from FITC uptake was observed using an Olympus BX-51 microscope with FITC filter. Four replicates of self-callusing mini carrot discs were infected with 125 of dsRNA-treated nematodes and harvested after five weeks for observation while the rest were kept in -80°C for RNA extraction and subsequently qPCR for quantification of gene expression.
Chapter 3: Optimising the protocol for uptake of double-stranded RNA by Pratylenchus species

Most of the results in this chapter were published in
3.0 Introduction

The discovery of RNAi in *C. elegans*, in which dsRNA induces the degradation of cognate endogenous mRNA and so prevents synthesis of the encoded protein, has provided a significant new tool to study gene function (Mello and Fire, 1995). RNAi has been investigated in many organisms including mammals, insects, fungi and plants (Romano and Macino, 1992, Kennerdell and Carthew, 1998, Ngô et al., 1998, Elbashir et al., 2001, Bucher et al., 2002). In *C. elegans*, RNAi can be triggered by exogenous dsRNA (100-500 bp long) delivered via soaking in buffered-solutions, by microinjection of dsRNA into the adult worm or by feeding on *Escherichia coli* engineered to produce dsRNA (Fire et al., 1998a, Tabara et al., 1998, Timmons and Fire, 1998, Fraser et al., 2000). This technology, and sequencing of the genome of *C. elegans*, has enabled functional analysis of almost all of its genes, making it the best annotated multicellular organism (*C. elegans* Sequencing Consortium, 1998, www.wormbase.org).

RNAi now provides new opportunities for research on plant parasitic nematodes (PPNs). Delivery of dsRNA to nematode juveniles via ‘soaking’ in solutions containing dsRNA has been used successfully to investigate the function of some genes in cyst nematodes (e.g. *G. pallida* and *H. glycines*), root knot nematodes (e.g. *M. incognita*, *M. hapla* and *M. javanica*) and migratory nematodes such as *R. similis* and *B. xylophilus* (Rosso et al., 2005b, Huang et al., 2006a, Adam et al., 2006, Haegeman et al., 2009, Cheng et al., 2010). For these obligate parasites, uptake of exogenous dsRNA is enhanced by the presence of neurostimulants such as octopamine, resorcinol or serotonin to the soaking solution (Bakhetia et al., 2005, Dubreuil et al., 2007). Presence of dsRNA in the stylet, pharynx and intestinal tract can be been monitored either with fluorescently labelled dsRNA or the fluorescent dye, fluorescein isothiocyanate (FITC), which is usually co-incubated with dsRNA in soaking solutions. Appropriate controls are needed because adverse effects on nematodes have occasionally been reported from FITC and some components of the soaking solution (Urwin et al., 2002, Rosso et al., 2005a, Huang et al., 2006a, Schroeder and MacGuidwin, 2006, Sukno et al., 2007, Adam et al., 2008). There is now good evidence which suggests that RNAi can be used as a control strategy for PPNs in that *in planta* delivery of siRNA/dsRNA reduces nematode establishment and development in culture or pot tests (Huang et al., 2006a, Yadav et al., 2006, Fairbairn et al., 2007, Sindhu et al., 2009). When this project was undertaken there was no published articles to suggest that RLNs were amenable to RNAi.

Although it is clear that *in vitro* RNAi can be a useful tool in functional studies of many plant parasitic nematodes, in some cases the effects elicited have been transient (Bakhetia et al., 2007). It was
postulated that for the effect of RNAi to be effective in the longer term, the nematodes have to be exposed to the dsRNA/siRNA each time they feed. Therefore, it is logical to expect that the best method to deliver dsRNA/siRNA to the nematodes to induce RNAi is via their food source (that is, their host plants). The first reported observation of RNAi-mediated gene silencing via plant delivery was by Yadav and colleagues (2006). Tobacco plants expressing hairpin constructs for the targeted nematode splicing factor and integrase gene not only demonstrated a near complete depletion of target mRNA but also resulted in effective host resistance against *M. incognita*, based on the number of egg masses and considerably smaller galls in transformed plants compared to control (Yadav et al., 2006). Although close examination of this publication suggest that the results presented were maybe not quite as clear cut, since ages of control and transgenic roots look different. This was followed closely by a publication by Huang and co-workers (2006a), also working on RKNs. A 16D10 sequence which is highly conserved among *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* was transformed into *Arabidopsis thaliana* and inoculation of these transgenic plants with all four *Meloidogyne* species convincingly demonstrated that uptake of either intact dsRNA or processed siRNAs can occur and is sufficient to induce an RNAi phenotype (Huang et al., 2006a). *M. incognita* has since been subjected to numerous *in vivo* RNAi experiments, all of which showed the amenability of *M. incognita* to various degrees of host-delivered silencing (Niu et al., 2012, de Souza Junior et al., 2013, Jaouannet et al., 2013, Papolu et al., 2013a).

Host-mediated RNAi has also been observed in cyst nematodes e.g. *H. glycines* and *H. schachtii* (Steeves et al., 2006, Sindhu et al., 2009, Patel et al., 2010). For *H. glycines*, transgenic soybeans expressing dsRNA homologous to the major sperm protein (MSP) gene led to a 68% reduction in egg production while the progenies obtained from MSP transgenic plants were unable to reproduce successfully (Steeves et al., 2006). For transgenic plant studies involving *H. schachtii*, there were fewer developing females after the J2s were fed on transgenic *A. thaliana* (Sindhu et al., 2009, Patel et al., 2010).

In addition to conventional transgenic plants which can take 4 to 6 months to regenerate, hairy root transformation after infection with *Agrobacterium rhizogenes* takes only a few weeks to produce transgenic roots. This approach provides a rapid alternative for functional studies of nematodes using RNAi (Ibrahim et al., 2011, Yang et al., 2013, Iberkleid et al., 2013, Eves-van den Akker et al., 2014). A study using transgenic soybean roots containing hairpins to the genes L-lactate dehydrogenase, mitochondrial stress-70 protein precursor, ATP synthase beta-chain mitochondrial precursor and tyrosine phosphatase individually, led to decreased gall production (2.7-, 2.3-, 12.4- and 19-fold) compared to controls, and retarded the development of *M. incognita* (Ibrahim et al., 2011).
hairy roots expressing dsRNA to Mj-far also attenuated the development of M. javanica J2s into females, when harvested 15 days post infection (Iberkleid et al., 2013). Hairy roots have also been generated with constructs containing 16D10. Like transgenic Arabidopsis with the same target gene (Huang et al., 2006a), transgenic hairy root lines were less susceptible to RKN infection (Yang et al., 2013).

In this chapter, the efficacy of RNAi in P. thornei and P. zeae has been studied using soaking to introduce dsRNA. Possible effects of different components of the soaking solution on the activity of nematodes have been examined. To investigate long term effects of RNAi on P. thornei, a modified carrot disc technique has been adapted for culture and extraction of RLNs for replicated high throughput experimentation. This system involves using sterile carrot mini discs in 24 well plates, for which only 50 nematodes are needed as an initial inoculum on each disc. This system has been used to assess how silencing calponin and troponin C genes delivered by soaking nematodes in dsRNA affected establishment and reproduction of P. thornei. In C. elegans, these two genes are required to maintain structure and contraction of muscles, and hence their orthologues in Pratylenchus spp. would be important genes in these migratory nematodes. Carrot hairy roots were also generated with the troponin C gene to monitor the effect of plant delivered dsRNA on P. thornei.

3.1 Materials and methods

3.1.1 Identification of target genes

P. thornei and P. zeae orthologues of the C. elegans troponin C (pat-10) and calponin (unc-87) genes were used to test the amenability of these RLNs to RNAi. The genes were obtained using amino acid sequences of C. elegans pat-10 (wormbase ID F54C1.7) and unc-87 (wormbase ID F08B6.4c) to query several databases including the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/), Nembase (www.nematode.org) and Nematode.net using TBLASTX 2.2.26+ (Altschul et al., 1997). Translated amino acid sequences of two ESTs from mixed stages P. penetrans with high identities and sequence coverage were selected: BQ627209 (557 nucleotides, nt) was 84% identical to C. elegans pat-10 whereas BQ626831 (524 nt) was 80% identical to C. elegans unc-87. Primers Pppat-10F and Pppat-10R, and Ppuncc-87F and Ppuncc-87R, were designed based on sequences BQ627209 (NCBI) and BQ626831 (NCBI) to amplify the equivalent sequences in P. thornei and P. zeae using cDNAs generated from mixed stages of both P. thornei and P. zeae. PCR products for both
genes differed in size and are designated with the prefixes Pt or Pz for *P. thornei* and *P. zeae* respectively (Table 2.1). Sequences of the ESTs have been deposited in GenBank: *Ptpat-10* (NCBI: JX122489), *Ptunc-87* (NCBI: JX122490), *Pzpat-10* (NCBI: JX122491) and *Pzunc-87* (NCBI: JX122492). cDNA synthesis, PCR and dsRNA synthesis were performed according to section 2.3.

### 3.1.2. Nematode soaking and dsRNA treatments

For all soaking experiments nematodes were incubated as in section 2.8.2, with some modifications. Different concentrations of the following compounds were added to optimise uptake or determine their effects on nematode activity: i) neurostimulants - octopamine or resorcinol; ii) spermidine derivatives - spermidine (Sigma Aldrich, 85561), spermidine trihydrochloride (STH, Sigma Aldrich, S2501) and spermidine phosphate salt hexahydrate (SPSH, Sigma Aldrich, S0381); and iii) Fluorescein isothiocyanate isomer I (FITC) (Sigma Aldrich, 46952). When dsRNA was added to the soaking solution, a final concentration of 1.0 or 2.0 mg/mL was used. Different numbers of nematodes were treated in 200 µL of soaking solution in replicates, covered and incubated in the dark at 21-23°C for the appropriate length of time (see specific experiments). After incubation nematodes were transferred to a 15 mL Falcon tube and washed three times with sterile water and centrifugation at 1, 200 g for 2 min each time to remove the soaking solution.

### 3.1.3. Analysis of RNAi phenotypes and FITC fluorescence intensity in nematodes

Nematodes were suspended in 100 µL of sterile water in wells of a 96 well plate; their vitality and RNAi phenotypes were assessed using an inverted light microscope (Olympus LH50A). Fluorescence from nematodes resulting from FITC uptake was observed using an Olympus BX-51 microscope with FITC filter at 10X magnification. Fluorescence intensities were quantified from captured images using the NIH software ImageJ (http://rsb.info.nih.gov/ij/) (Abramoff et al. 2004). Pixel counts were used to determine the differences in intensity of fluorescence. The fluorescent images were first converted to 8-bit greyscales and a threshold set to obtain the number of fluorescent pixels for each image. The threshold used was the level required to remove all background and auto-fluorescence in control non-fluorescing images, which were kept constant throughout the analysis. Fluorescent intensity, expressed
as percent fluorescence, for representative images (nematodes) was determined by dividing the number of fluorescent pixels for a given nematode by the total pixel area.

### 3.1.4 Carrot hairy root transformation

#### 3.1.4.1 Production of *P. thornei* pat-10 construct

The construct were digested from pDoubler and cloned into pCleaverA, an RNAi vector under the 35S Cauliflower Mosaic Virus (CaMV) gene promoter provided by Dr John Fosu-Nyarko (Murdoch University). Primers were designed to have recognition sites of *Xba*I/*Xho*I and *Bam*H*I/*Kpn*I enzymes for directional ligation into pDoubler. Sense strand was obtained by digesting pDoubler with *Xba*I and *Bam*H*I* while antisense strand was obtained by digesting with *Xho*I and *Kpn*I and cloned into either side of a bean catalase gene (BC) intron in pCleaverA (Fig. 3.1). hpRNA expression cassettes were subcloned into a plant binary vector, pART27 using a single *Not*I digestion. The linearised pART27 vector (5 ng) was dephosphorylated with Antarctic Alkaline Phosphatase according to the manufacturer’s protocol (New England Biolabs Inc. (NEB), Ipswich, UK). The cassettes were then ligated to pART 27 in a 3:1 insert:vector molar ratio. A description of the cloning steps is as shown in Fig. 3.1. The pART27 plant binary vector has the *nptII* gene for plant selection and a spectinomycin gene for bacteria selection.

**Figure 3.1** Construction of hairpin RNAi vectors. (A) The sense strand was generated by digesting pDoubler with *Xba*I and *Bam*H*I* while the antisense strand was obtained with *Xho*I and *Kpn*I digestion. The digested strands were then cloned into either sides of the bean catalase gene (BC) intron site in the pCleaverA vector which also contains the CaMV 35S promoter site, *Not*I restriction sites and a NosA terminator. (B) The *Not*I fragment from pCleaverA containing the hpRNA cassettes were then subcloned into a binary vector such as pART27 and used to transform carrot discs.
3.1.4.2 Confirmation of constructs in vectors

PCR was carried using the following primers (Table 3.1) to confirm the presence of constructs and arms of constructs in pCleaverA and subsequently in pART27. SIntron and S35S primers were used to check presence of sense fragments while ASIntron and ASNosA primers were for antisense fragments. 35SArt and SP6 were used to confirm the presence of the constructs in binary vector, pART27.

Table 3.1: Sequence of primers to check presence of sense and antisense fragments in pCleaverA

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIntronF</td>
<td>TCATCATCATAGACACAGA</td>
<td>320</td>
</tr>
<tr>
<td>S35S</td>
<td>GATTGATGTGACATCTCCACTGA</td>
<td></td>
</tr>
<tr>
<td>ASIntron</td>
<td>TCGTGCTATGATGATGATGA</td>
<td>210</td>
</tr>
<tr>
<td>ASNosA</td>
<td>CATCTCATAAAATACGTCATGCATT</td>
<td></td>
</tr>
<tr>
<td>35SArt</td>
<td>GTCTTGAGAGACCTGCTCGTA</td>
<td>681</td>
</tr>
<tr>
<td>SP6</td>
<td>CATACGATTAGGTGACACTAG</td>
<td></td>
</tr>
</tbody>
</table>

3.1.4.3 Preparation of Agrobacterium rhizogenes competent cells

K599 competent cells obtained from Dr. Leila Eshragi (Murdoch University) were streaked on an LB plate with 50 mg/L rifampicin and incubated overnight at 28°C. K599 was chosen because of its low virulence which could limit the number of Ri T-DNA transformed roots to a number comparable to normal roots. A single colony from the plate was inoculated in 5 mL of LB medium with 50 mg/L rifampicin and left on a shaker set at 225 rpm at 28°C overnight. The overnight culture was added to 50 mL LB medium and shaken for 6 h to reach cell density (OD<sub>600</sub>) of 0.34. When the desired OD was reached, the culture was pelleted at 4,000 rpm at 4°C for 10 min after which cells were resuspended with 10 mL of ice cold 20 mM CaCl<sub>2</sub>. Resuspended cells were pelleted again at 4,000 rpm at 4°C for 10 min and supernatant decanted. Cells were resuspended in 1 mL of ice cold 20 mM CaCl<sub>2</sub> and aliquoted into 100 μl in a 1.7 mL microfuge tube which is then snap frozen in liquid nitrogen and stored at -80°C until use.

3.1.4.4 Transformation of construct into Agrobacterium rhizogenes

After confirmation of constructs in pART27 vector, A. rhizogenes strain K599 was transformed with the constructs by heat-shock. Approximately 500 ng of plasmid DNA was added to 100 μL of competent cells on ice, mixed by gentle flicking and snap-frozen in liquid nitrogen for 5 min. Cells were then thawed in 37°C water bath for 5 min, added with 500 μL of LB medium and left on the shaker at
225 rpm at 28°C for 2 to 4 h. After shaking, the cell suspension was pelleted at 4, 000 g for 2 min and resuspended in 100 µL of LB medium. Resuspended cells were seeded on LB plate with 50 mg/L rifampicin and 150 mg/L spectinomycin and incubated at 28°C for two days. After two days, PCR was done with gene specific primers (Table 2.1) to screen for transformed colonies as section 2.5.3 with some modifications. Colonies picked were inoculated in 20 µL of sterile water of which 7 µL was incubated at 96°C for 10 min before PCR. Following screening, a single positive colony was used to inoculate 10 mL of LB with 10 µL of 150 mg/L spectinomycin and 50 mg/L rifampicin and grown for 2 days at 28°C with vigorous shaking (225 rpm). The bacteria were then centrifuged at 4000 g for 10 min and the supernatants were discarded. The pellets were resuspended in 25 mL of LB containing spectinomycin and left at 28°C on the shaker for approximately 2 h. Cell density (OD) was then measured at 600 nm using Perkin Elmer Lambda 25 UV/VIS Spectrometer using LB medium as blank. When the OD of 0.6 to 0.8 was achieved, the bacteria solutions were removed from 28°C and were used for plant transformation.

### 3.1.4.5 Carrot hairy root transformation

For each construct, 15 carrot discs of 10 mm thickness prepared as in section 2.1 were placed on 2.2 g/L MS basal solution with Gamborg's vitamins (Sigma Aldrich, Australia), 3% sucrose and 0.8 % agar. The pH of all media was adjusted to pH 5.8 before autoclaving. Before transforming the discs, bacterial cultures with the correct OD were centrifuged at 4,000 rpm for 15 min and the supernatant removed. Cell pellets were resuspended with 150 µL of LB and 100 µM of acetosyringone and 10 µL of each culture were used to infect each disc. Injection needles were used to lightly pierce the spot where the cultures were pipetted on the discs, to allow better penetration of the bacteria into the tissue. Controls consisted of carrot discs inoculated with K599 alone and K599 transformed with GFP. Infected carrot discs were incubated in the dark at 26°C for 3 days after which were transferred to moderate light in a culture room. Proliferating discs with 1 cm long roots were selected and these root segments were excised aseptically, put on MS medium with antibiotics (250 mg/L cefotaxime and 150 mg/L kanamycin) and incubated in darkness at 26°C. Roots transformed with K599 only were transferred to antibiotic-free medium. Before nematode infection, the hairy roots (3 per plate) which had been checked for transgenic status by PCR were transferred to MS medium without antibiotics for a week. The growth rate of the hairy roots were measured every two days after excision from carrot discs.
3.1.4.6 Extraction of hairy root DNA

Extraction buffer (2% CTAB, 100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0; adjusted to pH 5.0 with HCl) was used to extract genomic DNA from hairy roots. Before extraction, 1% polyvinylpyrrolidone and 1% β-mercaptoethanol were added to the extraction buffer and placed at 65°C. Approximately 3 cm of root was excised with a sterile blade and transferred into a 1.5 mL microfuge tube. The root was snap-frozen in liquid nitrogen and ground with three 5 mm metal beads in a tissue lyser set at 25 strokes per sec for 3 min. Macerated tissue was added with 500 µL of preheated extraction buffer, vortexed and placed back at 65°C for 45 min. After incubation, the microfuge was centrifuged at maximum speed for 5 min and the liquid phase transferred to a new 1.5 mL microfuge tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the liquid phase and mixed gently for 2 min after which was centrifuged at maximum speed for 2 min. The upper phase was transferred to a new 1.5 mL tube and precipitated with 0.1 volume of 7.5 M ammonium acetate and 2 volumes of ice cold 100% ethanol at -20°C overnight. Precipitated DNA was spun down at maximum speed for 20 min and the supernatant discarded. The pellet was washed by adding 1 mL of 70% ethanol and centrifuging at maximum speed for 5 min. After air-drying, the pellet was resuspended in 20 µL of sterile water and the concentration measured as in section 2.6. PCR was then performed using gene specific primers and 100 to 200 ng of DNA to check for presence of construct in pART27 (Table 2.1).

3.1.4.7 Infection and analysis of transgenic hairy roots.

Approximately 50 P. thornei J2s were used to inoculate each hairy root. Presence of nematodes in the hairy roots was checked after 2 weeks of infection using acid fuchsin staining. After 5 weeks, hairy roots were cut into 2 cm lengths and put in the mist chamber for nematode extraction (Section 2.2). The number of nematodes extracted from hairy roots containing gene of interests were compared to those of the controls. Messenger RNA transcript levels of the genes that were targeted with RNAi in roots at 2 and 5 weeks post infection were determine by qPCR using gene specific primers.
3.2 Results

3.2.1. Ingestion of soaking solution by *P. thornei* and *P. zeae* using FITC

To find an optimum concentration of FITC that enabled uptake of solutes through the stylet and into the gut with minimal detrimental effects on nematodes, six concentrations of FITC (0.1 mg/ml; 0.2 mg/ml; 0.4 mg/ml; 0.8 mg/ml; 1.0 mg/ml; 2.0 mg/ml) were added to the basic soaking solution and nematode behaviour and uptake was observed. The effect of FITC on nematodes was estimated by counting active and inactive or dead nematodes after incubation at 21 °C for 16 h. For each concentration of FITC, 500 mixed stages of *P. thornei* were soaked in basic soaking solution with 50 mM octopamine. For controls, a similar number of nematodes was soaked under the same conditions but without FITC. Two replicates were prepared for each soaking experiment. Nematodes soaked with FITC all fluoresced after 16 h of incubation (Fig. 3.2). With fluorescence localised in the stylet and eosophageal gland cells, it appeared that the nematodes ingested soaking solution via the stylet. The number of *P. thornei* with fluorescence increased with increasing concentration of FITC after 16 h of incubation, except that there was no obvious difference in fluorescence of nematodes incubated in solutions with 0.4, 0.8 and 1.0 mg/mL (Fig. 3.2). However, at 2 mg/mL, fluorescence was very high and similar in all nematodes (Fig. 3.2 K, L).
Figure 3.2 Fluorescence of mixed stages of *P. thornei* incubated for 16 h in basic soaking solution with different concentrations of FITC. (i) (ii) and (iii) Fluorescence resulting from FITC uptake in nematodes, soaked with 1 mg/ml FITC at 2, 4 and 16 h, respectively. A and B = 0.1 mg/ml; C and D = 0.2 mg/ml; E and F = 0.4 mg/ml; G and H = 0.8 mg/ml; I and J = 1.0 mg/ml; K and L = 2.0 mg/ml. Scale bar represents 200 µm.

Also at 2 mg/ml FITC, 98 % of the treated nematodes were motionless (Fig. 3.2 K, L and Fig. 3.3A). Motionless nematodes were considered dead if they did not move after exposure to light and swirling for 20 min. This observation indicates the fluorescent dye could be toxic to *P. thornei* at 2 mg/ml. However, there was no statistical difference between the average viability of *P. thornei* exposed to any of the other five FITC concentrations (0.1 - 1.0 mg/ml) (p<0.05), and there was only a 16 % reduction in viability at 1 mg/mL compared to nematodes soaked without FITC (Fig. 3.3A).

To examine possible effects of longer incubation of nematodes with FITC, a time course experiment was done where mixed stages of both *P. zeae* and *P. thornei* were separately incubated in basic soaking solution with 1.0 mg/mL FITC and the vitality of nematodes assessed at 4, 8 and 24 h. The number of nematodes which showed FITC fluorescence increased with time, with almost half of the nematodes exhibiting obvious fluorescence 8 h after incubation. After 24 h of incubation, almost all nematodes in soaking solutions with FITC fluoresced. On average, there was a 23 % reduction in vitality of *P. thornei* soaked with FITC at the three time points when compared with the controls (without FITC).
(Fig. 3.3B). However, for *P. zeae*, a significant loss of vitality (average of 37%) was observed when soaked in solutions with FITC after 4, 8 and 24 h indicating they were adversely affected by the fluorescent dye (*p* < 0.05, Fig. 3.3B). More than half of *P. zeae* incubated in the basic soaking solution with 1.0 mg/ml FITC were inactive after 24 h. All FITC used in experiments was dissolved in 70% of dimethylsulfoxide (DMSO) before addition to soaking solutions. When 2% dimethylformamide (DMF) was used instead of DMSO almost 100% loss in nematode activity was observed for *P. thornei* after 16 h incubation (data not shown).

![Bar chart A](image1.png)

**A**

![Bar chart B](image2.png)

**B**

**Figure 3.3** Effects of soaking mixed stages of *P. thornei* and *P. zeae* with FITC. (A) Average percentage of active *P. thornei* after soaking with different concentrations of FITC for 16 h. (B) Average percentage of active *P. thornei* and *P. zeae* after soaking with 1 mg/mL of FITC for 4, 8 and 24 h.
3.2.2 Octopamine but not resorcinol is effective in stimulating uptake of solution by *P. thornei* and *P. zeae*

In addition to the 50 mM octopamine used in soaking solutions to investigate optimum FITC levels, four other concentrations of octopamine (5 mM, 10 mM, 30 mM and 100 mM) and 1 % resorcinol were added to soaking solutions with 1 mg/mL FITC to test the possibility of enhancing ingestion by nematodes and to determine whether increased pharyngeal pumping caused by neurostimulants had adverse effects on nematode viability. For each concentration, the number and activity of fluorescing nematodes and the intensities from two replicates of 500 nematodes were observed and compared to nematodes incubated in basic soaking solution without neurostimulants. After 16 h of soaking, fluorescence was observed in almost all nematodes and eggs soaked at all the different concentrations of octopamine (Fig. 3.4). In contrast almost no fluorescence was observed in the stylet of nematodes when no octopamine was added to the soaking solution, although very weak fluorescence (autofluorescence) could be seen in the pharynx and esophageal glands of some *P. thornei* (data not shown). Fluorescence intensity in *P. thornei* increased with increasing concentration of octopamine. Strong FITC signals were observed in the stylet, pharynx and the excretory/secretory pore of *P. thornei* when soaked in the presence of octopamine.

![Figure 3.4 FITC fluorescence of mixed stages of *P. thornei* incubated for 16 h in soaking solution with different concentrations of octopamine: A = no octopamine; B = 5 mM; C = 10 mM; D = 30 mM; E = 50 mM; F = 100 mM. Scale bar represents 200 µm.](image-url)
Concentrations of up to 50 mM octopamine did not seem to affect viability of P. thornei since there was no significant difference in the activity of nematodes incubated with or without octopamine (p<0.05). Activity of P. thornei was only slightly reduced (by 12 %) when soaked with 100 mM octopamine compared to controls (p<0.05) (Fig. 3.5A).

As the number of active P. thornei soaked with 50 mM octopamine did not differ significantly from exposure to 5, 10 or 30 mM octopamine, the effect of longer incubations on viability of both P.thornei and P. zeae soaked with and without 50 mM octopamine was investigated 4, 8 and 24 h after incubation. For each time point, two replicates of 500 mixed stages of nematodes were set up separately and their activity monitored. Both P. thornei and P. zeae appeared normal after 8 hours of incubation with or without 50 mM octopamine (p<0.05) (Fig. 3.5A). A pronounced adverse effect of 50 mM octopamine on nematodes soaked for 24 h is indicated in that there was a 37 % and 41 % reduction in viability respectively for P. thornei and P. zeae (Fig. 3.5B). When 1 % resorcinol, another neurostimulant, was used in place of octopamine and incubated for 4, 8 and 24 h, a drastic reduction in viability of nematodes was observed. More than 90 % of both P. thornei and P. zeae were inactive, and scored as dead, after only 4 h of incubation (Fig. 3.6). Analysis of independent soaking experiments for the different time points indicated 1 % resorcinol is detrimental to both nematode species. This observation was confirmed using fluorescence microscopy where all treated nematodes showed very strong fluorescence indicating increased uptake of solutes compared to nematodes soaked without resorcinol (data not shown), possibly by diffusion through sphincters of dead nematodes. Lower concentrations of resorcinol in soaking solutions were not studied because addition of octopamine provided the desired levels of ingestion via the stylet.
Figure 3.5 Effects of soaking mixed stages of *P. thornei* with the neurostimulant octopamine. (A) Average percentage of active *P. thornei* after soaking with different concentrations of octopamine for 16 h. (B) Average percentage of active *P. thornei* and *P. zeae* after soaking with 50 mM octopamine for 4, 8 and 24 h.

Figure 3.6 Viability of *P. thornei* and *P. zeae* after soaking in a solution containing 1% resorcinol for 4, 8 and 24 h.
3.2.3. *Spermidine enhances FITC fluorescence intensity in P. thornei*

The effect of different concentrations and derivatives of spermidine on uptake by nematodes was also investigated. The intensity of fluorescence of *P. thornei* incubated for 16 h in basic soaking solution plus 1.0 mg/mL FITC and 50 mM octopamine with 5 different concentrations of spermidine (0.5 mM, 1 mM, 2mM, 3 mM and 6 mM), was compared to soaking without spermidine. Nematodes from all the treatments showed increased fluorescence compared to those soaked without spermidine, indicating enhanced uptake of the soaking solution. However, there was greater fluorescence intensity at 3 mM and 6 mM concentrations of spermidine. Similar results were obtained when two derivatives; spermidine trihydrochloride (STH) and spermidine phosphate salt hexahydrate (SPSH) were substituted for spermidine at 3 mM and 6 mM concentrations. Moreover, nematodes soaked with SPSH gave a characteristically sharper, slightly diffuse and intense fluorescence that outlined the shape and internal structures of nematodes more clearly (Fig. 3.7). Fluorescence intensities from representative *P. thornei* nematodes soaked in the three spermidine derivatives were quantified using the Image J software. Nematodes soaked in buffers with 3 mM spermidine displayed 33 % fluorescence intensity compared to 45 % in 3 mM STH and 86 % in 3 mM SPSH. When soaked in buffers with 6 mM of the compounds, *P. thornei* showed 38 % fluorescence for spermidine, 53 % for STH and 96 % for SPSH.

![Figure 3.7 FITC fluorescence from mixed stages of *P. thornei* soaked with 3 and 6 mM of three derivatives of spermidine for 16 h. (A and B) soaking with spermidine. (C and D) soaking with spermidine trihydrochloride. (E and F) soaking with spermidine phosphate salt hexahydrate. (G) soaking with no form of spermidine. (H) water control. Scale bar represents 200 µm.](image-url)
3.2.4. RNAi of calponin [unc-87] of *P. thornei*

To test the amenability of RLNs to RNAi and whether the differences in fluorescence intensities demonstrated by *P. thornei* soaked in buffers containing SPSH correlated with increased dsRNA uptake and reduction in mRNA, RNAi of the calponin gene (*Ptunc-87*) was undertaken for *P. thornei*. Three RNAi feeding experiments were set up, where for each, 2000 nematodes were fed with 2 mg/mL of dsRNA corresponding to *Ptunc-87, gfp* or with no dsRNA in a basic soaking solution that contained 10 mM octopamine. For each feeding experiment, three replicates were set up each with 3 mM spermidine, STH or SPSH and incubated at 21 °C for 16 h, after which nematodes’ viability and behaviour was observed. Nematodes soaked in dsRNA of *gfp* showed no behavioural differences from those soaked without dsRNA. About 90% of nematodes soaked with each of the three spermidine derivatives showed effects of dsRNA ingestion, and exhibited abnormal behaviour consistent with silencing of the *unc-87* gene, including twitching, slow movement, repeated banging of the head against the body and loss of orientation, in which treated nematodes moved in circles and sometimes in waves (Fig. 3.8).

**Figure 3.8** Phenotypes displayed by *P. thornei* and *P. zeae* following soaking in dsRNA of *Ptunc-87* and *Pzpat-10*. (A) loss of sense of direction in *P. zeae* caused by dsRNA of *Ptunc-87* (B) *P. thornei* banging its head onto body caused by dsRNA of *Ptunc-87* (C) Wavy movement in *P. zeae* exposed to dsRNA of *Pzpat-10* (D) Paralysis in *P. thornei* after exposure to dsRNA of *Pzpat-10*. Scale bar represents 200 µm.
The reduction in expression of mRNA of *Ptunc-87* in nematodes treated with dsRNA was quantified using qRT-PCR using expression of actin as an endogenous reference for normalisation. Reduction in transcript accumulation was calculated using the Comparative Ct method (ΔΔct) with reference to expression of the gene in nematodes fed with dsRNA of *gfp* and those soaked with no dsRNA. Nematodes soaked in dsRNA of *Ptunc-87* with all three spermidine derivatives showed drastic silencing of the gene. When compared to expression of *Ptunc-87* in *P. thornei* soaked with no dsRNA, there was 3,373-fold, 3,983-fold and 32,541-fold reduction in transcript accumulation respectively for soaking solutions containing spermidine, STH and SPSH. The trend was similar when reduction in transcript accumulation in *P. thornei* was calculated relative to nematodes treated with dsRNA of *gfp*: with gene knockdown of 10,085-fold, 11,910-fold and 97,289-fold in nematodes soaked in solutions containing spermidine, STH and SPSH respectively (Table 3.2). When expression was normalised using 18s rRNA, the extremely high fold reduction in *unc-87* transcripts in dsRNA-fed nematodes did not differ from results obtained when actin was used. From both analyses, there is a strong indication that increased fluorescence intensity correlated with greater gene silencing when soaked in SPSH, with about a 10-fold larger effect than soaking with STH and spermidine.
Table 3.2: Quantification of reduction in transcript accumulation after dsRNA-mediated gene silencing.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Concentration of dsRNA</th>
<th>Target nematode</th>
<th>Treatment</th>
<th>Fold decrease in transcript levels after dsRNA soaking</th>
<th>compared to expression in nematodes fed without dsRNA</th>
<th>compared to expression in nematodes fed with no dsRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzpat-10</td>
<td>2 mg/mL</td>
<td>P. thornei</td>
<td>Soaking for 16 hr</td>
<td>155.4</td>
<td>261.4</td>
<td></td>
</tr>
<tr>
<td>Pzpat-10</td>
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<td>P. thornei</td>
<td>Soaking for 16 hr + 5 days in water</td>
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<td></td>
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<tr>
<td>Pzpat-10</td>
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<td>P. thornei</td>
<td>Soaking for 72 hr + 2 days in water</td>
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<td>765.4</td>
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<td>Ptunc-87</td>
<td>2 mg/mL</td>
<td>P. thornei</td>
<td>Soaking solution with spermidine for 16 hr</td>
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<td>3373.4</td>
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<tr>
<td>Ptunc-87</td>
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<td>P. thornei</td>
<td>Soaking solution with spermidine</td>
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<td>3983.9</td>
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<tr>
<td>Ptunc-87</td>
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<td>P. thornei</td>
<td>Soaking solution with spermidine phosphate salt hexahydrate for 16 hr</td>
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<td>Pzpat-10</td>
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<td>P. thornei</td>
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<td>Soaking for 16 hr</td>
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3.2.5. RNAi of troponin C [pat-10] of *P. thornei*

When dsRNA corresponding to *pat-10* gene of *P. zeae*, *Pzpat-10*, was fed to 2000 vigorous mixed stages of *P. thornei*, the effect on nematode behaviour was monitored over time by observing the percentage of nematodes exhibiting abnormal behaviour and quantifying the extent of gene silencing. Nematodes were fed with 2 mg/ml *Pzpat-10* dsRNA in a basic soaking solution with 10 mM octopamine, 0.1 mg/ml FITC and the effects compared with those incubated under the same conditions with 2 mg/ml GFP dsRNA and with no dsRNA. The effect of ingesting *Pzpat-10* dsRNA was estimated by observing three samples of nematodes, approximately 650 nematodes at a time, using an inverted light microscope, and counting the number of active nematodes after 2, 4, 8, 10, 12, 16, 24, 30, 36, 48, 60 and 72 h. *P. thornei* nematodes exhibited clear and observable effects on ingesting dsRNA of *Pzpat-10* eight hours after incubation. This included decreased activity manifested in very slow or wavy movement compared to their vigorous activity before feeding (Fig. 3.8). In extreme cases nematodes were paralysed and did not move even after exposure to intense light. No such observations were made for nematodes fed with dsRNA of *gfp* and with no dsRNA. The number of active *P. thornei* fed with *Pzpat-10* dsRNA decreased significantly with time: from 100/650 (about 15 %) at 8 h to almost 85 % (550/650) 72 h after incubation (Fig. 3.9). There was no significant effect of soaking nematodes in dsRNA of *gfp* and without dsRNA (p<0.05) (Fig. 3.9).

![Figure 3.9 Reduction in activity of *P. thornei* after soaking with dsRNA of *Pzpat-10*.](image)
Evidence of *Pzpat-10* transcript knockdown was obtained from assessing transcript levels 16 h after feeding, normalised against expression of 18S rRNA. In addition, to determine the effect of longer incubations in dsRNA and possible recovery of gene expression when the dsRNA trigger was removed, transcript levels were quantified for nematodes kept in water for 5 days in the dark at 25°C following 16 h of incubation in dsRNA, and for nematodes kept in water for 2 days in the dark at 25°C following 72 h of incubation. After 16 h, expression of *Pzpat-10* in dsRNA-treated nematodes showed 155-fold and 261-fold reduction respectively compared to expression in nematodes fed with dsRNA of *gfp* and with no dsRNA (Table 3.1). Gene expression in similar nematodes left in water for 5 days (after initial soaking for 16 h in dsRNA) was 365-fold and 194-fold lower compared to expression in nematodes treated similarly with *gfp* dsRNA and with no dsRNA (Table 3.1). In general, after 72 hours of feeding on dsRNA, nematodes were alive but very weak compared to controls, and ingestion of dsRNA for the duration resulted in higher levels of gene silencing than shorter incubation times (16 h): 826-fold and 765-fold decrease compared to expression of nematodes fed with *gfp*-dsRNA and no dsRNA treatments respectively (Table 3.1).

### 3.2.6. Relative sensitivity of *P. thornei* and *P. zeae* to RNAi

To assess the relative sensitivity of *P. thornei* and *P. zeae* to RNAi, dsRNA of *Pzpat-10* and *Ptunc-87* were fed to both nematode species under the same conditions, their behaviour observed and the extent of transcript reduction assessed. About 500 active nematodes of each species (in triplicate) were incubated for 16 h at 22 °C in a basic soaking solution with 50 mM octopamine, 1 mg/mL FITC and 1 mg/mL dsRNA of either *Pzpat-10* or *Ptunc-87*. The fold decrease in transcripts in dsRNA-treated nematodes was compared to the level of expression in nematodes soaked with dsRNA of *gfp*. Nucleotides corresponding to dsRNA of *Pzpat-10* were 94% similar to *Ptpat-10* whereas the *Ptunc-87* fragment used to construct dsRNA was 87% similar to *Pzunc-87*.

The effects of RNAi on the two genes, both of which were associated with locomotion, were consistent with previous observations where nematodes became sluggish and paralysed (mainly for *pat-10*) and uncoordinated (Fig. 3.8). In both cases the effects on RNAi were more pronounced for *P. thornei* than for *P. zeae*, with about 70% of *P. thornei* becoming inactive compared to 50% for *P. zeae* when fed with dsRNA of *Pzpat-10*. Similarly, about three times more *P. thornei* were observed to have been affected by feeding on dsRNA of *Ptunc-87*. 
The RNAi phenotypic observations were confirmed when reduction of transcript accumulation of both genes was assessed using expression of 18s RNA for each species for normalisation and the fold decrease quantified against expression of both genes from each nematode species fed dsRNA of gfp. Ingestion of Pzpat-10 dsRNA resulted in 50.6-fold reduction in gene expression in P. thornei compared to only 3.6-fold reduction in P. zeae (Table 3.2) Similarly, there was a greater down-regulation of unc-87 for P. thornei (29.9-fold) than for P. zeae (7.8-fold). These results, combined with the observed phenotypes indicate that P. thornei could be more susceptible to RNAi than P. zeae (Table 3.2).

3.2.7. Silencing of pat-10 and unc-87 reduces establishment and reproduction of P. thornei and P. zeae

The mini disc culture system was then used to assess the fitness of nematodes treated with dsRNA of pat-10 and unc-87, to determine their ability to establish and reproduce on the carrot host. To assess the effects of silencing pat-10, four replicates of self-callusing mini carrot discs were each infected with 125 mixed stages P. thornei previously fed with 2 mg/mL dsRNA of Pzpat-10 in a basic soaking solution with 50 mM octopamine for 16 h. The infected discs were kept at 21°C for six weeks and the number of nematodes, extracted using a mist apparatus were compared to that of similar nematodes fed with dsRNA of gfp or no dsRNA. Whilst there was no significant difference in the average number of nematodes extracted from discs infected with nematodes fed with gfp dsRNA and no dsRNA, on average, there was an 81 % reduction in the number of nematodes retrieved from discs infected with Pzpat-10 dsRNA-treated nematodes (p<0.05) (Fig. 3.10A).

In the case of unc-87, the infection assays were also used to assess whether the greater reduction of transcript expression in nematodes fed in the presence of SPSH would affect their establishment and reproduction to a greater extent than those fed in the presence of spermidine and STH. Four replicates of self-callusing carrot discs were each infected with 50 mixed stages P. thornei previously fed with 2 mg/mL dsRNA for 16 h. Like pat-10, the effect of reduction in transcript accumulation of unc-87 in dsRNA-fed nematodes persisted, and strongly reduced establishment and reproduction on the carrot host. This is evidenced in the significantly lower average number of nematodes (29 %) retrieved from discs infected with dsRNA-treated nematodes (from all spermidine derivative feeding solutions) compared to those obtained from discs infected with gfp dsRNA-treated and no dsRNA-treated nematode infection
assays (p<0.05) (Fig. 3.10B). However, there was no significant difference in the number of nematodes retrieved from discs infected with nematodes previously soaked with the three spermidine types (p<0.05). This result indicates that the increased fluorescence intensities and the corresponding greater down-regulation in nematodes soaked with SPSH did not translate into lower establishment and reproduction of nematodes compared to those soaked in STH and spermidine.

**Figure** 3.10 Reduction in reproduction, on carrot mini discs over a 5 week period, of *P. thornei* after treatment with dsRNA of *pat*-10 and *unc*-87. **(A)** Average number of *P. thornei* extracted from carrot mini discs, nematodes were previously soaked with 2 mg/ml dsRNA of *Pzpat*-10 **(B)** Average number of *P. thornei* extracted from carrot mini discs, nematodes were previously soaked in 1 mg/ml dsRNA of *Ptunc*-87 with 3 mM spermidine, spermidine trihydrochloride and spermidine phosphate salt hexahydrate for 16 h.
3.2.8. Silencing of pat-10 via carrot hairy roots diminishes replication capability of P. thornei

Carrot hairy roots were generated with a construct expressing dsRNA of pat-10 to observe the effect of plant-delivered dsRNA to P. thornei. An average of two roots of 1 cm long were observed from each carrot disc, three weeks after the carrot discs were inoculated with A. rhizogenes. The roots were excised from the carrot discs and transferred to MS medium containing 150 mg/ml kanamycin. After 21 days on the MS medium, individual roots grew similarly, showing a mean length of 18.5 cm with a low coefficient of variation 2.7% (Table 3.3). There were significant differences in root elongation rate on different days (p<0.05) after subculture in all three types of transgenic roots. The root elongation was greatest on day 14 (increase by 2.65 cm) and the least on day 2 (increase by 0.06 cm) (Fig. 3.11).

Table 3.3 Comparison of the root elongation means over 20 days culture on medium

<table>
<thead>
<tr>
<th>Day</th>
<th>Root elongation mean (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ptpat-10 RNAi vector</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1.06</td>
</tr>
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<td>16.04</td>
</tr>
<tr>
<td>20</td>
<td>18.45</td>
</tr>
</tbody>
</table>

Figure 3.11 Comparison of the root elongation rate means over 20 days culture on medium
Phenotypically, *Ptpat-10* expressing root lines did not appear any different in terms of development and root morphology or growth compared to control hairy roots (Fig. 3.12). The expression of the kanamycin resistance gene in the different transformed root lines, was also confirmed, where ‘hairy roots’ without a construct did not survive. The transgenic status of carrot hairy roots was confirmed by PCR using gene specific primers, which resulted in a 393 bp product for *pat-10* and a 500 bp product for *gfp* (Fig. 3.12E). Acid fuchsin staining showed that nematodes have entered the experimental and control transgenic hairy roots (Fig. 3.12 i and ii).

**Figure 3.12** Carrot hairy roots on MS medium containing (A) *Ptpat-10* construct (B) *gfp* construct (D) K599 only. These were plated on MS medium with cefotaxime and kanamycin. Growth of hairy roots transformed with K599 only was retarded when plated on MS medium with cefotaxime and kanamycin. (C) K599 only-roots plated on MS medium without kanamycin (i) and (ii) acid fuchsin staining of *P. thornei* inside hairy roots containing *Ptpat-10* construct and hairy roots without construct. Scale bar represents 200 µM. (E) PCR to show the presence of *pat-10* and *gfp* in hairy root containing *Ptpat-10* construct. Lane 46 and 47 represented positive control PCR using *Ptpat-10* primers and *gfp* primers.
To evaluate the effects of *Ptpat-10* on *P. thornei* inoculation, 50 nematodes were used to infect 45 transgenic roots (15 events, 3 replicates) of *Ptpat-10* and *gfp*, as well as K599 only, for five weeks, one week after transfer to antibiotics-free MS medium. After five weeks, reproduction rate of *P. thornei* which fed on roots expressing *Ptpat-10* declined by a factor of 4 to 5 and was significantly different compared to those which infested control roots (Tukey-Kramer HSD test, p<0.05) (Fig. 3.13). On average, the number of nematodes collected from K599 only hairy roots range from 390 to 500 while 320 to 440 nematodes were harvested from *gfp* roots. There was no significant difference between the mean numbers of nematodes from K599 only and *gfp* roots between events (Fig. 3.13). When the average number of nematodes collected from K599 were compared to those from the other control roots, *gfp*, their means were not significantly different from nematodes collected from *gfp* event 1, 3, 9, 10, 13 and 14 but were significantly higher than those from events 2, 4, 5, 6, 7, 8, 11, 12 and 15 (Tukey-Kramer HSD, p≤0.05). From *Ptpat-10* roots, an average of 50 to 121 nematodes were collected after five weeks from fifteen events. *Ptpat-10* event 5 and 8 were the least susceptible with only 50 nematodes harvested while the highest average of nematodes collected was from event 3 with 121. The number of nematodes harvested from events of *pat-10* were not significantly different from each other except from event 3. While the average number of nematodes harvested from event 3 was not statistically higher than those from events 1, 2 and 6, it was different from the rest of the events (Tukey-Kramer HSD, p≤0.05) (Fig. 3.13).

![Figure 3.13](image)

**Figure 3.13** Significant decrease in reproduction of *P. thornei* (Tukey-Kramer HSD, p≤0.05) was seen in 15 events of carrot hairy roots expressing *pat-10* when compared to those in *gfp*-transformed roots and K599 only hairy roots. Error bars indicate the standard error of the mean. Different lower case letters within bars indicate statistically significant differences between means of *pat-10*, *gfp* and K599 only hairy roots between different events, by a one-way ANOVA pairwise multiple-comparison Tukey-Kramer test (p≤0.05).
To determine if the decrease in reproduction rate corresponded with knockdown of *Ptpat-10* gene expression, qPCR was conducted on RNA extracted from *P. thornei* harvested from 15 events, 2 weeks and 5 weeks post infection (wpi). At week 2, there was a 1168-fold (K599 only) and 216-fold (*gfp*) reduction in transcript abundance of *Ptpat-10* in relation to controls. However, the silencing effect dropped to just 121-fold and 86-fold, respectively when harvested five weeks post infection (Fig. 3.14).

![Graph showing qPCR analysis](image)

**Figure 3.14** qPCR analysis show the fold reduction of *Ptpat-10* expression in *P. thornei* two and five weeks after inoculation from 15 different events.

### 3.3. Discussion

This study provides the first detailed report of dsRNA-mediated gene silencing in RLNs by artificial feeding. Significant silencing of two genes, calponin and troponic C was observed in *P. thornei* and *P. zeae* following optimisation of soaking conditions with dsRNA sequences from these two genes. Both genes are essential for structural integrity and proper muscle contraction in nematodes (Goetinck and Waterston, 1994, Terami et al., 1999, Matthews et al., 2004, Ono and Ono, 2004). Vermiform/infected stages of RLNs migrate and feed from cell to cell largely in the root cortex of their hosts and any paralysis or immobility will affect their feeding ability and subsequent survival or reproduction in a host.

RLNs, like most parasitic nematodes, can ingest external solutions with the help of neurostimulants. Basic soaking solutions containing M9 buffer, 0.05% gelatine, 0.5-6 mM spermidine, 10-50 mM octopamine and 0.1 – 1.0 mg/mL FITC, were optimal for uptake by mixed stages of *P. thornei* and *P. zeae*, and allowed fluorescence to be observed in the stylet and intestine without significant detrimental effects on nematode activity after incubation for up to
16 hours. However, concentrations of some components of the soaking medium and incubation times did affect their viability. Resorcinol was not a good neurostimulant to promote dsRNA uptake by these nematodes, because soaking mixed stages of both P. thornei and P. zeae in 1 % resorcinol for only 4 hours reduced their viability by almost 100 % and 80 % respectively. A less detrimental effect has been reported for M. incognita where, although incubation of J2s for up to 4 hours in 1 % resorcinol induced uptake of dsRNA without reducing viability, incubation over 6 hours results in only 10 % lethality (Rosso et al., 2005a). However, for M. javanica overnight incubation of J2s in lower concentrations (0.5 %) killed nematodes without uptake of dsRNA (Adam et al., 2008). When FITC is included in dsRNA soaking solutions for PPNs, concentrations at 1.0 mg/mL or lower are used to trace uptake of external solutions or dsRNA for between 4 and 24 hours. It is known that even at these low concentrations some nematodes are affected. For example, the cumulative hatching rate of H. glycines was reduced when soaked with 0.1 mg/mL FITC (Schroeder and MacGuidwin, 2007). For the RLNs used in our study, we did not observe any significant phenotypic effects of FITC at lower concentrations, but soaking in 1.0 mg/mL for longer than 16 hours appeared to affect viability of P. zeae more than P. thornei.

Addition of spermidine to soaking solutions for dsRNA for RNAi significantly increased target gene knockdown. One explanation is that it neutralises the negative charge of nucleic acids thereby facilitating uptake of dsRNA through the intestine (Maeda et al., 2001). In the experiment done here, addition of the three spermidine derivatives to the soaking solution increased the fluorescence intensified in P. thornei, which was highest at 3 and 6 mM concentrations. A significant observation was that soaking solutions containing spermidine phosphate salt hexahydrate resulted in a more intense fluorescence than was the case for spermidine and spermidine trihydrochloride, and this also suggested increase in dsRNA uptake by nematodes which was confirmed with a 10-fold decrease in transcript accumulation of Ptunc-87. However, the greater reduction in transcript was not associated with a corresponding reduction in reproduction of P. thornei on carrot discs compared to nematodes soaked with spermidine and spermidine trihydrochloride. This is partly because RNAi is not a stochastic process, and it is possible that a threshold level of dsRNA/siRNA is needed to trigger silencing (of this particular gene) and any additional dsRNA molecules taken up may not necessarily increase the effect. Such a threshold level may have been achieved in each of the three experiments with the spermidine derivatives. Despite this observation, substituting spermidine phosphate salt hexahydrate could provide better phenotypic characterisation of nematodes in RNAi soaking experiments and also enhance uptake of dsRNA, which could enhance RNAi effects depending on the target gene.
The effectiveness of RNAi in nematodes depends on several factors, including the turnover of the target gene, susceptibility of the organism to RNAi, the amount of dsRNA delivered, the base composition and position of dsRNA construct in a target gene, the length of dsRNA sequence, mode of delivery and type of target tissue (Fire, 1999, Orii et al., 2003, Huang et al., 2006a, Kimber et al., 2007, Haegeman et al., 2009, Jones and Fosu-Nyarko, 2014). This study showed that gene silencing induced by soaking nematodes with dsRNA effectively reduced the expression levels of both troponin C and calponin of P. thornei and P. zeae, but the extent depended on the nematode species, the target gene and the concentration of dsRNA used. Transcript knockdown of Ptpat-10 in P. thornei induced by 2 mg/mL after 16 hours was three times higher than when 1 mg/mL of dsRNA was used. Similarly for unc-87, an increase in dsRNA concentration from 1 mg/mL to 2 mg/mL resulted in a more effective gene knockdown than for the pat-10 gene. Increasing the level of dsRNA of Ptunc-87 resulted in an almost complete absence of target gene expression: results were similar whether expression was normalised with 18s rRNA or actin. However, feeding with a higher concentration of dsRNA does not always ensure an increase in RNAi effect. In a similar experiment when expression of a parasitism gene in H. glycines was downregulated using 2.5 mg/mL and 5 mg/mL of dsRNA, the two-fold increase in concentrations of the dsRNA trigger did not improve the level of gene silencing, an observation attributed to the nature of the gene and possibly the position of the dsRNA construct in relation to the target gene sequence (Sukno et al., 2007).

In our experiments there was a clear difference in susceptibility between P. thornei and P. zeae to RNAi. When both nematodes were fed with the same amounts of dsRNA constructs of pat-10 and unc-87 under the same conditions, the behaviour of more P. thornei was affected and there was also a greater reduction in transcript for both genes in this species. The sequence of the dsRNA of Pzpat-10 used for the soaking experiments was 94 % similar to Ptpat-10 whereas that of Ptunc-87 was 87 % similar to Pzunc-87. To find a possible explanation for this result, we used si-Fi [http://labtools.ipk-gatersleben.de/] to determine the number of possible siRNAs of 18-25 nucleotides that could be generated from each dsRNA, and the number of efficient siRNAs that could target the ESTs of P. thornei and P. zeae corresponding to the genes. The parameters used were: (1) A siRNA must have a G/C content of between 35-60 (2) the antisense strand should start with an A/U and (3) at least 3 of the first 7 nucleotides at the 5’ end of the antisense strand are A/U (Lück and Douchkov, 2009). Because the Ptunc-87 dsRNA sequence differed by 13 % from that of P. zeae, it was expected that more siRNAs with perfect matches would be generated for P. thornei (Table 3.4). This is consistent with the greater knockdown of unc-87 in P. thornei (29.9-fold reduction) compared to the 7.8 fold reduction in
mRNA transcript in *P. zeae*. The sequence of *Pzpat-10* dsRNA used in the soaking experiments was 100 % and 94% similar to the ESTs of *P. zeae* and *P. thornei*. Consequently, there were consistently more significant total and efficient siRNAs of all sizes generated as possible triggers for RNAi of the *P. zeae* target gene (Table 3.4)(p<0.05). However, this result was not consistent with the response of *P. zeae* to soaking in *Pzpat-10* dsRNA. A possible explanation is that there is a higher siRNA threshold for gene knockdown in *P. zeae*, at least for the *pat-10* gene. Such results are not surprising since differences in susceptibility to RNAi have been reported for species of *Caenorhabditis*, and it cannot be assumed that RNAi will be equally effective in even closely related nematode species (Descotte and Montgomery, 2003, Nuez and Felix, 2012). Such differences could reflect the diversity in RNAi effectors/genes in different nematode groups and that RNAi pathways may differ between nematode species (Dalzell et al., 2011, Rosso et al., 2009). One conclusion is that it provides experimental support that sequences used for dsRNA constructs to silence genes in nematodes do not need to be 100 % identical to a target gene. It also follows that, depending on the gene targeted, a sequence from one nematode species (of a similar genus) may be used to silence orthologous genes in another nematode species irrespective of whether the gene sequence(s) of the latter is known.
Table 3.4 Predicted siRNAs generated from dsRNA of Ptunc-87 and Pzpat-10 for silencing the corresponding genes in *P. thornei* and *P. zeae*

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA size (bp)</th>
<th>Number of siRNAs generated</th>
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<tr>
<td></td>
<td></td>
<td><em>P. thornei</em></td>
<td></td>
<td><em>P. zeae</em></td>
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<tr>
<td></td>
<td></td>
<td>All hits</td>
<td>Efficient hits</td>
<td>All hits</td>
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<tr>
<td>Ptunc-87</td>
<td>18</td>
<td>153</td>
<td>40</td>
<td>290</td>
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<td></td>
<td>19</td>
<td>136</td>
<td>51</td>
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<td>25</td>
<td>125</td>
<td>30</td>
<td>282</td>
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<tr>
<td>Pzpat-10</td>
<td>18</td>
<td>116</td>
<td>26</td>
<td>12</td>
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<td>19</td>
<td>103</td>
<td>33</td>
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<td>25</td>
<td>113</td>
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Soaking *P. thornei* in dsRNA of *pat*-10 for 24 h resulted in a 3 fold reduction in transcript level compared to expression after soaking for 16 h, indicating that longer soaking times can increase gene knockdown. This must be balanced by possible deleterious effects of longer soaking times on the viability of nematodes. Although there appears to be some recovery in *pat*-10 transcript levels when nematodes soaked for 16 h were removed from the dsRNA trigger, the effect of the target knockdown persisted and affected the reproduction of the treated nematodes cultured on carrot discs such that there was an 81 % reduction in numbers 5 weeks after infecting the carrot discs. Similarly, *P. thornei* soaked with dsRNA of Ptunc-87 failed to replicate normally after soaking, and only 29 % of control values were obtained 5 weeks after infection. The mini carrot disc method for culturing and extracting all stages of both *P. thornei* and *P. zeae* was convenient and suitable for assessing the longer term effects of gene silencing after soaking in dsRNA. The culturing on mini discs will be a useful tool for functional analysis of RLN genes, for which two transcriptomes have recently been published (Haegeman et al., 2011, Nicol et al., 2012).
A hairy root system was also used as an ‘in vivo’ approach to silence the expression of pat-10 in *P. thornei* and to evaluate the consequential effects on parasitism. Carrot hairy root lines possessing the RNAi constructs led to a 77% reduction in reproduction of *P. thornei* when harvested five weeks after inoculation. The percentage of replication of *P. thornei* growing on the hairy root system was slightly higher than that obtained from the soaking experiments (85%). This could be because in the soaking experiments, nematodes were ‘forced’ to take in the components of the soaking medium using octopamine, whilst in the hairy root system, nematodes were free to feed at will. Also, in the *in vitro* system, *P. thornei* fed with *pat-10* showed some recovery after the dsRNA trigger was removed. However, the silencing of *Ptpat-10* gene expression of transgenic hairy roots containing RNAi constructs measured at week 5 post infection decreased from 1168-fold to 121-fold compared with no dsRNA control. A possible explanation for this observation is that at week 2, there were more active nematodes, therefore the target gene silencing was more pronounced. At week 5, the only nematodes remaining were those which managed to escape the RNAi effect, hence the extent of silencing was less. However, more work e.g. developmental expression of *pat10* has to be done to explain the extent of silencing as it depends on the inherent stage specific expression of target gene. Alternatively, the decrease in silencing effect at week 5 could be due to the fact that the nematodes have adapted and adjusted their production levels of *pat-10* accordingly to negate the effects of RNAi. At week 2, the nematodes were not prepared against *pat-10* silencing hence a stronger RNAi effect was observed. Nevertheless, this study demonstrates that the hairy root system can also be used for functional studies of the effects of target gene silencing for root lesion nematodes.

This work clearly shows that the RLNs *P. thornei* and *P. zeae* are amenable to dsRNA-mediated RNAi via soaking in dsRNA. In addition, it also shows that *P. thornei* is also susceptible to RNAi after *in vivo* delivery of siRNAs/dsRNA from cells in hairy roots. Therefore, RLNs which are migratory endoparasites, do not differ in this respect from other plant parasitic nematodes studied. The results provided here are also consistent with the recently published transcriptomes of *P. thornei* and *P. coffeae*, in which evidence is provided for the presence transcripts involved in the exogenous RNAi pathway (Haegeman et al., 2011, Nicol et al., 2012). The significance of the results presented here is that they show that RNAi can be used to study gene function in root lesion nematodes, and that gene silencing of vital genes has the potential to be used as a control strategy for this important group of crop pests.
Chapter 4: Putative parasitism genes of *Pratylenchus coffeae*, *Pratylenchus thornei* and *Pratylenchus zeae*
4.0 Introduction

Until recently, study of root lesion nematodes had been relatively neglected. This is despite their economic importance and destructive nature, in that their entry into host roots not only damages the root tissues directly, but also encourages secondary infestations by other soil pathogens. The lifestyle of root lesion nematodes inside a host differs from that of sedentary endoparasites because they do not induce elaborate modifications of host cells in the form of syncytia or giant cells. However, the repertoire of proteins which they may possess and use may be similar for host invasion and suppression or evasion of host defences. The identification of putative parasitism genes will aid in understanding the molecular interactions of host and phytoparasitic nematodes – especially which ones are involved in entry, migration and evading host defences. Secretory proteins produced in the oesophageal, amphidial, and rectal glands and in the hypodermis and intestine, are thought to play essential roles in plant parasitism (Davis et al., 2000).

Before the advent of next generation sequencing, only about 2000 ESTs were present in NCBI for root lesion nematodes, with the majority from Pratylenchus penetrans (Mitrev et al., 2004). However the recent completion of transcriptomes of three species of root lesion nematodes, Pratylenchus coffeae (Haegeman et al., 2011), Pratylenchus thornei (Nicol et al., 2012) and Pratylenchus zeae (Fosu-Nyarko et al., 2014) has shed more light on these nematodes and how they interact with their hosts. From these research projects, new and known parasitism genes have been identified. The information obtained is of great importance because these genes may serve as targets for potential development of new strategies for nematode control. Although belonging to the same genus, there are variations in the types and numbers of genes in different plant nematode species. The objective was to mine these resources for candidate parasitism genes by identifying and comparing members of secreted products from these transcriptomes.
4.1 Material and Methods

4.1.1 Annotation of transcripts for putative functions in parasitism

Contigs and singletons of the Pratylenchus coffeae and P. thornei transcriptomes were assembled according to data obtained from NCBI (P. coffeae sequence read archive, SRA028814; P. thornei transcriptome shotgun assembly, JO845319-JO845338 and JL859810-JL866456) (Haegeman et al., 2011, Nicol et al., 2012) while data from P. zeae were obtained from Dr. John Fosu-Nyarko (Murdoch University, Western Australia). After assembly using SoftGenetics NextGENe V2.16 software and the Condensation Tool, sequences which had matches to genes previously characterised as being involved in parasitism were searched for in the three transcriptomes. Putative parasitism genes were determined from published literature (Haegeman et al., 2011, 2012). Searches of transcripts encoding parasitism genes were performed mostly on the annotated sequences of plant parasitic nematodes- six root-knot nematodes: Meloidogyne arenaria (Ma), M. chitwoodi (Mc), M. hapla (Mh), M. incognita (Mi), M. javanica (Mj), M. paranaensis (Mp), five cyst nematodes: Globodera mexicana (Gm), G. pallida (Gp), G. rostochiensis (Gr), Heterodera glycines (Hg) and H. schachtii (Hs), migratory nematodes: P. penetrans (Pp), P. vulnus (Pv) and Radopholus similis (Rs), stem nematode Ditylenchus africanus (Da), the mycophagous nematode A. avenae (Aa), and one animal parasitic nematode, A. suum (As). Transcripts were subjected to tBLASTx to confirm the identity of these annotated sequences and those with matches to a particular gene of interest with an E-value of $1E^{-05}$ or lesser, were aligned using ClustalW to determine if a full-length gene could be obtained. Selected sequences were further analysed using the pipeline in Fig. 4.1. Aligned sequences were used as input for the ExPASy Translate tool (http://web.expasy.org/translate/) which generates polypeptide sequences in different reading frames. These amino acid sequences were subjected to SignalP analysis (http://www.cbs.dtu.dk/services/SignalP/) to determine the presence and cleavage sites of signal peptides characteristic of a protein secreted via the classical pathway (Bendtsen et al. 2004). Sequences were also screened with transmembrane hidden Markov models (TMHMM v.2) (http://www.cbs.dtu.dk/services/TMHMM-2.0/) to exclude proteins with predicted transmembrane regions (Krogh et al. 2001). Sequences with no transmembrane regions were submitted to SecretomeP analysis (http://www.cbs.dtu.dk/services/SecretomeP/) to identify proteins that could be secreted via the non-classical secretory pathway (Bendtsen et al. 2004). TargetP (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT analysis were performed.
to predict the subcellular localisation of putatively secreted proteins (Emanuelsson et al. 2000, Horton et al. 2006) (Fig. 4.1).

Figure 4.1 Processing pipeline to identify potential secreted proteins from *Pratylenchus* spp. transcriptomes. Secreted proteins are characterised by the presence of a signal peptide (predicted using SignalP) and lack of transmembrane domain (predicted using TMHMM). However, proteins could also be secreted via an alternative pathway. The identity of such proteins could be predicted using SecretomeP. Transcripts which are annotated as localised extracellularly by TargetP and WoLF PSORT were also considered to be secreted.

**4.1.2. Spatial expression of the genes amplified**

**4.1.2.1 Probe generation**

As *P. thornei* is the focus of this study, downstream functional characterisation of putative parasitism genes was undertaken for *P. thornei*. *In situ* hybridisation was also done to determine the spatial expression of the genes of interest. To generate template for *in situ* hybridisation probes, target genes were digested with *EcoR*I and *Xho*I from pDoubler (Fig. 4.2). Probes were generated by adding 1 µg of template DNA, 1 µL of 50 U/µL of T7 RNA polymerase, 2 µL of 10X rNTP (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP and 3.5 mM DIG-UTP) (Roche, Switzerland), 2 µL of 10X transcription buffer, 1 µL of 10 U/ µL Rnase Inhibitor and topped up with DEPC-treated water for a final volume of 20 µL which were incubated at 37°C for
2 h. Purification of probe and yield estimation were then performed according to The DiG System User’s Guide for Filter Hybridization (Boehringer Mannheim).

Figure 4.2 Target genes amplified with primers containing restriction sites XbaI and XhoI on one primer and BamHI and KpnI on the other primer were digested and then ligated into pDoubler.

4.1.2.2 In situ hybridization

In situ hybridisation was done according to de Boer et al. (1998) with some modifications. Briefly, approximately 1000 P. thornei for each probe were fixed with 2% paraformaldehyde for 18 h at 4°C, followed by 4 h at 22°C. Fixed nematodes were then resuspended in 0.1x paraformaldehyde and an aliquot of 150 µL was placed on a slide and cut with a razor blade under a dissection microscope. Overnight hybridisation was done in hybridisation buffer with approximately 300 ng/mL probe at 55°C. Labelling was then performed by incubating the nematode fragments in 0.5 mL blocking solution containing 750 mU/mL alkaline-phosphatase-conjugated sheep anti-digoxigenin (Roche) for 2 h. Alkaline phosphatase activity was detected using 5 µL of 18.75 mg/mL nitroblue tetrazolium chloride/9.4 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67% DMSO stock solution (Roche) at 4°C overnight. The nematodes were then examined using a differential interference contrast microscope.
4.2 Results

4.2.1. Identification of nematode parasitism genes

After processing and assembly of reads, 14,409 contigs and 95,005 singletons were obtained from *P. coffeae*, 17,323 contigs and 255, 853 singletons from *P. thornei* and 10,163 contigs and 139,014 singletons from *P. zeae*. Out of these, 179 contigs and 211 singletons of *P. coffeae*, 121 contigs and 406 singletons for *P. thornei* and 94 contigs and 679 singletons for *P. zeae* had significant matches to 26 sequences previously linked to parasitism processes (NCBI BLASTP E-value of ≤ 1E-05) (Fig. 4.4, Table 4.1). From these 26 putative parasitism genes (PPGs), a total of 150 contigs of *P. coffeae* have significant matches (E-value ≤ 1E-05 from tBLASTx) to EST sequences from cyst nematodes (CNs), 143 matches to EST sequences of root knot nematodes (RKNs), 139 matches to ESTs from migratory nematodes (MNs) and only 86 matches to EST sequences from *A. suum* (Fig. 4.3). Singletons of *P. coffeae* also have the most hits with ESTs of PPGs from CNs with 322 matches, followed by 169 matches with ESTs from RKNs, 117 with ESTs from MNs and 101 ESTs from *A. suum*. Conversely, the number of matching parasitism transcripts from *P. thornei* were similar for CNs (88, 335), RKNs (89, 362) and migratory nematodes (83, 336). For *P. zeae*, 70 contigs matched ESTs of PPGs from *Meloidogyne* spp., 60 and 61 ESTs matches to CNs and migratory nematodes and 35 ESTs to *A. suum*. The highest number of singletons matching to PPGs belonged to the EST sequences from CNs, with 421 matches, followed by RKN (387), MNs (355) and *A. suum* (195) (Fig. 4.3).

![Figure 4.3 Number of Pratylenchus contigs and singletons matching to other parasitic nematodes. CN: cyst nematodes; RKN: root knot nematodes; MN: migratory nematodes, Pc: P. coffeae, Pt: P. thornei, Pz: P. zeae.](image-url)
All of the genes identified from the *P. coffeae* transcriptome (Fig. 4.4) had matches to contigs and singletons, except for ubiquitin extension protein and Skp-1 which only matched contigs most similar to those of *G. rostochiensis* and *R. similis*, respectively. From the *P. thornei* transcriptome, four genes (cellulose binding protein, xylanase, probable protein kinase C substrate, Hgg-23 and *M. incognita* putative esophageal gland cell secretory protein 2, msp2) were found to match only singletons while a contig-only match was found for Skp-1, which was most similar to that of *Meloidogyne* spp. and *H. glycines* putative esophageal gland cell secretory protein 13, hsp13. Although chorismate mutase (*cm-1*), a gene whose product is thought to be used by PPNs to suppress host defences, by manipulation of the endogenous shikimate pathway, was identified in *P. thornei*, it has an *E*-value which is higher than 1E-05, and therefore not considered to be a definite match. However, tBLASTx searches showed that the singleton matched the *G. pallida* *cm-1* gene, alternatively spliced (gi HM148923.1) and *G. tabacum* *cm-1* gene, alternatively spliced (gi HM148927.1), both with 43% amino acid identity. Similarly, Skp-1 was the only gene in the *P. zeae* transcriptome that had no match to singletons. Conversely five parasitism genes were found only in singletons from the *P. zeae* transcriptome, which were calreticulin/calnexin, glutathione synthetase, peroxiredoxin, pectate lyase and polygalacturonase (Fig. 4.4). The *P. zeae* transcriptome also encodes a homologue of polygalacturonase, which is most similar to the nucleotide sequence of *M. incognita* (AY098646.1) (*E*-value 4E-23, total score: 361). Polygalacturonase was not found in the other two transcriptomes. In all three transcriptomes, there were (groups of) genes which had abundant matching transcripts e.g. the group of proteases which are secreted by all plant parasitic nematodes: aminopeptidase, cysteine proteases (cathepsin B, D, L, S, Z) and serine protease, and products possibly utilised by PPNs to suppress host defences e.g. glutathione peroxidase (*Gpx*), glutathione reductase (*Gr*), glutathione-S-transferase (*Gst*), peroxiredoxin (*Prx*), RANBP, superoxide dismutase (*SOD*), thioredoxin (*Trx*). Another multiple-transcript matching gene identified, which also have putative functions in the evasion of host defences, is the fatty acid and retinol binding protein (*Sec-2/Far-1*) (Prior et al., 2001, Iberkleid et al., 2013) (Table 4.1).

Cell wall degrading enzymes (β-1, 4-endoglucanase, pectate lyase, polygalacturonase, xylanase) secreted by plant parasitic nematodes during tissue migration, were also identified with significant similarity (*E*-value ≤ 1E-05) to those characterised for *Meloidogyne*, *Globodera*, *Heterodera*, *R. similis* and *P. penetrans* (Smant et al., 1998, Uehara et al., 2001, Gao et al., 2002, Haegeman et al., 2008). One of the highest number of matching transcripts for a gene in all three transcriptomes was for transthyretin-like protein (*ttl*) and precursors, with a total of 30, 97
and 121 transcripts of *P. coffeae*, *P. thornei* and *P. zeae*, respectively, all of which have the best matches to *R. similis* ttl-1 to ttl-4 (*E*-value ≤ 1E-05) (Table 4.1). Other genes that have matching transcripts in the *Pratylenchus* transcriptomes, which have high similarity to those of sedentary endoparasitic nematodes are 14-3-3/b protein (total score – Pc: 824, Pt: 1720, Pz: 1354), calreticulin or calnexin (total score – Pc: 864, Pt: 1094, Pz: 701), annexins (total score – Pc: 798, Pt: 885, Pz: 1357), galectin (total score – Pc: 718, Pt: 1859, Pz: 1563) and FMRFamides (total score – Pc: 872, Pt: 1081, Pz: 1430) (Table 4.1).
Figure 4.4 Number of contigs (A) and singletons (B) in the *P. coffeae*, *P. thornei* and *P. zeae* transcriptomes that matches putative parasitism genes in other phytoparasitic nematodes and *A. suum*. Number of contigs (C) and singletons (D) in the *P. coffeae*, *P. thornei* and *P. zeae* transcriptomes that matches genes identified from oesophageal gland cells of *H. glycines* and *M. incognita* (Gao et al., 2001, Wang et al., 2001, Huang et al., 2003). Note: Ampep: Aminopeptidase; Crt: calreticulin; UbiExPro: ubiquitin extension protein; Skp-1: S-phase associated kinase protein; Gpx: glutathione peroxidase; Trx: thioredoxin; Sod: superoxide dismutase; Nex: annexin; GST: glutathione-S-transferase; Grx: glutathione reductase; GS: glutathione synthetase; Prx: peroxiredoxin; Gal: galectin; Ctp: cathepsin; Far: fatty acid and retinol binding protein; Ttl: transthyretin-like protein; Vap: venom allergen-like protein; Pel: pectate lyase; Eng: cellulase; Pg: polygalacturonase; Cbp: cellulose binding protein; Rbp: Ran binding protein; Sp: serine protease and Xyl: xylanase. Hsp11: hypothetical oesophageal gland cell secretory protein 11; hsp13: *H. glycines* putative esophageal gland cell secretory protein 13; hgg-14: *H. glycines* probable polyprotein allergen; Hgg-23: probable protein kinase C substrate; msp2:*M. incognita* putative esophageal gland cell secretory protein 2.
Analysis was also performed to identify if sequences from the transcriptomes have matches to ‘pioneer’ genes identified from oesophageal gland cells of *H. glycines* and *M. incognita* (Gao et al., 2001, Wang et al., 2001, Huang et al., 2003). A total of 15 transcripts of *P. coffeae*, 35 of *P. thornei* and 38 of *P. zeae* were found to have matches to *H. glycines* hypothetical oesophageal gland cell secretory protein 11 (hsp-11) (E-values ≤ 1E-05) when entered in NCBI tBLASTx. The transcriptomes also contained transcripts that have similarities to hsp13 (AF343567), Hgg-14 (AF344866), Hgg-23 (AF344868) and msp2 (AF531161) (Table 4.1). However only the *P. coffeae* transcriptomes have transcripts which match Msp-31 (AY142121) and Msp-32 (AY142116) (Huang et al., 2003). No matching transcripts were found in the transcriptomes for the remaining ‘pioneer’ gene sequences identified (Gao et al., 2001, Wang et al., 2001, Huang et al., 2003). Other genes with no significant matches to transcripts in the *P. coffeae, P. thornei* and *P. zeae* transcriptomes include cyst nematode effector proteins, 19C07 and 10A06, *Meloidogyne* parasitism protein, 16D10, amphid-associated effectors, MAP-1 and HYP effector-like, as well as CLE-related proteins (E-values ≤ 1E-05).
Table 4.1: *P. coffeae*, *P. thornei* and *P. zeae* transcripts with the most significant similarities to PPGs of PPNs, the length of best matching transcript and their E-values.

<table>
<thead>
<tr>
<th>Nematode parasitism genes</th>
<th>Total score and accession number of best matching gene*</th>
<th>Length of best matching transcript (bp)</th>
<th>E-value</th>
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<td>14-3-3</td>
<td>824 (CB931457.1)</td>
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<td>Calnexin / Calreticulin</td>
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<td>Endoglucanases (and precursors)</td>
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<td>296 (AF531161.1)</td>
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4.2.2. In silico characterisation of putative parasitism genes of the three Pratylenchus spp.

Transcripts putatively encoding secreted proteins were identified using an in silico analysis of the consensus sequences of the 26 PPGs (Table 4.1), after which were analysed using a series of bioinformatics algorithms, including ExPASy Translate, SignalP, SecretomeP, TargetP, WoLF PSORT and TMHMM. In this context, to be considered a potential secretory protein, the transcript must fulfil at least two of the five criteria: predicted to have signal peptide by SignalP, possess no transmembrane domains, NN-score exceeding the normal threshold by 0.5 when entered into SecretomeP, predicted to contain a secretory pathway SP in TargetP and/or have extracellular localisation predicted by WoLF PSORT. It should be noted that the transcripts used for analysis in this study do not necessarily have full-length and were consensus sequences of PPGs which could be different regions of the genes.

Seventy six amino acid sequences: 26 from *P. coffeae*, 24 from *P. thornei* and 26 from *P. zeae* (Fig. 4.4), were fed into the SignalP (NN) algorithm to search for signal peptides (SP) for protein secretion and cleavage sites. A total of five proteins from the three transcriptomes was identified with a signal peptide (SP) (D-score probability of 0.450 or higher in the NN algorithm): aminopeptidase (*P. coffeae*), TTL (*P. thornei* and *P. zeae*) and FMRFamide and SEC-2 (*P. zeae*). The 76 amino acid sequences (with and without predicted SP) were also analysed by TMHMM to predict any transmembrane domains. None of the amino acid sequences were predicted to have a transmembrane domain. Transcripts without SPs were then fed into SecretomeP to find out if they were secreted via the non-classical pathway. Of these, 39 sequences (15 from *P. coffeae*, 11 from *P. thornei* and 13 from *P. zeae*) were identified with an NN-score exceeding the normal threshold by 0.5 (Table 4.2), indicating that they could possibly be secreted via another channel besides the classical pathway. To further confirm the identity of the proteins, TargetP was employed to predict their subcellular localisation, based on the predicted presence of the N-terminal pre-sequences: mitochondrial transit peptide, secretory pathway signal peptide or other locations. The result of TargetP analysis reflected the findings from SignalP in that aminopeptidase (*P. coffeae*), TTL (*P. thornei*) and FMRFamide, SEC-2 and TTL (*P. zeae*) were all predicted to contain an SP (Reliability Class, RC>0.8). The only variation was that transcripts encoding aminopeptidase from *P. thornei* and cellulose binding protein from *P. coffeae* were also predicted to contain secretory pathway SP (RC>0.8) by TargetP. Since the reliability class of all predictions exceeds 0.8, the predictions were considered the safest by TargetP. Finally, WoLF
PSORT was used to predict the localisation of the proteins. Extracellular localisation was predicted for six proteins of *P. coffeae* and nine proteins for both *P. thornei* and *P. zeae* (Table 4.2). Based on the bioinformatics analyses, all transcripts encoding cell wall modifying enzymes: β-1,4-endoglucanase, CBP, pectate lyase, polygalacturonase and xylanase, were indicated as putatively secreted when present, for all three species of *Pratylenchus*.

Some proteins which are thought to be involved in suppression/evasion of host defences were also predicted to be secreted according to the bioinformatics workflow, namely SEC-2, VAP-1 (MSP-1), by *P. coffeae*, *P. thornei* and *P. zeae* (Table 4.2). The transcript encoding *Pc*-SEC-2 was predicted to have a SP, an extracellular localisation and no transmembrane domains while transcripts encoding *Pt*-SEC-2 and *Pz*-SEC-2 potentially have an SP according to TargetP, were localised extracellularly according to WoLF PSORT and do not have transmembrane domains (TMHMM). All three transcripts encoding *Pratylenchus* VAPs were predicted to be secreted because they have NN-score exceeding the normal threshold by 0.5 when entered into SecretomeP and absence of transmembrane domains. In addition, TargetP predicted the potential presence of secretory pathway SP in all transcripts encoding VAPs in this study. Other proteins which could be secreted and which were found in all three *Pratylenchus* species included calreticulin (CRT) and cathepsins (CTP). Both CRT and CTP were predicted to be secreted by SecretomeP (NN score > 0.5) and they lacked transmembrane domains. In addition, an extracellular localisation was predicted for the *P. zeae* transcript matching to CRT and CTPB entered into WoLF PSORT.

There were six proteins that were predicted to be secreted in one or two transcriptomes but not in all: these included RANBPM, serine protease (Sp) and SOD in *P. coffeae*, aminopeptidase (AMP) in *P. thornei* and *P. coffeae*, 14-3-3b and SXP-1 in *P. coffeae* and *P. zeae* (Table 4.2). The transcripts encoding *Pc*-RANBPM, *Pc*-SOD, *Pc*-AMP, *Pc*-Sp, *Pc*-SXP-1 and *Pz*-SXP-1 could putatively be secreted via a non-classical pathway because they have NN score > 0.5 (SecretomeP). Additionally, *Pc*-RANBPM, *Pt*-Sp, *Pt*-FMRFamide and *Pz*-FMRFamide were also predicted to be localised extracellularly by WoLF PSORT. For *Pt*-AMP, it was predicted by TargetP to contain a secretory pathway SP, alongside *Pt*-FMRFamide and *Pz*-FMRFamide. According to the bioinformatics workflow, ten proteins were not predicted to be secreted in the three *Pratylenchus* transcriptomes, as they do not meet at least two of the five criteria to be a secreted protein. These products are those with possible involvement in mitigating oxidative stress e.g. glutathione reductase, glutathione-S-transferase, glutathione synthetase,
peroxiredoxin and thioredoxin, as well as annexin, galectin, serine protease, Skp-1 and ubiquitin extension protein.
Table 4.2 Results from the processing pipeline for identification of secreted proteins from *Pratylenchus* spp. transcriptomes.

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<tr>
<th>Name of effectors</th>
<th>SignalP predict signal peptide</th>
<th>NN Score &gt;0.5 by SecretomeP?</th>
<th>WoLF PSORT predict secreted protein?</th>
<th>TargetP predict secreted protein?</th>
<th>TMHMM predict no transmembrane regions?</th>
<th>Conclusion</th>
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### Antioxidants

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### Modifiers of plant defense system

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### Miscellaneous functions

| Compound                        | Y | N | N | N | N | Y | Y | N | Y | Y | Y | PS | NS | NS | NS |
|---------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 14-3-3b                         | N | N | N | Y | Y | Y | N* | N* | N* | N | N | N | Y | Y | Y | PS | PS | PS |
| FMRFamide                       | N | N | Y | Y | Y | Y | N | N | Y | Y | Y | PS | PS | S |    |    |    |
| Galectin                        | N | N | N | N | N | N | N | N | Y | Y | Y | PS | NS | NS | NS |
| Transthyretin-like protein      | Y | Y | Y | Y | Y | Y | Y* | Y* | Y* | Y | Y | Y | N | N | N | PS | PS | S  |

Note: Y: met the criteria set by the program; N: does not meet the criteria set by the program; -: sequence not available; * in cytoplasm; ' in extracellular; N*M in mitochondria; N*N in nuclear, - not present, PS: possibly secreted, S: secreted, NS: not secreted
4.2.3 Spatial expression of some putative parasitism genes

Putative parasitism genes may be expressed in organs and tissues capable of secreting proteins to the host-parasite interface, i.e. oesophageal, intestine, amphidial and rectal glands and the hypodermis. *In situ* hybridisation was carried out on *P. thornei* to determine the spatial expression of transcripts putatively encoding cell wall degrading enzymes (β-1,4-endoglucanase, pectate lyase and polygalacturonase), proteases (aminopeptidase, cathepsin L and Z), genes thought to be involved in suppression of host defences (thioredoxin, glutathione-S-transferase, *vap-1, sec-2*) and genes with other putative parasitism functions such as galectin and *ttl*. Although not found in the transcriptome data, a sequence identical to polygalacturonase was amplified from *P. thornei* using a primer which was designed based on *P. zeae* sequence. The analyses were performed only on transcripts from the *P. thornei* infective stages since transcripts exclusively expressed in the egg stage are unlikely to play a direct role in nematode-plant interactions. Five probes hybridised to the oesophageal gland cells: β-1,4-endoglucanase, pectate lyase and polygalacturonase, *gst, vap-1*; whilst four were localised to the intestinal region (aminopeptidase, cathepsin L, galectin and thioredoxin). *Pt-ttl, Pt-sec-2* and *Pt-ctpZ* expression was localised in the hypodermis (Fig. 4.6). No hybridisation signal was detected in the nematodes when control sense probes generated from *PtctpL* and *Pt-eng* were used (Fig. 4.5G and H).
Figure 4.5 Tissue localisation of putative parasitism gene mRNA in *P. thornei* using in situ hybridisation. (A) β-1,4-endoglucanase; (B) glutathione-S-transferase; (C) pectate lyase; (D) polygalacturonase; (E) venom allergen-like protein; (F) cathepsin L; (G and H) No staining was detected with sense probe at the posterior (G) and anterior (H) region of *P. thornei*; (I) cathepsin Z; (J) galectin (K) transthyretin-like protein (L) fatty acid and retinol binding protein; (M) thioredoxin; (N) aminopeptidase. Note: S: stylet; M: median bulb; SvG: subventral gland; H: hypodermis; I: intestine. Scale bar represents 200 μM.
4.3 Discussion

To gain insight into the molecular basis of the host-parasite interaction, three *Pratylenchus* spp., *P. coffeae*, *P. thornei* and *P. zeae*, were subjected to EST analysis to mine for PPGs. Orthologues to genes encoding cell wall modifying enzymes (e.g. β-1,4-endoglucanase and pectate lyase), which are the best characterised genes in sedentary endoparasites, were identified (Table 1.1). In addition, transcripts homologous to xylanases were present in *P. coffeae* and *P. thornei*: these are thought to facilitate the migration of nematodes in plant tissues by breaking down xylans, the major polysaccharide component of hemicellulose matrix component of plant cell walls. However, no sequences which matched xylanase were found in the *P. zeae* transcriptome. It is not clear whether xylanase is actually absent because it is not required by *P. zeae* in its interaction with host plants, or whether its absence was a consequence of how the reads were assembled. Similarly, polygalacturonase was not identified in this study for *P. coffeae*, but transcripts encoding polygalacturonase were isolated from the same dataset in another study (Haegeman et al., 2011). This demonstrates that differences in results could be obtained from the same dataset when different assembly programs (SoftGenetics NextGENe vs. CLC Genomics Workbench 4.02 software) are used. Results from a transcriptome analysis could be improved by using at least two different assembly programs on a dataset to minimise assembly errors.

All three *Pratylenchus* transcriptomes also contained many transcripts putatively encoding proteins identified as antioxidants, such as *Gr*, *Gpx*, *Gst*, *Prx*, *SOD* and *Trx*. The putative proteins in this study were not predicted to be secreted except for superoxide dismutase of *P. coffeae*. In plant parasitic nematodes, SOD exists in two forms: Cu/Zn SOD and MnSOD. The sequence from *P. coffeae* entered for bioinformatics analysis matched Cu/Zn SOD, which is cytosolic and has been shown to be secreted in other parasitic nematodes (Tang et al., 1994). In contrast, sequences from *P. thornei* and *P. zeae* matched MnSOD which has a mitochondrial location, and is therefore not secreted (Lee, 2003). *In situ* hybridisation undertaken on glutathione-S-transferase and thioredoxin indicated that that they were expressed in different tissues: *Pt-gst* in the oesophageal gland cells and thioredoxin in the intestinal region. Thus far, all thioredoxins isolated from nematodes have been expressed in the intestine, but this localisation is not exclusive to parasitic nematodes (Jee et al., 2005, Jiménez-Hidalgo et al., 2014). However in *C. elegans*, thioredoxin was postulated to have roles in prevention of bacterial and fungal infections, instead of the anti-oxidising activities proposed for parasitic nematodes. Although not predicted to be secreted in all three
Pratylenchus transcriptomes, Pt-gst was hybridised to a secretory organ similar to that for M. incognita (Dubreuil et al., 2007). Nematode proteins lacking a signal peptide may in fact be secreted (Robertson et al., 2000, Fioretti et al., 2001, Jaubert et al., 2002), probably through another secretory pathway other than the endoplasmic reticulum (ER)- Golgi network (Rubartelli and Sitia, 1991, Reinhardt and Hubbard, 1998, Bendtsen et al., 2004).

Transcripts homologous to other genes which may have functions in modulation of host responses were also identified, for example annexin, sxp-1, sec-2 (far-1) and vap-1 (msp-1). SEC-2, SXP-1 and VAP-1 are potentially secreted products, based on bioinformatics analyses, but the putative protein encoding annexin in this study was not predicted to be secreted (Table 4.2). Like those of sedentary nematodes, Pt-sec-2 and Pt-vap-1 have also been expressed at the hypodermis and oesophageal glands, respectively. Based on bioinformatics analyses and spatial expression, these transcripts in Pratylenchus could putatively have similar roles as those in sedentary nematodes which is to down-regulate host innate immunity (Prior et al., 2001, Iberkleid et al., 2013, Lozano-Torres et al., 2014). In sedentary nematodes, chorismate mutase was also secreted, and prevents hosts from mounting defence responses during infection (Lambert et al., 1999, Jones et al., 2003). Transcripts encoding chorismate mutase, which restricts the synthesis of chorismate-derived salicylic acid in host plants, was also present in the transcriptomes of P. coffeae and P. thornei, but at an insignificant E-value (>1E-05) and percentage identity (43%). Because of this, it is difficult to decide whether chorismate mutase is in fact really present in root lesion nematodes. Furthermore, the recently completed Pratylenchus genome of P. coffeae also did not indicate the presence of cm within this nematode (Burke et al., 2015). Nevertheless, cm is present in a migratory nematode, albeit in a different genus. Like CMs of sedentary nematodes, CM of Hirschmanniella oryzae (Ho-CM) has a putative signal peptide and is expressed in the pharyngeal gland cells, indicating functions possibly similar to those of sedentary nematodes. Ho-CM also belonged to the same clade as CMs of other plant parasitic nematodes but is most closely related to CM of P. coffeae (Bauters et al., 2014). This indicated that CMs found in the transcriptomes of Pratylenchus could indeed be present.

Other well-represented genes in all three transcriptomes are the proteases (aminopeptidase, cysteine proteases and serine protease). Pt-ampep and Pt-ctpl were expressed in the intestinal region of P. thornei, supporting the hypothesis that they could be involved in digestion of host proteins for nutrition, similar to the function proposed for other parasitic nematodes (Urwin et al., 1997a, Samarasinghe et al., 2011). In contrast, the antisense probe of Pt-ctpZ hybridised to the hypodermis of P. thornei. All nematodes are encased in a
multi-layered cuticle, which is crucial for their development and survival. This exoskeleton is composed primarily of collagen, which is secreted by the hypodermis. Cathepsin Z has been shown to be present in the hypodermis, and it is thought that it may be involved in the activation of peptide moulting hormone (Hashmi et al., 2004). Because Pt-ctpZ was also expressed in the hypodermis, it could putatively have similar role in moulting.

One of the largest group of genes found in all three Pratylenchus transcriptomes is the transthyretin-like proteins and precursors. Like fatty acid and retinol binding protein, ttls are nematode-specific and thus represent a good target for nematode control. Yet, not much is known about this family of genes in plant parasitic nematodes apart from the study on R. similis (Jacob et al., 2007). Numerous roles have been postulated for ttl in R. similis, from ligand binding to functions in nervous system. In this study, transcripts encoding TTLs of P. coffeae, P. thornei and P. zeae were predicted to have extracellular functions. This coupled with the hybridisation of Pt-ttl to a secretory organ, the hypodermis region of P. thornei, supports the involvement of these proteins in parasitism by migratory nematodes. Other nematode proteins with putative functions in host plants included 14-3-3/b, calreticulin, FMRFamide, galectin, RANBPM, SKP1, and ubiquitin extension protein. Of these, only galectin and ubiquitination related proteins (ubiquitin extension protein and SKP1) were not predicted to be secreted.

Analysis on the transcriptomes also revealed transcripts matching to genes identified from oesophageal gland cells of H. glycines, namely hsp-11, hsp13, Hgg-14 and Hgg-23 as well as msp2 of M. incognita (Huang et al., 2003). Some of these genes have since been further characterised and renamed, for example hsp13, which has been identified and characterised as a venom allergen-like protein in H. glycines (Gao et al., 2001). In addition, Hgg-14 has also been identified as a probable polypeptide allergen (PA). In animal parasitic nematodes (APNs), it was postulated that nematodes use PA and fatty acid binding protein to acquire fatty acids from the environment or host due to their inability to synthesis fatty acids de novo (Kennedy et al., 1995, Moore et al., 1999). However, the gene encoding this protein was only expressed in the intestines of G. pallida, with no proof of expression in the gland cells or in the hypodermis (Jones et al., 2009). Nevertheless, the localisation of this gene could vary depending on its life stage as seen in Ostertagia ostertagi where PA was expressed in the intestine in L3 stage and in the hypodermis in L4 and adult stages (Vercauteren et al., 2004). It will be of interest to further characterise Hgg-14 in PPNs including Pratylenchus spp. to determine its localisation in different life stages and their implications in host-parasite interactions.
Similarly, although hsp11 was also isolated from oesophageal gland cells of H. glycines, it showed a considerable match to ttl-1 of R. similis (E-value 4E-63) which have been reported to hybridise to the tissues around the vulva of R. similis (Jacob et al., 2007). More work has to be done to determine if hsp11 is indeed ttl-1 or otherwise, and if hsp11 is expressed in more than one location within the nematode. Another gene with significantly matching transcripts in the Pratylenchus transcriptomes is Hgg-23, a probable protein kinase C substrate. Protein kinase C (PKC) plays a major role in cellular signalling, including cell-cycle progression, transcription and is achieved by phosphorylation of other proteins (Newton, 1995). However, since protein expression varies between different kinds of cells and the effects of PKC are cell-type-specific, it is important to verify the localisation of Hgg-23 in Pratylenchus spp. before the function of this gene could be determined. The transcriptomes also contained transcripts with similarities with M. incognita putative oesophageal gland cell secretory protein 2 (msp2), msp31 and msp32 but the functions of these genes have yet to be elucidated. However, these genes were reported to be hybridised to oesophageal gland cells of M. incognita which is often associated with plant parasitism by nematodes (Huang et al., 2003).

Some genes including 7E12, 10A06, 16D10, 19C07, CLE peptides and MAP-1 do not have matching transcripts in all three Pratylenchus transcriptomes (Gao et al., 2001, Wang et al., 2001a, Huang et al., 2003, Huang et al., 2006a, Lu et al., 2009, Hewezi et al., 2010, Lee et al., 2010, Tomalova et al., 2012, Eves-van den Akker et al., 2014). Since Pratylenchus spp. feed briefly and intermittently from host cells and do not induce a single, permanent feeding site, it is possible that they have less extensive repertoire of effectors compared to sedentary endoparasites. The genes missing from the Pratylenchus transcriptomes but are present in sedentary phytoparasitic nematodes could be those required for plant signalling pathways during reactivation of cell cycle, cell re-differentiation during feeding site initiation and feeding site maintenance.

The Pratylenchus transcriptomes also lacked transcripts that significantly match HYP effector-like protein of G. pallida (Eves-van den Akker et al., 2014). The absence of HYP effector-like sequences in the Pratylenchus transcriptomes is not surprising as these effectors were only present in all CNs studied and in the closely related reniform nematode Rotylenchus reniformis and not identifiable from the EST database of R. similis and the genome sequence of M. incognita or M. hapla (Eves-van den Akker et al., 2014). Since HYP effector-like sequences are not found in RKNs which form giant cells and R. similis which do not establish permanent feeding sites, it is hypothesised that HYP effectors could have a role in the establishment of syncytium. Given that both CNs and R. reniformis form syncytial feeding sites (Jones et al.,
2013) and that they are phylogenetically closely related (van Megen et al., 2009), it is likely that this hypothesis could be correct.

The results provided here indicate that, despite sharing the same parasitism strategy, there appear to be differences in the gene complements present in the three *Pratylenchus* spp. This was not surprising as similar observations were made for the genomes of *M. incognita* and *M. hapla*. Nevertheless, these three transcriptomes have revealed a commonality of parasitism effectors for *Pratylenchus* spp. that could be targeted for further functional studies. In addition, these transcriptomes have also provided many unannotated genes and genes with unknown functions. It will be interesting to characterise these genes to find out if some have a function in parasitism, and if so, are they exclusive to migratory nematodes? Thus far, RNA interference (RNAi) has proved to be a powerful method for functional studies of parasitic nematodes, including for *Pratylenchus* spp. The three *Pratylenchus* spp. studied here as well as *P. vulnus* are clearly susceptible to RNA interference manipulation when soaked in dsRNA of some target genes (Joseph et al., 2012, Tan et al., 2013, Fanelli et al., 2014). In addition, by selecting parasitism genes which have sequences with high percentage of identities with each other, broad spectrum control against these three species of migratory nematodes (and possibly others) could well be possible. Cross-species RNAi has been shown to be possible, where the reproduction of *P. zeae* was reduced to up to 81% when fed dsRNA derived from *P. thornei* (Tan et al., 2013). Although initially neglected, molecular studies on *Pratylenchus* spp. are now advancing rapidly. Information on the transcriptomes of *Pratylenchus* spp. are adding to the understanding of the parasitic strategy of both migratory and sedentary endoparasitic nematodes.
Chapter 5: Functional analysis of genes encoding putative cell wall degrading enzymes of *P. thornei*
5.0 Introduction

Beta-1, 4-endoglucanases (cellulases) are one of the earliest and most commonly isolated cell wall degrading enzymes of plant parasitic nematodes (Smant et al., 1998). These enzymes are thought to facilitate the intracellular migration of plant parasitic nematodes through plant roots by degrading polysaccharides possessing beta-1, 4-glucan backbones such as cellulose and xyloglucan in the cell walls (Smant et al., 1998). Endoglucanases have been identified in well-studied sedentary nematode genera including Meloidogyne, Heterodera and Globodera (Smant et al., 1998, Bera-Maillet et al., 2000, Gao et al., 2002, Abad et al., 2008) as well as in the migratory nematodes Radopholus similis, Ditylenchus africanus and Pratylenchus penetrans and Pratylenchus vulnus (Uehara et al., 2001, Haegeman et al., 2008, Kyndt et al., 2008, Fanelli et al., 2014). Recently, an endoglucanase was also identified in the fungivorous nematode Aphelenchus avenae (Karim et al., 2009). All of these endoglucanases belong to the glycosyl hydrolase family 5 (GHF5). Endoglucanases belonging to other GH families have also been found in plant parasitic nematodes, e.g. Bursaphelenchus xylophilus (GHF45) and Xiphinema index (GHF12) (Kikuchi et al., 2004, Jones et al., 2005).

Together with cellulose and hemicelluloses, pectin is a major structural component of the plant cell walls. Pectin degradation requires the joint action of pectin esterases, which remove the methoxyl groups from pectin, and depolymerases (hydrolases and lyases), which cleave the backbone chain (Tamaru and Doi, 2001). Phytoparasitic nematodes possess two types of depolymerase for pectin degradation: pectate lyase and polygalacturonase. Pectate lyase (pectate transeliminase, EC 4.2.2.2), which catalyses the cleavage of the internal α-1, 4-linkages of unesterified polygalacturonate (pectate) by β-elimination, plays a key role in pectin degradation (Barras et al., 1994). Genes encoding pectate lyases have been cloned from several sedentary phytoparasitic nematodes, including species of Heterodera, Globodera, and Meloidogyne (Popeijus et al., 2000, de Boer et al., 2002, Doyle and Lambert, 2002, Huang et al., 2005, Kudla et al., 2005), and phytoparasitic nematodes of the Aphelenchoidea superfamily, B. xylophilus, Bursaphelenchus mucronatus and A. avenae (Kikuchi et al., 2006, Karim et al., 2009). Several of these pectate lyases have also been further characterised by in situ hybridisation (Doyle and Lambert, 2002, Huang et al., 2005, Fanelli et al., 2014). The importance of pectate lyase for phytoparasitic nematodes parasitism of plants was demonstrated with RNAi experiments. Silencing of a pectate lyase gene by RNAi in J2s of H. schachtii resulted in reduced infection (Vanholme et al., 2007) while transient expression of a
*G. rostochiensis* pectate lyase in *Nicotiana benthamiana* leaves led to severe deformities of the infiltrated tissues (Kudla et al., 2007).

Polygalacturonases function as catalysts in the hydrolysis of pectic polygalacturonic acid to release oligogalacturonides. These are categorised into two classes depending on their mode of action: endo-polygalacturonases and exo-polygalacturonases (Jaubert et al., 2002). Thus far, polygalacturonase-like sequences have been identified in EST data for *Meloidogyne* spp. (including *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita* and *M. javanica*), *P. coffeae* and the fungivore nematode *A. avenae*. Only the polygalacturonase in *M. incognita* has been further characterised. Mi-PG isolated from *M. incognita* was predicted to be an exo-polygalacturonase from GHF28 based on its amino acid sequence (Jaubert et al., 2002). Like pectate lyases, there is good evidence to suggest that polygalacturonases are produced in the oesophageal gland cells and secreted from the stylet of the nematode into plant tissue, thus facilitating the penetration and intercellular migration of the nematode (Jaubert et al., 2002).

To study if genes encoding cell wall degrading enzymes are also expressed in *P. thornei*, we have analysed the available data from the *P. thornei* transcriptome for sequences encoding CWMPs. This resulted in the identification of putative β-1, 4-endoglucanase, pectate lyase and polygalacturonase. Here, the characteristics of each CWMPs identified from *P. thornei*, including their possible roles during migration and how they may influence the hosts of *P. thornei* were discussed.

### 5.1. Materials and methods

#### 5.1.1. Analysis of sequences

Alignments, translations of DNA sequences and analysis of protein sequences were done as described in chapter 4. Identities of genes sequenced were confirmed using BLASTP analyses on the non-redundant (*nr*) and est_others (non-mouse and non-human) databases at NCBI (Altschul et al., 1997). One hundred best BLASTP hits (E-value 1e-05) were aligned with ClustalW2 and the alignment was used to construct a phylogenetic tree with Bayesian statistics using the software MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). A mixed model for protein evolution with 1,000,000 generations (sample frequency = 100) was chosen and the
first 250,000 generations was discarded as ‘burn-in’. The resulting tree was visualised in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

5.1.2. Temporal expression pattern analysis of genes encoding cell wall degradation proteins

To investigate the patterns of expression of target gene transcripts in different developmental stages, RT-PCR experiments were undertaken on eggs, juveniles and adults of *P. thornei*. The mixed stages of nematodes extracted from carrot discs using the mist chamber, were isolated under the microscope according to their length and sizes which reflects the differences in life stages. RT-PCR was performed with primers listed in Table 2.1 with 200 eggs, juveniles or adults. 18S rRNA was used as an internal control to standardise RNA levels. The PCR was carried out in a total of 60 µl using this thermal profile: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s followed by 72°C for 30 s. After 25 cycles, a small aliquot (15 µl) was removed from each sample. This was repeated every three cycles to detect the exponential phase of the reaction. The resulting PCR fragments were analysed on a 1.2% 1x TAE agarose gel.

5.2. Results

5.2.1. Characteristics of the putative cell wall modifying enzymes

5.2.1.1. Beta-1, 4-endoglucanase

Examination of the *P. thornei* dataset for sequences with similarity to β-1, 4-endoglucanases led to the identification of two contigs and 15 singletons. Primer pairs were designed based on the β-1, 4-endoglucanases sequence with the highest total score in the *P. thornei* transcriptome. After amplification and sequencing, a sequence of 409 nt was obtained for *Pt-eng*. Because the transcript of Pt-ENG used in this study is a partial sequence, it could not be predicted if it has an N-terminal signal peptide for secretion. Nevertheless, the full-length sequence should contain a signal peptide, like the endoglucanases of all PPNs studied thus far (Smant et al., 1998, Haegeman et al., 2008, Kyndt et al., 2008). The Pt-ENG transcript was however predicted to be secreted by SecretomeP, having an N-N score which exceeds 0.5.
In addition, Pt-ENG also does not have a transmembrane domain which is one of the characteristics of a standard secreted protein. Although the length of Pt-eng obtained in this study is only a fraction of a full-length β-1, 4-endoglucanase of nematodes, for example, of *P. coffeae* which is 1846 nt, it is sufficient for further characterisation of this gene in *P. thornei*.

Searches for conserved domains of Pt-ENG revealed that it belonged to the glycoside hydrolase family with a catalytic domain of 59 amino acids (http://www.ebi.ac.uk/interpro/). Although Interpro analysis only indicated that Pt-ENG belongs to a glycosyl hydrolase family, it did not specify the GH family. However, BLASTP analysis and alignments indicated that there is a high possibility that the Pt-ENG found here also belongs to the GHFS family. In its catalytic domain, Pt-ENG shares 19% similarity at the amino acid level with another Pt-ENG sequence (accession number AER27782) submitted in the NCBI GenBank database. When compared to Pp-ENG which is the closest match to our Pt-ENG in BLASTP, Pt-eng showed 79% similarity to amino acid regions 260 to 329 of Pp-ENG-1 (accession number BAB68523), which is part of the catalytic domain of Pp-ENG (Fig. 5.1). The other Pt-eng found in NCBI (gi AER27782) was also similar to Pp-ENG-1, but unlike the Pt-ENG identified in this study, the other Pt-ENG was specifically similar with amino acids 34 to 276 of Pp-ENG-1. In addition to a catalytic domain, Pt-ENG contained a putative glycine-rich linker and a partial putative carbohydrate binding domain (CBM), which is characterised by the conserved tryptophan residue (W) located at amino acid 600 (Fig. 5.1, green star).
Figure 5.1 Translated amino acid sequence alignment of putative *P. thornei* endoglucanase Pt-ENG and endoglucanases of migratory endoparasitic nematodes, *P. coffeae* Pc-ENG-1, *P. penetrans* Pp-ENG-1, *R. similis* Rs-ENG-1A and Rs-ENG-1B, sedentary nematodes *M. incognita* Mi-ENG-1, *G. rostochiensis* Gr-ENG-1, *H. glycines* Hg-ENG and bacterial endoglucanases *Hymenobacter swuensis* Hsw-ENG and *Escherichia coli* Ec-ENG. The location of catalytic domain, linker and partial carbohydrate binding domain (CBM) is indicated. The red dot indicates the conserved cysteine which is not found in bacterial endoglucanase while the green star represents the tryptophan residue usually conserved in family II carbohydrate binding domain (CBM) (Bera-Maillet et al., 2000)
BLASTP searches performed on the *nr* database with Pt-ENG protein sequence as query revealed similarity to glycoside hydrolase family 5 (GHF5) cellulases from eukaryotic origins and bacterial genera (Fig. 5.2B). The eukaryotic matches were from the plant parasitic nematode genera *Pratylenchus*, *Meloidogyne*, *Globodera*, *Heterodera*, *Radopholus*, *Ditylenchus* and *Aphelenchus* and the longhorn beetle species *Psacothea hilaris* and *Apriona japonica*. To deduce the phylogeny of root lesion nematode β-1, 4-endoglucanases, the amino acid sequence of Pt-ENG was included in the analysis. The β-1, 4-endoglucanase sequence (gi ABX79356) for *P. coffeae* was retrieved from NCBI Genbank, while the following sequences were obtained for *P. penetrans* (gi BAB68523, AER27773 and AER27774). Seven *P. vulnus* β-1, 4-endoglucanase sequences (gi AER27790, AER27788, AER27792, AER27789, AER27783, AER27791, AER27785) and two *P. neglectus* sequences (gi AER27770, AER27768) were also included to generate the phylogenetic tree (Fig. 5.2A). The phylogenetic analysis of Pt-ENG indicates that it is most closely related to the cellulase of *P. coffeae* (ABX79356) and Pp-ENG-2 (BAB68523) (Fig. 5.2). Analysis also showed that Pt-eng clustered with other *Pratylenchus* β-1, 4-endoglucanases except for endoglucanases of *P. vulnus* (Fig. 5.2). From the tree it was observed that β-1, 4-endoglucanases from the phylum Nematoda differ from bacterial cellulases and endoglucanases of longhorn beetles which clustered in the middle of the tree. It also shows that *Pratylenchus* β-1, 4-endoglucanases are more closely related to root knot nematodes β-1, 4-endoglucanases than to those of other nematode species.
Figure 5.2 Phylogenetic tree of *Pratylenchus* spp. β-1, 4-endoglucanase. The tree was constructed using Bayesian analysis based on ClustalW2 alignment of the deduced catalytic domain of β-1, 4-endoglucanase from *P. coffeae*, *P. penetrans*, *P. thornei* and *P. vulnus* and 100 closest homologous sequences to Pt-ENG from BLASTP. Location of Pt-ENG in the tree was marked by a red star. The scale bars show the length of branch that represents an amount genetic change of 0.1.
5.2.1.2. Pectate lyase

The $Pt$-pel sequence identified in the transcriptome consisted of 350 nt which encode a partial protein of 111 amino acids. The translated amino acid of Pt-PEL did not have a predicted signal peptide for secretion, but could still be a secreted product according to analysis of SecretomeP (NN Score >0.5). In addition, this putative protein does not contain a transmembrane helix, which is another hallmark of a secreted product.

A conserved domain search with Pt-PEL indicated that amino acids in the region 141 to 246 matched protein family pfam03211 of pectate lyases (E-value 3.8e-18, Marchler-Bauer et al., 2007). Within the catalytic site, a suite of closely spaced cysteines, $C_{185}$ and $C_{188}$, that are present in most members of the PL3 family was observed, as well as a consensus site for N-linked glycosylation, at $N_{200}TVA_{203}$ (http://prosite.expasy.org/scanprosite/) (Fig. 5.3). The Pt-PEL in this study also contain part of a integrally conserved region designated the BF domain, found only in the pectate lyase PL3 family (Hatada et al., 2000) within which included some well conserved amino acid residues, $D_{157}$, $K_{158}$, $K_{180}$ and $R_{183}$ (Fig. 5.3).
Figure 5.3 Alignment of the putative partial sequence of *P. thornei* pectate lyase and its closest homologs from BLASTP: sequences of *M. enterolobii*, *M. javanica*, *M. incognita*, *Amycolatopsis mediterranei*, *Actinoplanes friuliensis* and *Bacillus* spp. Identical residues are shaded black and residues that are similar are shaded grey. Putative N-glycosylation site found in Pt-pel of *P. thornei* at amino acid 200 (red rectangle) while the red stars represent the location of charged amino acid residues. Closely located cysteines that are present in most members of the PL3 family are marked with black dot.

A BLASTP search on the *nr* database with Pt-pel revealed the highest similarity with the family 3 pectate lyases (PL3) MJ-PEL-1 of *M. javanica* (Genbank accession number AAL66022.1) and Me-PEL-1 of *M. enterolobii* (Genbank accession number ADN87334.1), with 52 and 53% identity. In addition, Pt-PEL also shares amino acid similarity with PL3 pectate lyases from a range of bacterial and fungal species, and other nematode species such as *M. incognita*, *H. glycines*, *Globodera*, *B. xylophilus* and *A. avenae* (Fig. 5.4).

A phylogenetic tree was then constructed using Bayesian analysis based on ClustalW2 alignment of 100 protein sequences from NCBI BLASTP analysis which are most similar to Pt-PEL. The phylogeny of members of the PL3 family revealed that Pt-PEL is clustered with pectate lyases of root knot and cyst nematodes (Fig. 5.4). Phylogenetic analysis also showed that pectate lyases of
endoparasitic nematodes are distantly related from those of ectoparasitic nematodes such as *Bursaphelenchus* spp. Pt-PEL is least related to pectate lyase of other pathogens e.g. fungi and bacteria.

**Figure 5.4** Phylogenetic analysis constructed from ClustalW alignments of *P. thornei* pectate lyase, *H. glycines* Hg-PEL-1 and Hg-PEL-2, *G. rostochiensis* Gr-PEL-1 and 100 protein sequences of bacteria, fungi and plant parasitic nematodes with closest similarity to Pt-pel in BLASTP. Pt-PEL is indicated with a star while PEL of plant parasitic nematodes are shaded. The scale bars show the length of branch that represents an amount genetic change of 0.05.
5.2.1.3. Polygalacturonase

Analysis of available data led to the identification of a singleton which had similarity to exo-polygalacturonase PehB (AAC46001; EC3.2.1.82.) from the plant pathogenic bacteria *Ralstonia solanacearum* with E-values ranging from 7e-14 to 6e-22 in BLAST search output. Following amplification and sequencing of *Pt- pg*, a fragment of 329 nt was obtained and the ORF encodes a protein of 109 amino acids. SignalP analysis did not predict the presence of an N-terminal signal peptide for secretion, but SecretomeP analysis imply that Pt-PG could be secreted. This was further validated with *in situ* localisation for *P. thornei*, in which *Pt- pg* expression was seen confined to the subventral oesophageal secretory gland cells (Fig. 4.4). A consensus site for N-linked glycosylation was not predicted for Pt-PG but a polygalacturonase active site was identified (Fig. 5.5).

**Figure 5.5** Alignment of the translated amino acid sequence of *Pt- pg* with some of its closest homologs from BLASTP: sequences of *Ralstonia solanacearum* (YP_003745669), *Yersinia mollaretti* (WP_004877071), *Yersinia similis* (AHK19399), *Klebsiella oxytoca* (EWF75323), *Pectobacterium carotovorum* (WP_010300150), *Pseudomonas putida* (YP_008096117), *Erwinia chrysanthemi* (CAB99320) and the plant parasitic nematodes, *M. incognita* and *Aphelenchus avenae*. The polygalacturonase active site was present at position 44-60 of Pt-PG.

BLASTP search of the *nr* database with *Pt- pg* showed that it is most similar to the exo- and endo-polygalacturonases of several bacterial species (31 to 65% identity) (Fig. 5.7). *Pt- pg* also matched the polygalacturonase Mi-PG-1 (gi AAM28240) of *M. incognita* (E-value 1e-23; 52% identity).
The identity of the protein being a polygalacturonase is further confirmed with Interpro analysis where it is shown to have signature matches to glycosyl hydrolase family 28 (GH28) between amino acid region 8 to 107, polygalacturonase between aa region 42 to 56 and pectin lyase fold/virulence factor between aa region 1 to 105 (Fig. 5.6).

![Figure 5.6 Overview of Pt-PG domain and its signature matches in InterPro (Hunter et al. 2011).](image)

BLASTP searches also revealed the presence of three domains in Pt-PG; glycosyl hydrolase family 28 (pfam00295) (E-value 8.62e-06), PLN02793 (probable polygalacturonase) (E-value 4.10e-05) and the COG5434 endopolygalacturonase domain PGU1 (E-value 2.05e-26). COG5434, classified as a model that may span more than one domain, is not attached to any particular domain superfamily and is made up of a consensus built on twelve protein sequences to annotate bacterial endopolygalacturonase (Marchler-Bauer et al., 2013). Although all twelve sequences underlying COG5434 are classified as polygalacturonases of GH28, only for two of the sequences (gi AAD35522, gi CAA89686) enzyme activity has been tested experimentally (Parisot et al., 2003). The protein sequence AAD35522 categorised in the EC 3.2.1.67 nomenclature, represents exo-polygalacturonases whereas CAA89686, classified as an EC 3.2.1.15 enzyme, represents polygalacturonases. BLASTP analysis also showed that the Pt-PG sequence matches to mostly bacteria polygalacturonase sequences from the Proteobacteria (Ralstonia, Pseudomonas, Pectobacterium, Klebsiella, Dickeya and Yersinia spp.) and Firmicutes (Bacillus, Clostridium, Pelosinus and Zymophilus spp.) phylum. However, Pt-PG is most similar at amino acid level to exo-polygalacturonase (EC 3.2.1.82) of R. solanacearum (WP_0132060619.1) (65%), Yersinia similis (AHK19399.1) (62%) and Klebsiella oxytoca (EWF75323.1) (55%). Based on these results, it can be concluded that there is a strong possibility that Pt-PG codes for an exo-polygalacturonase.

A phylogenetic tree was then constructed from ClustalW2 alignment of 100 protein sequences from NCBI BLASTP analysis which are most similar to Pt-PG. A phylogenetic analysis demonstrated that Pt-PG is most closely related to polygalacturonases from the Ralstonia spp. However, Pt-PG also belong to the same cluster as polygalacturonases from closely related plant parasitic nematodes, M. incognita and A. avenae (Fig. 5.7).
Figure 5.7 Phylogenetic analysis constructed from ClustalW alignments of polygalacturonase of *P. thornei* (Pt-PG), *M. incognita* (Mi-PG), *A. avenae* (Aa-PG) and 100 sequences from NCBI BLASTP which are most similar to Pt-PG. Pt-PG is marked with a star while polygalacturonase of plant parasitic nematodes are in orange fonts. The scale bars show the length of branch that represents an amount genetic change of 0.05.
5.2.2. *Temporal expression of genes encoding cell wall modifying proteins*

Temporal expression of β-1,4-endoglucanase, pectate lyase and polygalacturonase of *P. thornei* was assessed using semi-quantitative PCR on cDNA derived from different life stages. Gene specific primers were used for amplification of *Pt-eng*, *Pt-pel* and *Pt-pg* while 18s rRNA primers was used as control PCR. In eggs, no expression of *Pt-pel* and *Pt-pg* was detected although some *eng* expression was observed at cycle 34. In contrast, all three genes were highly expressed in the juveniles and adult *P. thornei*. Expression of *Pt-pg* was the highest in the juvenile stage, followed by *Pt-pel* and *Pt-eng*. In comparison, *Pt-eng* has the highest expression in the adult stages compared to the other two genes encoding CWMPs. The expression of *Pt-pg* was the lowest in the adult stage, indicated by the less bright band at cycle 25, compared to that of *Pt-eng* and *Pt-pel*.

![Figure 5.8](image.png)

*Figure* 5.8 Expression of β-1,4-endoglucanase, pectate lyase, polygalacturonase and 18S rRNA in eggs, juveniles and adults of *P. thornei*.

5.2.3. *Silencing of genes involved in plant cell wall degradation reduces the replication rate of P. thornei*

The mini disc culture system was used to assess the establishment and reproduction capability of *P. thornei* treated with dsRNA of *Pt-eng*, *Pt-pel* and *Pt-pg*. To assess the effects of silencing genes which encodes cell wall modifying enzymes, four replicates of self-callusing mini carrot discs were each infected with 50 mixed stages *P. thornei* previously fed with 2 mg/mL dsRNA of *Pt-
Eng, Pt-pel or Pt-pg in a basic soaking solution with 50 mM octopamine for 16 h. After soaking, phenotypes were observed and the expression of these genes checked by qRT-PCR. Phenotypically, only nematodes treated with dsRNA of pectate lyase did not display the typical sigmoidal shape or wavy movements of nematodes in the controls (Fig. 5.9). Nematodes treated with dsRNA of Pt-eng and Pt-pg demonstrated similar body shape and movement as the controls.

![Figure 5.9](image)

Figure 5.9 Phenotypes displayed by P. thornei after soaking in medium containing dsRNA of target genes for 16 h. (A) β-1,4-endoglucanase (B) pectate lyase (C) polygalacturonase (D) gfp and (E) no dsRNA. Scale bar represents 200 µm.

The reduction in expression of mRNA of Pt-eng, Pt-pel and Pt-pg in nematodes treated with dsRNA were then quantified using qRT-PCR using expression of 18S rRNA as an endogenous reference for normalisation. Reduction in transcript accumulation was calculated using the Comparative Ct method (ΔΔCt) with reference to expression of the gene in nematodes fed with dsRNA of gfp and those soaked with no dsRNA. P. thornei soaked for 16 h in dsRNA of the target genes involved in plant cell wall degradation did show various degrees of silencing (Table 5.1). The expression of Pt-eng in dsRNA-treated nematodes showed 849-fold and 151-fold reduction respectively compared to expression in nematodes fed with dsRNA of gfp and with no dsRNA (Table 3.1). A similar trend of reduction in transcript abundance was found for nematodes treated with dsRNA of pectate lyase: higher degree of gene knockdown was observed relative to the nematodes soaked without dsRNA (23-fold) compared to those treated with dsRNA of gfp (11-fold). Similarly, RNAi of Pt-pg resulted in a high reduction of mRNA levels in nematodes when compared to no dsRNA. The mRNA expression of polygalacturonase was also affected by gfp dsRNA treatment (Table 5.1).
Table 5.1 Quantification of transcripts of plant cell wall degrading enzymes of *P. thornei* after dsRNA-mediated gene silencing.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold knockdown (No dsRNA)</th>
<th>Fold knockdown (dsGFP control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-1,4-endoglucanase</td>
<td>849±0.53</td>
<td>151±0.59</td>
</tr>
<tr>
<td>Pectate lyase</td>
<td>23±0.73</td>
<td>11±0.09</td>
</tr>
<tr>
<td>Polygalacturonase</td>
<td>302±0.07</td>
<td>151±0.23</td>
</tr>
</tbody>
</table>

Following 16h of soaking in dsRNA, mixed stage nematodes were used to infect carrot mini discs. After five weeks, nematodes were harvested from the discs and their numbers counted. Whilst there was no significant difference in the average number of nematodes extracted from discs infected with nematodes fed with *gfp* dsRNA and no dsRNA, there was a 91% reduction in the number of nematodes retrieved from discs infected with *Pt-eng* dsRNA-treated nematodes (*p*<0.05) (Fig. 5.10). In the case of pectate lyase, although its transcript reduction measured by qRT-PCR was comparatively lower than that for β-1, 4-endoglucanase, the treatment was sufficient for 86% reduction in the average number of nematode extracted after 5 weeks of culture. For polygalacturonase, on average, there was a 95% reduction in nematodes harvested from the discs.

**Figure 5.10** Reduction in reproduction of *P. thornei* on carrot mini discs over a 5 week period, after treatment with dsRNA of genes involved in the modification of host cell wall: beta-1, 4-endoglucanase (*Pt-eng*), pectate lyase (*Pt-pel*) and polygalacturonase (*Pt-pg*), compared with treatment with dsRNA of *gfp* and with no dsRNA.
5.3. Discussion

Categorisation of a glycosyl hydrolase into families is based on similarities between amino acid sequence in the catalytic domain and hydrophobic cluster analysis (Henrissat, 1991). Presently, there are 133 families (http://www.cazy.org/Glycoside-Hydrolases.html) of which only fourteen contain cellulases. All endogenous β-1, 4-endoglucanases isolated from plant parasitic nematodes belong to family GHF5, except for the GHF45-cellulases from the pine wood nematode B. xylophilus and a GHF12-cellulase from X. index (de Meutter et al., 1998, Smant et al., 1998, Rosso et al., 1999, Kikuchi et al., 2004, Jones et al., 2005, Haegeman et al., 2008, Fanelli et al., 2014). Cellulases of GHF5 from phytoparasitic nematodes have variable structures: some have a linker and a carbohydrate binding module (CBM), while others only have a linker but lack a CBM (Smant et al., 1998, Rosso et al., 1999, Uehara et al., 2001, Ledger et al., 2006). However, all cellulases described have a catalytic domain. Here we report a partial sequence of β-1, 4-endoglucanase identified from P. thornei, which appears to belong to the family GHF5. The sequence of β-1, 4-endoglucanase, Pt-ENG, consist of a catalytic domain, a linker and could putatively have a CBM according to the alignment with Pp-ENG-1, Pc-ENG and Rs-ENG-1A which have such a module. In addition, Pt-ENG also have one conserved tryptophan residue within the CBM which have been shown to be involved in carbohydrate binding in G. rostochiensis (Bera-Maillet et al., 2000).

Like other phytoparasitic nematodes, Pt-ENG has a C-terminal linker region (Kyndt et al., 2008). However, the amino acid composition of the linker between the catalytic domain and the CBM is not conserved between taxa. In root lesion nematodes, the putative linkers are either glycine rich (as in the Pt-ENG and Pc-ENG reported here) or threonine rich (Pp-ENG-1) (Fig. 5.1). The same variation was observed in linkers identified in cyst (glycine-serine or alanine-lysine-proline rich) and root knot nematodes (glycine-serine and asparagine rich) (Kyndt et al., 2008) ENGs. This could be due to the minimal evolutionary pressure on the linker since this segment of the protein may only have a structural function as an extended, flexible joint between domains (Gilkes et al., 1991). If there has been little selection pressure on this linkage, then there will have been fewer constraints on its sequence (Gilkes et al., 1991).

The catalytic domain of Pt-eng was very similar to that of Pp-eng-1 and Pc-eng-1 (79 and 81% respectively). Comparison of catalytic domains with those of another migratory nematode, R. similis revealed that Pt-ENG was relatively identical to two endoglucanases, Rs-ENG-1A and Rs-ENG-2 (64 and 59%). However, the phylogenetic analysis undertaken here revealed that although both are migratory nematodes, endoglucanase from P. thornei are more similar to those of root knot nematodes whilst
endoglucanases of *R. similis* are more similar to those of cyst nematodes. Since phytoparasitic nematodes could possess up to 21 endoglucanase genes (Abad et al., 2008), there may be additional endoglucanases to be discovered for *P. thornei*. Nevertheless, from the phylogenetic tree, it was observed that GHS cellulases are conserved in Pratylenchidae, root-knot and cyst nematodes and the genera *Radopholus* and *Aphelenchus*, indicating that these cellulases originated from the same gene and resulted from horizontal gene transfer from a common ancestor of *Tylenchida* and *Aphelenchida* clades. The catalytic domain of Pt-eng also showed a low percentage similarity (19%) with another Pt-eng sequence (gi AER27782) deposited in the NCBI GenBank database. It could not determine if the Pt-eng sequence (gi AER27782) and the Pt-eng identified in this study are the same gene but belong to different segments or they are from a different cellulase. Annotation of both sequences would be better if full-length sequences were available for each of the genes.

Expression of Pt-eng was assessed in eggs, J2s and adults. Elevated expression in the juvenile and adult vermiform is not surprising because all vermiform stages of the life cycle of *P. thornei* are infective. However, the observation that some cellulase expression was present in *P. thornei* eggs was surprising since eggs are not an infective life-stage for this nematode. Cellulase expression in eggs was also reported for the cyst nematodes, *H. glycines*, Hg-eng-1 and Hg-eng-4 (Gao et al., 2004b) and for several *Meloidogyne* cellulases (Rosso et al., 1999, Ledger et al., 2006). The likelihood that some of the embryos in the eggs were more developed and closer to hatching could not be excluded, thus accounting for the low expression of Pt-eng in the egg sample. In such cases, the hatching nematode may have been becoming ‘primed’ for infection.

Polygalacturonases are the other member of the glycosyl hydrolase family identified in this study. The partial sequence for *P. thornei* identified, Pt-pg, belongs to GHF28, and encoded a 97 amino acid protein that is more similar to microbial exo-PGs. Nevertheless, localisation of Pt-pg expression specifically in the subventral glands of *P. thornei* via *in situ* hybridisation indicated that Pt-pg is not a contaminant or of symbiont origin. Although inconclusive, the high similarity between Pt-PG and bacterial PGs provides evidence to support the hypothesis that this nematode parasitism gene may have been acquired by horizontal gene transfer from bacteria. GH28 polygalacturonases of bacterial origin have also been identified in other phytoparasitic nematodes, *A. avenae, M. incognita* (Jaubert et al., 2002) and the closely related *P. coffeae* (Haegeman et al., 2011). However, only *M. incognita* polygalacturonase has been characterised and is known to be expressed only in the juvenile stage (Jaubert et al., 2002). Unlike Mi-pg (Jaubert et al., 2002), Pt-pg was expressed in the juvenile and adult stages of *P. thornei*. As indicated above, this is logical since *M. incognita* probably does not require polygalacturonase after establishing its feeding site because it does not need to migrate...
further in the plant tissue, whereas migratory nematodes like *P. thornei*, following entry, move and feed from cell-to-cell throughout their life cycle.

Although lacking biochemical evidence, based on sequence similarity searches Pt-PG appears to be an exo-polygalacturonase, like Mi-PG. In general, exo-acting enzymes cleave at the non-reducing end of the homogalacturonans to release D-galacturonic monomers. Endo-acting enzymes cleave a polysaccharide at random positions internally and cause a more rapid decrease in average chain length, hence, an endo-polygalacturonase will have more effect on the integrity of a pectin polymer than an exo-polygalacturonase (ten Have et al., 2002). Endo-polygalacturonase activity on pectic substances also releases oligomeric galacturonosyl fragments, which may stimulate plant defence responses (Cervone et al., 1989). Biotrophic pathogens, like *Pratylenchus* species, must avoid or neutralise host defence responses and therefore it seems likely that they will benefit from a cell wall degrading enzyme repertoire that does not release oligomeric galacturonosyl fragments. Therefore, it would be interesting to study the enzymatic properties of *Pt-pg* to determine which class of polygalacturonases it belongs, its impact on cell wall integrity and role in modulating plant defence response.

In addition to GH28 polygalacturonase, degradation of the pectin backbone also depends on polysaccharide lyase family 1 and polysaccharide family 3 (PL3). Based on amino acid sequence similarities and phylogenetic analysis, Pt-PEL identified is assigned to PL3 family, to which most phytoparasitic nematode pectate lyases belong. In addition, Pt-PEL contain some charged residues and closed spaced cysteines which are characteristics of PL3 pectate lyases. These charged residues are important for the catalytic activity, calcium binding, substrate binding, and/or maintenance of structural integrity of pectate lyases (Hatada et al., 2000). Like *Pt-eng*, expression of *Pt-pel* is high in both juvenile and adult vermiform stages confirming this enzyme is required for plant parasitism.

The functional significance of the putative endoglucanase, pectate lyase and polygalacturonase was established by RNAi and mini carrot discs infection experiments. Although no morphological changes were observed after soaking, the results showed clearly that *in vitro* RNAi induced by soaking nematodes with dsRNA effectively reduced the expression levels of *Pt-eng* and *Pt-pg* of *P. thornei*. For *Pt-pel* dsRNA-treated nematodes, the abnormal body shape correlated with reduced transcript accumulation. Despite the significantly higher fold knockdown for *Pt-eng* and *Pt-pg* compared to *Pt-pel*, knockdown of *Pt-pel* was sufficient to result in an 86% reduction in nematode replication. The aberrant phenotype observed in *P. thornei* after RNAi with dsRNA of *Pt-pel* has not been reported for *pel* of other PPNs (Bakhetia et al., 2007, Sukno et al., 2007, Vanholme et al., 2007). This could be because for some genes with known functions like *pel*, observation of the phenotypes is
only done briefly after RNAi treatment using the microscope under low magnification to check the viability of nematodes before infection. It would be interesting to see if the same phenotype is observed in other PPNs after RNAi treatment with dsRNA of pel when higher magnification is used. However, it could be due to differences in the type of PEL. In the genome of P. coffeae (Burke et al., 2015), three PELs were identified while as many as 22 PELs were found from M. hapla genome (Opperman et al., 2008). Depending on which pel was chosen, there could be a possibility of differences in phenotypes observed after RNAi. More work has to be done to verify this statement. In vitro RNAi by soaking on dsRNA derived from cellulase and polygalacturonase of P. thornei led to 91 and 95% success rate in preventing nematodes from replicating. Because the amount of dsRNA and incubation time were standardised, differences in silencing efficiency can be attributed to these factors, i.e. conformation of dsRNA, amount of target genes within the organism and the protein turnover rate, et cetera. It is worth determining the exact amount of a particular gene product in a single nematode to know the minimal amount of dsRNAs/siRNAs which can be applied to silence it. Nevertheless, silencing of Pt-eng, Pt-pel and Pt-pg decreased the ability of the nematodes to effectively invade, survive and reproduce in host tissues, hence suggesting that they are all required for invasion and movement of the nematodes in plant roots. In conclusion, these results clearly show that in vitro RNAi via soaking can be applied to the migratory nematode P. thornei both to determine the functional role of target genes in plant parasitism and as a potential strategy of RLN control.
Chapter 6: Cysteine proteases: target or targeted
6.0 Introduction

In addition to cell wall degrading enzymes, many putative parasitic secretory proteins are produced from plant nematode gland cells, such as venom allergen-like protein (vap) (Ding et al., 2000), chorismate mutase (Jones et al., 2003), chitinase (Gao et al., 2003), calreticulin (Jaubert et al., 2002) and ubiquitin extension protein (Tytgat et al., 2004). The functions of these genes have been studied to varying degrees, with the exception being the vap family, although additional data is now available (Ding et al., 2000, Gao et al., 2001, Wang et al., 2007).

Vap proteins are secreted from the SVGs and belong to the SCP/TAPs protein family (Pfam accession number PF00188) that have a SCP/Tpx-1/Ag-5/PR-1/Sc7 domain. This domain encodes several functional classes of proteins such as sperm-coating proteins (SCP), mammalian testis-specific extracellular proteins (Tpx), venom-allergens from wasps and ants (Ag), and glioma and plant pathogenesis-related (PR) proteins. Although their physiological functions remain elusive, vap proteins have been proposed to play a role in reproduction, immunity, development and host invasion in some parasitic nematodes (Alexander et al., 1993, Murphy et al., 1995, Hawdon et al., 1996, Murray et al., 2001, Kovalick and Griffin, 2005). Parasitic nematodes from the orders Strongylida, Rhabditida, Tylenchida, Spirurida and Ascaridida (Clades V, IV and III of the phylum Nematoda) have all been reported to possess the vap proteins (Hawdon et al., 1996, Schallig et al., 1997, Bin et al., 1999, Ding et al., 2000, Tawe et al., 2000, Gao et al., 2001, Wang et al., 2007, Kang et al., 2012). In addition, Lozano-Torres and co-workers (2014) recently reported that secreted venom allergen like protein of a phytoparasitic nematode could function as an inhibitor of the extracellular cysteine protease receptor in plants. This is important as these receptors operate as a signalling node in innate immunity of tomato plants. In addition, RNAi of Gr-vap-1 reduces infection, whereas overexpression increases infection levels, and significantly susceptibility to fungal pathogens as well (Lozano-Torres et al., 2014), further suggesting that they down-regulate plant innate immunity.

In addition to plants, cysteine proteases are also found in many different organisms including in parasitic nematodes. Cysteine proteases, belonging to subfamily C1 of clan A, display catalytic activity because of the presence of extra shell electrons on the sulphur of thiol group (Atkinson et al., 2009). Within the C1 family, thiol-dependent cathepsin-like cysteine proteases can be divided into 10 subfamilies on the basis of primary sequence and substrate preferences (B, C, F, H, K, L, O, S, W, Z) (Berti and Storer, 1995, Barrett and Rawlings, 1996). Various functions have been linked to proteases in host-parasite interactions, ranging from parasite immuno-evasion, to essential nutrient uptake, enzyme activation, virulence, tissue and cellular invasion, as well as embryogenesis and moulting (Cox
et al., 1990, Carmona et al., 1993, Lustigman et al., 2004). However these roles have been hypothesised from their activities against substrates in vitro and spatio-temporal expression patterns of the enzymes. Without gene or chemical knockout, the exact physiological functions of parasite enzymes, and hence, which ones are important targets of control, remain unknown. Given the significance of this ubiquitous class of enzymes involving a wide array of essential metabolic functions in host-parasite interactions, these proteases could be important targets for bio-engineering of novel crop plants with increased tolerance towards nematode parasitism. In this chapter, the aim was to isolate cysteine proteases, cathepsin L and cathepsin Z, from the root lesion nematode *P. thornei* and to assess the effects of RNA interference on them. An additional aim was to study its vap protein(s) and to evaluate their contribution to *P. thornei* virulence in host plants.

6.1. Materials and methods

Identification and amplification of target sequences were done as described in Chapter 2. Similarities of amino acid sequence deduced from ORFs with known cathepsin L and Z cysteine proteases and venom allergen-like protein sequences of other species were determined using the NCBI BLASTP program. Phylogenetic analysis of cathepsin L and venom allergen-like protein sequences aligned with CLUSTALW2 were conducted using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). A mixed model for protein evolution with 1,000,000 generations (sample frequency = 100) was chosen with the first 250,000 generations cast-off as burn-in. The tree generated was viewed in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). To compare the relationship between different cathepsin families, a phylogenetic network was generated using the BioNJ method (bootstrap = 1000) in the Splitstree 4.13.1 software (http://www.splitstree.org/) (Hudson and Bryant, 2006). To identify if target proteins were predicted to be secreted, protein sequences were entered into SignalP, SecretomeP, TargetP, WoLF PSORT and TMHMM. Temporal expression of target genes were determined by PCR as described in Chapter 5. Pre-parasitic *P. thornei* were obtained by hatching eggs in vitro. *In vitro* RNAi of genes and its effects in *P. thornei* was performed as described in section 2.8 of Chapter 2.
6.2. Results

6.2.1. Cathepsin L

Cathepsin L is one of the most common cysteine proteases in phytonematodes, often associated with digestion. To identify sequences encoding cathepsin L in *P. thornei*, the transcriptome was analysed, which resulted in the identification of five contigs and 34 singletons. The putative cathepsin L (Pt-ctpL) sequence was obtained by PCR after analysis of the *P. thornei* transcriptome. This partial sequence comprised of 414 bp with an ORF encoding a polypeptide of 138 amino acids. The translated sequence, Pt-ctpL, was then fed into a bioinformatics workflow and the transcript was predicted to be secreted by SecretomeP (NN score >0.5) and WoLF PSORT and the TMHMM analysis showed it does not have a transmembrane region.

The BLASTP program was used to compare the Pt-ctpL with all protein sequences in the databases, and the most significant matches were with cathepsin L of *H. schachtii* (ACJ13098.1) (81% identity; E-value 3e-76), *G. pallida* (ACJ13079.1) (79% identity; E-value 4e-76), *Globodera tabacum* (ACJ13092.1) (79% identity; E-value 6e-76) and *M. incognita* (CAD89795.1) (76% identity; E-value 1e-69). The Pt-ctpL sequence analysed encodes a peptidase C1A subfamily protein and domains similar to the papain family cysteine protease (pfam00112) (E-value 7.40e-60), PTZ00021 (Falcipain-2; provisional) (E-value 5.6e-32) and COG4870 cysteine protease (E-value 3.47e-10). Falcipain-2 (FP2) is a principal trophozoite cysteine protease of *Plasmodium falciparum*, a human malarial parasite, for which inhibition of FP2 leads to arrested development of the parasite (Pandey et al. 2005). This domain is also present in cathepsin L of the cyst nematodes (CNs) *G. pallida* and *G. tabacum* and the stem nematode *Ditylenchus destructor*, but not identified in the sequences of the free-living nematodes *Caenorhabditis remanei*, *C. elegans*, the root knot nematode *M. incognita*, cyst nematodes *H. schachtii*, *H. glycines*, the pine wilt nematode *B. xylophilus* and hookworm nematode *Ancylostoma ceylanicum*.

Because the Pt-CTPL sequence was incomplete (only 41% coverage of the full-length CTPL from *G. rostochiensis*), the ERFNIN and GNFD motifs which are characteristic of cathepsin L were not present (Karrer et al. 1993, Vernet et al. 1995). Nevertheless, the GCNGG motif was observed in Pt-CTPL, between amino acid 231 to 235. With the exception of the central asparagine residue, this GCNGG motif is invariant in all ERFNIN motif-containing proteases (Karrer et al. 1993). Like other papain-like cysteine proteases, Pt-CTPL sequence also contained a highly conserved catalytic cysteine residue at amino acid 190 and a glutamine (Gln) residue at amino acid 184. The cysteine residue in this motif is predicted to be involved in the formation of a disulphide bridge while the conserved Gln...
residue is involved in maintaining an active enzyme conformation (Kamphuis et al., 1985). In addition, Pt-CTPL has a putative N-glycosylation site located at residue position 152 and S2 subsites at amino acids 237 and 238 (Fig. 6.1). The S2 subsites have a dominant effect on the overall specificity of cysteine proteases (Turk et al., 1998). Analysis of the S2 subsites of the nematode proteases shows that they are typical cathepsin Ls and can be expected to show activity against CPL-like substrates.

Figure 6.1 Alignment of Pt-ctpL with the matched region of cathepsin L from other plant parasitic nematodes. The N-glycosylation site and cysteine active site are boxed in red and green while the conserved GCNGG sequence is boxed in blue. S2 subsets located at the conserved LMD sequence are represented by S2. The red arrow indicates the pro-peptide cleavage site.

Phylogenetic analysis of Pt-CTPL and 100 sequences which are most similar to Pt-CTPL sequence after BLASTP analysis, revealed that Pt-CTPL appeared to cluster together with Cathepsin Ls of other plant parasitic nematodes. However, cathepsin L of B. xylophilus (ACH56225) and D. destructor (ACT35690) appear very different from the other sequences used in the analysis. Proteins from animal and human parasitic nematodes seem to be more related to free living nematodes compared to plant parasitic nematodes. Cathepsin Ls of mammals, insects, fish and reptiles are divergent from cathepsin Ls of plant parasitic nematodes (Fig. 6.2).
Figure 6.2 Phylogenetic relationships of Pt-CTPL and 100 most similar sequences to Pt-CTPL from BLASTP constructed using MrBayes program. Pt-CTPL is in orange font. The scale bar represents a genetic change of 0.05.
6.2.2. Cathepsin Z (also known as cathepsin X)

Cathepsin Z is another member of the cysteine protease family which could play an important role in host-nematode interaction. Analysis of the *P. thornei* transcriptome resulted in two contigs and six singletons which matched cathepsin Z, of which a 243 bp fragment encoding 87 amino acids was amplified from *P. thornei* cDNA. This partial Pt-CTPZ sequence (only 41% coverage of the full-length CTPZ from *Toxocara canis*) does not encode a signal peptide, but analysis with SecretomeP (NN score exceeding 0.5), WoLF PSORT and TMHMM (no transmembrane helices predict that Pt-CTPZ could be secreted). The amino acid sequence did not have an ERFNIN motif that was highly conserved and interspersed in the cathepsin L family, but rather contained a complete repeat of the conserved CGSCW motif around the active site cysteine (Fig. 6.3, green box). However, a HIP motif, which is a unique characteristic of cathepsin Z, was found in Pt-CTPZ, at position 65-67, shortly before the cysteine active site (Fig. 6.3). Alignment of Pt-CTPZ with sequences of cathepsin Ls, cathepsin Bs and cathepsin Zs showed that Pt-CTPZ shared a high degree of identity with other cathepsin Zs (51-83%) while little identity could be seen with cathepsin L-like cysteine protease (19-25%) and cathepsin B (17-27%).

Performing a BLASTP search against the GenBank non-redundant protein database identified that Pt-ctpZ shared the highest identity with cathepsin Z of *O. volvulus* (AAT00789.1, e-value 3e-44). BLASTP analysis also revealed that Pt-ctpZ belongs to the peptidase C1 superfamily (cd02698, e-value 1e-49) and contained the domains papain family cysteine protein (pfam00112, e-value 8.08e-18) and dipeptidyl-peptidase I precursor; provisional (PTZ00364, e-value 3.21e-10). The Falcipain-2 domain (PTZ00021, e-value1.87e-07), also predicted for Pt-CTPL, was present in all the cathepsin Zs of the organisms used in this analysis.
Figure 6.3 Alignment of Pt-CTPZ with the matched region of cathepsin Z from other organisms. Conserved CGSCW and HIP sequence are boxed in green and blue. Cysteine active site is marked with a red circle while S2 subsets are represented by S2. Genbank accessions of organisms used in this alignment: A. suum (As-ctpz) (ERG79842.1), Brugia malayi (Bm-ctpz) (AAT07061.1), Caenorhabditis briggsae (CBB-CTPZ-1) (CAP24890.1), Caenorhabditis elegans (Ce-ctpz) (NP_491023.2), Caenorhabditis remanei (CRE-CTPZ-1) (EF090122.1), (CRE-CTPZ-2) (XP_003114210.1), Dictyocaulus viviparus (Dv-ctpz) (AFM37367.1), Dictyostelium discoideum (Dd-ctpz) (EAL65685.1), Homo sapiens (Hs-ctpz) (NP_001327.2), L. loa (Lo-ctpz) (EFO21073.2), Onchocerca volvulus (Ov-ctpz) (AAT00789.1), Trichinella spiralis (Ts-ctpz) (EFV52934.1), Toxocara canis (Tc-ctpz) (AAD30154.1), Wuchereria bancrofti (Wb-ctpz) (EJW82660.1).

A phylogenetic network was generated using BIONJ with an alignment of the protein sequences of various papain-like cathepsins using the software SplitsTree 4.13.1 (Hudson and Bryant, 2006). The results showed that Pt-CTPZ was clustered together with all the other members of the cathepsin Z family and was most closely related to cathepsin Z from animal parasitic nematodes O. volvulus (AAT00789.1), B. malayi (AAT07061.1), W. bancrofti (EJW82660.1) and L. loa (EFO21073.2) (Fig. 6.4). Analysis also revealed that cathepsin Zs are more closely related to cathepsin B than to other cathepsins. Cathepsin L, S and F which belong to the cathepsin L-like family were clustered together (Fig. 6.4).
6.2.3 Venom allergen-like protein

The putative Pt-vap contained 350 nt with a putative open reading frame (ORF) of 79 bp. This partial sequence was not predicted to have a signal peptide and N-glycosylation site. BLASTP analysis revealed the predicted Pt-VAP had highest similarities with extracellular venom allergen proteins from G. rostochiensis VAP1 (AEL16453.1) (65% identity; E-value 4e-28), H. glycines VAP-1 (AAK60209.1) (65% identity; E-value 7e-28), M. incognita VAP-1 (ABO38109.1) (42% identity; E-value 4e-11), C. elegans VAP-1 (NP_741951.1) (49% identity; E-value 6e-13) and A. caninum VAP-1 (AAC47001.1) (45% identity; E-value 2e-16).

Manual alignment of the translated Pt-vap sequence with venom allergen-like proteins of G. rostochiensis (AEL16453), H. glycines (AAK60209), D. ditylenchus (ADC35399), M. arenaria (ABL61274), M. incognita (ABO38110), Bursaphelenchus mucronatus (ADV57661), B. xylophilus (ADG86238), C. elegans (NP_741951) and A. caninum (AAC47001) made with CLUSTALW2 in the BioEdit program revealed that these sequences were generally conserved. Most venom allergen-like proteins have eight conserved cysteine residues and a HYTQ amino acid region, although Y is replaced by W in VAPs described from plant endoparasitic nematodes up to now (Hawdon et al. 1996) (Fig. 6.5). Pt-VAP contain five of the conserved cysteine residues and the sequence HWTQ (amino acids 375-378), which is a conserved variation of the HYTQ sequence. Although B. xylophilus and B. mucronatus are also plant parasitic nematodes, the Y in the HYTQ amino acid region was instead replaced by F, possibly due to them feeding on wood or fungal hyphae growing in it instead of plant roots. Free-living nematodes, C. elegans and animal parasitic nematodes, A. caninum both have HYTQ region in their VAP sequences. Cys85, Cys162, Cys163, and Cys175 are known to be involved in disulphide bond formation in other cysteine rich proteins (Hawdon et al. 1999). The Pt-VAP sequence has all these cysteine residues (amino acid 389, 394 and 413) except for Cys85.
Figure 6.5 Alignment of the putative protein sequence of *P. thornei* venom allergen-like protein and that of other nematode species: *G. rostochiensis*, GrVAP-1 (AE16453), *H. glycines*, Hg-VAP-1 (AAK60209), *D. destructor*, Dd-VAP-2 (ADC5399), *M. arenaria*, Ma-VAP-1 (ABL61274), *M. incognita*, Mi-VAP-1 (ABO38110), *B. mucronatus*, Bm-VAP-1 (ADV57661), *B. xylophilus*, Bx-VAP-2 (ADG86238), *C. elegans*, Ce-VAP-1 (NP_741951) and animal parasitic nematode *A. caninum* Ac-ASP-2 (AAC47001). Identical residues are shaded black. Grey arrows and red box represent the Allergen V5/Tpx-1 family signature and conserved sites respectively.

To deduce the phylogeny of VAPs, amino acid sequence of Pt-VAP and VAPs from free-living nematodes (*C. briggsae*, *C. brenneri*, *C. elegans*, *C. remanei*), plant parasitic nematodes (*M. arenaria*, *M. incognita*, *G. rostochiensis*, *H. glycines*, *D. destructor*, *B. mucronatus*, *B. xylophilus*), and animal parasitic nematodes (*A. caninum*, *N. americanus*, *Ancylostoma duodenale*, *Haemonchus contortus*, *Heligmosomoides bakeri*, *Ostertagia ostertagi*, *A. ceylanicum*) aligned with CLUSTALW2 were used to generate the phylogenetic tree using MrBayes 3.2.2 (Fig. 6.6). Phylogenetic analysis of Pt-VAP revealed that it is closely related to VAPs of cyst nematodes, *H. glycines* and *G. rostochiensis* (Fig. 6.6). Analysis also show that VAPs from the animal parasitic nematode (APN) *Toxocara canis* formed a group nested in the paraphyletic plant parasitic nematode cluster while VAPs from other APNs are clustered with each other in other clades.
Figure 6.6 Phylogenetic analysis constructed from ClustalW alignments of venom allergen-like proteins of *P. thornei* with VAPs from plant parasitic nematodes, animal parasitic nematodes and free-living nematodes. Pt-VAP is in red font. The scale bars show the amount of genetic change of 0.05.
6.2.4 Temporal expression of genes encoding cathepsins and venom allergen-like protein.

Temporal expression of cathepsin L, cathepsin Z and venom allergen-like protein of *P. thornei* was assessed using semi-quantitative PCR on cDNA derived from different life stages. Total RNA was extracted from eggs, pre-parasitic juveniles, parasitic juveniles and adults. RNA from pre-parasitic *P. thornei* were obtained from eggs hatched *in vitro* in water. For Pt-ctpZ, gene expression was observed throughout the life cycle, whilst transcript accumulations for Pt-ctpL and Pt-vap were absent in the egg and pre-parasitic juvenile stages. Transcript accumulation for all target genes were the highest during the parasitic juvenile stage. Expression of Pt-vap was the highest in the J2 and adult stages compared to Pt-ctpL and Pt-ctpZ. For Pt-ctpL, there was more transcripts in the J2 stage compared to Pt-ctpZ.

![Image of PCR gel showing expression levels of Pt-ctpL, Pt-ctpZ, Pt-vap, and 18S rRNA in different life stages of *P. thornei*.]

**Figure 6.7** Expression of Pt-ctpL, Pt-ctpZ, Pt-vap and 18S rRNA in eggs, pre-parasitic juveniles, juveniles and adults of *P. thornei*. As a positive control, all cDNA templates were amplified with the primers for 18S rRNA from *P. thornei*.

6.2.5 RNA interference of putative genes encoding cathepsins and a venom allergen-like protein.

*In vitro* RNAi was performed to observe the changes in the reproduction ability of *P. thornei* after exposure to dsRNA of cathepsin L, cathepsin Z and venom allergen-like protein. DsRNA of cathepsin L used was 414 bp while dsRNA of Pt-ctpZ and Pt-vap generated were 243 bp and 350 bp respectively. After 16 h of soaking mixed populations of *P. thornei* with dsRNA of these target genes, the nematodes were examined for changes in body shape and viability. Nematodes soaked in dsRNA of target genes, Pt-ctpL, Pt-ctpZ and Pt-vap maintained the same sigmoidal body shape and move in
similar wavy pattern as the control nematodes (nematodes treated in no dsRNA and dsRNA of \textit{gfp})
(Fig. 6.8).

\textbf{Figure} 6.8 Phenotypes displayed by \textit{P. thornei} after soaking in medium containing dsRNA of target genes for 16 h. (A) cathepsin L (B) cathepsin Z (C) venom allergen protein (D) \textit{gfp} and (E) no dsRNA. Activities of nematodes fed with target gene dsRNAs did not differ from those of controls (D and E).

Evidence of \textit{Pt-ctpL} transcript knockdown was obtained from assessing transcript levels 16 h after feeding, normalised against expression of 18S rRNA (Table 6.1). Ingestion of \textit{ctpL} dsRNA resulted in 54-fold and 43-fold reduction in transcript accumulation relative to nematodes treated with no dsRNA and with \textit{gfp} dsRNA. A greater down-regulation of gene expression was noted with \textit{Pt-ctpZ} dsRNA-fed nematodes, its transcript accumulation was reduced by 288-fold after dsRNA treatment in relation to no dsRNA. The abundance of message for venom allergen-like protein in \textit{P. thornei} was also decreased (101.13-fold) when the nematodes were exposed to \textit{Pt-vap} dsRNA. In general, the fold knockdown was less when transcript abundance of nematodes fed with dsRNA of target genes were compared to those soaked in dsRNA of \textit{gfp} than to those soaked without dsRNA (Table 6.1).
Table 6.1 Quantification of reduction in cathepsins and venom allergen-like protein transcript accumulation 16 hr after dsRNA-mediated gene silencing.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold knockdown (No dsRNA control)</th>
<th>Fold knockdown (GFP dsRNA control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin L</td>
<td>53.82±0.10</td>
<td>42.81±0.06</td>
</tr>
<tr>
<td>Cathepsin Z</td>
<td>288.02±0.17</td>
<td>135.30±0.05</td>
</tr>
<tr>
<td>Venom allergen protein</td>
<td>101.13±0.30</td>
<td>41.07±0.16</td>
</tr>
</tbody>
</table>

Following soaking for 16 h, the nematodes were used to infect mini carrot discs: after five weeks, all the nematodes present were extracted from the mini carrot discs. Reproduction of the nematodes on carrot hosts treated with dsRNA derived from genes encoding cathepsins were not as severely affected by RNAi compared to Pt-vap dsRNA-treated nematodes: there were only 35% and 54% of nematodes respectively for Pt-ctpL and Pt-ctpZ compared to the numbers present in controls after five weeks (Fig. 6.9). Notably there was no increase in the number of nematodes soaked in Pt-vap dsRNA over the five weeks culture period.

![Graph showing reduction in reproduction of P. thornei on carrot mini discs after 5 week culture](image)

**Figure 6.9** Reduction in reproduction of *P. thornei* on carrot mini discs after a 5 week culture, following treatment with dsRNA of cathepsins and venom allergen-like protein for 16 h; cathepsin L (*Pt-ctpL*), cathepsin Z (*Pt-ctpZ*) and venom allergen-like protein (*Pt-vap*), compared with treatment with dsRNA of *gfp* and with no dsRNA.
6.3. Discussion

A series of studies have been undertaken to analyse cysteine proteases of a number of parasitic nematode species. At present seven cathepsins have been identified for these nematodes at the sequence level (B, C, D, F, L, S and Z). Most cathepsins are endopeptidases whilst only cathepsin B, C and cathepsin Z act as carboxypeptidases (Guha and Padh, 2008). This study is the first to investigate cysteine proteases in *P. thornei*: partial sequences of cathepsin L and Z cysteine protease from *P. thornei*, were successfully identified and cloned in this study. According to their deduced amino acid sequences both proteases, Pt-CTPL and Pt-CTPZ, can be classified as belonging to the sub-family C1A of papain-like cysteine proteinases (MEROPS peptidase database, http://merops.sanger.ac.uk/index.shtml). Phylogenetic analysis demonstrated that Pt-CTPL was more closely related to those of plant parasitic nematodes such as *M. incognita*, *G. pallida* and *H. glycines*, than to proteins from animal parasitic nematodes, *Drosophila* and mammals. Cathepsin L of the pine wilt nematode *B. xylophilus* and the stem nematode *D. destructor*, however, differ from those of *P. thornei* and other plant parasitic nematodes, possibly as a result of their rather different lifestyles and more distant phylogenetic relationships. Although *D. destructor* is an endoparasitic nematode, it attacks stems and bulbs instead of roots whereas pine wilt nematode, *B. xylophilus*, feeds on pine wood or fungal hyphae living in it.

Whilst cathepsin Ls are endopeptidases, cathepsin Z acts as carboxy-monopeptidase or carboxy-dipeptidase, both have numerous postulated functions ranging from protein maturation to catabolism (Guncar et al., 2000). Analysis showed that Pt-CTPZ, like all cathepsin Zs, has the carboxy-dipeptidase domain. In addition, the presence of the HIP motif in the mature region and the absence of the ERFNIN and GFND motif in the pro-region of Pt-CTPZ clearly demonstrate its relation to cathepsin Z group, and distinguished it from the cathepsin L and B subfamilies. Pt-CTPZ also contained a three residue insertion, termed the mini loop, which is found only in cathepsin Zs. This ‘loop’ blocks substrate access into the active site in the primed binding regions and provides the H residue, His23 which bind the carboxyl group at the C-terminus of the substrate (Guncar et al., 2000). Although phylogenetic analysis indicated that Pt-CTPZ was most closely related to cathepsin Zs of *O. volvulus*, *B. malayi*, *W. bancrofti* and *L. loa*, they do not belong to the same clade. Currently, cathepsin Z sequences of PPNs are not available from public databases, therefore relationship of Pt-CTPZ could not be established between them.

In phytoparasitic nematodes, a main role for intestinal cathepsin L-like cysteine proteases may be the extracellular digestion of dietary proteins (Lilley et al., 1996b, Neveu et al., 2003a, Shingles et
Localisation of *Pt-ctpL* to the intestine of the parasitic juvenile and adult stages suggested that this protease may also have a role in nutrition or digestion. Based on sequence alignments, cathepsin L of *P. thornei* clustered with those of PPNs that have functions in digestion (Lilley et al., 1996b, Neveu et al., 2003a, Shingles et al., 2007). In contrast, in Strongylid nematodes, cysteine proteases are responsible for degradation of host haemoglobin, as shown for *Necator americanus* (Ranjit et al., 2009) and *Haemonchus contortus* (Rhoads and Fetterer, 1995). These data suggested some substrate specificity for the enzymes, depending on the organism and its target. However because the *Pt-ctpL* transcript was only expressed in parasitic stages, this may indicate that some function of the cysteine protease is actually more directly related to the parasitic aspects of the plant-nematode relationship. Moreover, knockdown of *Pt-ctpL* retarded the replication of *P. thornei*. RNAi of *Mi-cpl-1* also resulted in lowered establishment rate of nematodes. This could be due to changes in shape of the female nematodes which subsequently decreased their egg-laying capability (Atkinson et al., 1996). For *C. elegans*, RNAi of a cathepsin L caused a 95% embryonic lethality (Hashmi et al., 2002). However, silencing of cathepsin L does not necessarily lead to reduction in the total number of nematodes: dsRNA of cathepsin L with *H. glycines* and *G. pallida* resulted in an increased male: female ratio (Urwin et al., 2002). The differences in observations after RNAi treatment for different organisms have made it difficult to deduce the precise function of cathepsin L. However, from all these experiments, it is clear that activity of cathepsin L is essential for normal development of nematodes, and therefore it represents an attractive target for more permanent, *in planta* RNAi based host resistance.

Unlike *Pt-ctpL*, transcripts of *Pt-ctpZ* were present in all nematode stages, but with significant differences between them: higher transcript levels were expressed in egg and parasitic J2s than in pre-parasitic J2 and adult stages. Although *in situ* hybridisation analysis indicated that the *Pt-ctpZ* transcript was localised to the cuticle of *P. thornei* and RNAi treatment of *Pt-ctpZ* leads to decline in reproduction, the function of *Pt-ctpZ* could not yet be determined. However, the understanding of the function of *Pt-ctpZ* could be aided by the studies done on animal parasitic nematodes and free living nematodes. RNAi of cathepsin Z was reported to retard moulting processes of *O. volvulus* and *C. elegans* (Hashmi et al., 2004, Lustigman et al., 2004). The authors hypothesised that cathepsin Zs of nematodes are involved in the digestion of the old cuticle, degradation of the cuticular anchoring proteins, and/or the activation of peptide moulting hormones or other moulting enzymes by processing their pro-enzymes (Hashmi et al., 2004, Lustigman et al., 2004). However, to determine if moulting processes of *P. thornei* were really affected when *Pt-ctpZ* is silenced, future experiment could incorporate the use of scanning/transmission electron microscope to observe the changes in cuticle structure at different days post infection after RNAi treatment against cathepsin Z of *P. thornei*.
As for *C. elegans*, RNAi failed to completely arrest the moulting process of *P. thornei* as the nematodes were still able to establish to a certain extent in the mini carrot discs. This may indicate that other proteases/genes are active during the moulting process in *P. thornei* and that could compensate for the absence of the *Pt-ctpZ* silenced in the RNAi treated worms.

In phytoparasitic nematodes, VAPs have been identified in *H. glycines*, *M. incognita*, *D. africanus* and in *B. xylophilus*, and now *P. thornei*. Genes encoding VAPs are specifically up-regulated in infective juveniles during the onset of parasitism in plants (Ding et al., 2000, Gao et al., 2001, Kang et al., 2012). Transcript accumulation of *Pt-vap* showed the same pattern as for the other phytoparasitic nematodes, with presence only in the parasitic and adult stages. The absence of transcript accumulation in non-parasitic stages of *P. thornei*, coupled with localisation of *Pt-vap* expression in the subventral gland cells, indicates a possible involvement in plant parasitism. Moreover, after RNAi against *Pt-vap*, reproduction and increase in the number of nematodes on carrot discs was completely inhibited. Although it is challenging to predict the exact function for venom allergen-like proteins of phytoparasitic nematodes, some ideas could be obtained from the research of animal parasitic nematodes. VAP proteins are thought to be essential for the establishment and persistence of infections by nematodes in animals, possibly as a modulator of the host defense system (Hawdon et al., 1996, Bower et al., 2008). A recent report from Smant’s laboratory (Lozano-Torres et al., 2014) suggested that this function could be similar for VAPs of phytoparasitic nematodes as well. Secreted VAP of *G. rostochiensis* (Gr-VAP-1) was observed to trigger a defence response in tomato plants by interacting with the apoplastic cysteine papain-like proteases Rcr3pim of *Solanum pimpinellifolium*. Gr-VAP1 and Rcr3pim are both required to activate defence-related programmed cell death and resistance to nematodes mediated by the extracellular plant immune receptor Cf-2 in tomato (Lozano-Torres et al., 2014). To determine if that is also the case for *P. thornei*, a VAP protein could be artificially generated and localised in plants to see if they interact with cysteine papain-like proteases similar to that found in tomato. Smant’s group also suggest that cyst nematode VAPs down-regulate the plant’s innate immunity to nematode infection, since overexpression in plants increases nematode infection, and susceptibility to fungal pathogens as well. Similar overexpression of the *Pt-vap* in host plants would aid in further understanding of the role for Pt VAP in plant-nematode interaction. In addition, other genes or metabolites of plants which could be involve in innate immunity of host plants could be measured after exposure to *Pt-vap* followed by a metabolomic or RNAseq analysis. From such experiments, it should be possible to determine if *Pt-vap* modulates innate plant defences in the same way as that of *G. rostochiensis* or in a different manner.
Chapter 7: Analysis of transcripts of fatty acid binding protein and antioxidants of *P. thornei*
7.0 Introduction

One of the earliest induced responses of a plant following successful recognition of a pathogen is to produce reactive oxygen species (ROS) quickly (Doke, 1983, Auh and Murphy, 1995). ROS, particularly superoxide anion (O$_2^-$), hydroxyl radicals (OH$^-$) and hydrogen peroxide (H$_2$O$_2$), can create a ‘barrier’ against the spread of the pathogen to other parts of the plant by strengthening the host cell walls via cross-linking of glycoproteins (Bradley et al., 1992, Lamb and Dixon, 1997). In addition, ROS can also cause lipid peroxidation and cellular damage by oxidising cell components such as DNA, proteins and lipids (Lamb and Dixon, 1997, Montillet et al., 2005). Consequently, a repertoire of ROS scavenging enzymes such as glutathione reductase (grx), glutathione-S-transferase (gst), peroxiredoxin (prx) and thioredoxin (trx), is needed by plant pathogens to mitigate the damaging effects of increased levels of host-induced ROS (Molina and Kahmann, 2007, Blackman et al., 2005, Dubreuil et al., 2011). Although genes encoding antioxidants are also present in free-living nematodes, their potential importance in parasitism have been shown in animal parasitic nematodes (Brophy et al., 1995a, Brophy et al., 1995b, Zhan et al., 2010) and some PPNs (Lu et al., 1998, Jones et al., 2004a, Dubreuil et al., 2007). Because of this, it is of interest to observe whether the genes with probable function in detoxification of ROS, also has a role in plant parasitism by root lesion nematodes.

Some genes encoding ROS scavenging enzymes have been identified in PPNs but only few have been characterised, for example glutathione peroxidase, peroxiredoxin and glutathione-S-transferase (Robertson et al., 2000, Jones et al., 2004a, Dubreuil et al., 2007, 2011). Studies of peroxiredoxin and glutathione peroxidase of G. rostochiensis, and glutathione-S-transferase of Rotylenchus reniformis showed that these enzymes are indeed involved in detoxification of ROS, being involved in the catalysis of the breakdown of H$_2$O$_2$ and other larger hydroperoxides (Robertson et al., 2000, Jones et al., 2004a, Hou, 2013). In addition, the localisation of these genes on the surface of invasive juveniles, e.g. peroxiredoxin of G. rostochiensis (Gr-prx) and subventral gland cells e.g. glutathione-S-transferase of M. incognita (Mi-gst-1), indicate a role for these proteins at host-parasite interface, possibly against ROS produced by plants. The significance of peroxiredoxins and glutathione-S-transferase for plant parasitism by nematodes was further proven with RNA interference where silencing of prx and Mi-gst-1 genes in the presence of H$_2$O$_2$ reduced the egg mass formation and viability of M. incognita (Dubreuil et al., 2007, 2011). The study on R. reniformis also showed the importance of R. reniformis gst (Rr-gst) as transgenic soybean roots carrying the constructs containing Rr-gst have significantly higher H$_2$O$_2$ levels than control roots (Hou, 2013). Although yet to be characterised for PPNs, genes like thioredoxin and glutathione reductase may also...
have potential roles in detoxification of ROS, based on study on animal parasitic nematodes (Muller et al., 1997). For example, thioredoxin of *Brugia malayi* has been shown to protect it from DNA nicking activity of oxygen radicals and like other genes encoding antioxidants, *Bm-trx-1* was also found in the secretory-excretory products of parasitic stages of the nematodes (Kunchithapautham et al., 2003).

There are other proteins present at the nematode surface that may aid the nematode in avoiding/inhibiting host defence responses. One such protein group comprises the highly conserved nematode-specific fatty acid and retinol binding (FAR) proteins which bind a wide range of fatty acids including linoleic and linolenic acids, precursors of the plant defence compound, jasmonic acid (Prior et al., 2001, Delker et al., 2006). FAR proteins of *M. javanica*, *G. pallida* and *R. similis* have been shown to inhibit the lipoxygenase-mediated metabolism of unsaturated linoleic and linolenic acids and allene oxide synthase of the host plants (Prior et al., 2001, Haegeman et al., 2012, Iberkleid et al., 2013, Zhang et al., 2015). This inhibition may lead to suppression of jasmonate synthesis and of downstream signalling pathways, thereby compromising host defences (Prior et al., 2001, Iberkleid et al., 2013, 2015).

The aim of the research in this chapter was to carry out preliminary characterisation of genes encoding glutathione reductase, glutathione-S-transferase, peroxiredoxin, thioredoxin and fatty acid and retinoid binding protein of *P. thornei*.

### 7.1 Experimental procedures

PCR was done as in section 2.3.3 using primers, PtGluRed, PtGST, PtPrx, PtTrx and PtFar with XbaI.XhoI restriction sites appended to one primer and BamHl.KpnI attached to the other primer (Table 2.1). Sequence homology comparisons were conducted on fatty acid and retinoid binding protein (Pt-far), glutathione reductase (Pt-grx), glutathione-S-transferase (Pt-gst), peroxiredoxin (Pt-prx) and thioredoxin (Pt-trx) transcripts of *P. thornei*, using BLASTX and BLASTN. Transmembrane regions and glycosylation sites of these transcripts were predicted using ExPaSy bioinformatics resource portal (http://www.expasy.org/tools/). Predictions of a signal peptide for secretion and the cleavage site in the five transcripts in this chapter were performed with SignalP while secondary structures of the putative Pt-FAR were predicted by PROFphd in the ProteinPredict server. Phylogenetic analysis of FAR proteins aligned with CLUSTALW2 were conducted using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). A mixed model for protein evolution with 1,000,000 generations (sample frequency = 100) was chosen with the first 250,000 generations cast-off as burn-in. The tree generated was viewed in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Expression of *Pt-
grx, Pt-gst, Pt-prx, Pt-trx and Pt-far genes in different stages of *P. thornei* were undertaken as in section 5.1.2 while *in vitro* RNAi of these genes was performed as in section 2.8. After RNAi, qPCR was performed on four biological replicates and three technical replicates with primers listed on Table 2.1.

### 7.2 Results

#### 7.2.1 Fatty acid binding protein of *P. thornei*

A total of four contigs and 10 singletons were identified in the *P. thornei* transcriptome which matched to fatty acid and retinoid binding protein (FAR) of other PPNs e.g. *G. rostochiensis*, *H. glycines*, *M. incognita* and *R. similis*. PCR amplification of the FAR consensus sequence using cDNA of *P. thornei* resulted in a 609 bp Pt-far which translate to 187 amino acids, is 95% of a full-length sequence of *R. similis* FAR (AFI80890.1). The putative Pt-FAR amino acid sequence includes a hydrophobic signal leader that is cleaved between Ala-17 and Gly-18, as predicted by SignalP. No N-glycosylation site was predicted but a conserved casein kinase II phosphorylation site is present at residues 47-51 (Fig 7.1). Like FARs from animal parasitic nematodes and *G. pallida*, secondary structure analysis of Pt-FAR predicts an α-helix-rich structure, with several amphipathic helical stretches (Fig. 7.1). Another structural property shared with FAR proteins of animal parasites is that a coiled coil structure is strongly predicted for Pt-FAR by the Coils algorithm, between positions 42-99 and 136-154.
Figure 7.1 Multiple alignment of selected partial FAR sequences from *R. similis* (Rs-FAR), *M. javanica* (Mj-FAR), *G. pallida* (Gp-FAR), *H. schachtii* (Hs-FAR), *Onchocerca ochengi* (Oo-FAR), *Ancylostoma caninum* (Ac-FAR), *C. elegans* (Ce-FAR) with putative fatty acid binding protein of *P. thornei* (Pt-FAR) to illustrate the differences of the sequences of the nematodes and the region and sites of greatest conservation. Identical amino acids are highlighted in black. The green boxes indicate potential signal peptides, and the potential casein kinase II site is indicated by the light blue box. The secondary structure line is derived from an analysis of the sequences by PROFphd in PredictProtein server (https://www.predictprotein.org/html), showing the presence of 12 α-helices.

Parasitic nematodes possess one or two structurally novel classes of FAR proteins (Prior et al., 2001, Garofalo et al., 2002, Cheng et al., 2013) whilst eight FAR proteins have been identified in the genome of *C. elegans* which are divided into three classes: class I (Ce-FAR-1, 2, 3 and 6), class II (Ce-FAR-4 and 5) and class III (Ce-FAR-7 and 8) (Garofalo et al., 2002). Pt-FAR and FAR of other parasitic nematodes, such as *G. pallida* (Gp-FAR-1), *M. javanica* (Mj-FAR-1), *Heligmosomoides polygyrus* (Hp-FAR-1) and *O. volvulus* (Ov-FAR-1), share the highest sequence similarity with class I of *C. elegans* (Table 7.1).
Table 7.1 Comparison of sequences between some FAR proteins of plant and animal parasitic nematodes with that of *C. elegans*, PPNs: *P. thornei* (Pt-FAR), *R. similis* (Rs-FAR), *M. incognita* (Mi-FAR-1), *M. javanica* (Mj-FAR-1), *G. pallida* (GpFAR-1), *A. besseyi* Ab-FAR-1), APNs: *O. volvulus* (Ov-FAR-1), *A. caninum* (Ac-FAR-1), *B. malayi* (Bm-FAR-1), *H. polygyrus* (Hp-FAR-1).

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To deduce the phylogeny of FAR proteins, Pt-FAR and FAR sequences from plant parasitic nematodes (order Tylenchida), animal parasitic nematodes (order Spirurida and Strongylida) and free-living nematodes (order Rhabditida) were used in the analysis. The results showed that Pt-FAR is clustered with nematodes from the order Tylenchida (*M. arenaria*, *M. javanica*, *G. pallida*, *R. similis* and *A. besseyi*) and is most closely related to *R. similis*. Analysis also demonstrated that FAR proteins are generally conserved within the order of nematodes, for example PPNs within the order Tylenchida and *Caenorhabditis* spp. within the order Rhabditida (Fig. 7.2), possibly due to their specific targets e.g. plants would contain different fatty acids and retinoids for binding by FARs of PPNs compared to those in humans targeted by human FARs.
Figure 7.2 Phylogenetic analysis of fatty acid and retinol binding (FAR) proteins of *P. thornei* with some FAR protein homologues of plant parasitic nematodes (order Tylenchida), animal parasitic nematodes (order Strongylida, Spirurida) and free-living nematodes (order Rhabditida). *X. laevis* retinol binding protein (NP_001084682) was used as the outgroup.
7.2.2 Transcripts of *P. thornei* potentially involved in detoxification of oxygen reactive species

Analysis of the available sequence data from the *P. thornei* transcriptome led to the identification of partial transcripts which could be involved in the detoxification of ROS from *P. thornei*, namely glutathione-S-transferase (*Pt-gst*), glutathione reductase (*Pt-gr*), peroxiredoxin (*Pt-prx*) and thioredoxin (*Pt-trx*). PCR with primers generated from consensus sequences of putative *Pt-gst*, *Pt-grx*, and *Pt-trx* and a contig sequence of peroxiredoxin in the *P. thornei* transcriptome resulted in the amplification of transcripts which putatively encode *Pt-gst* (68 aa), *Pt-grx* (80 aa), *Pt-prx* (171 aa) and *Pt-trx* (81 aa) respectively. Only *Pt-gst* was predicted to be secreted, albeit via a non-classical secretory pathway because it has an NN score exceeding 0.5 according to SecretomeP analysis and it lacks a transmembrane region.

Analysis with *Pt-prx* in BLASTP revealed that it is most similar to peroxiredoxins of *G. rostochiensis* (CAB48391.1) (86% identity, E-value 8e-107 and *M. incognita* (ACZ67203.1) (84% identity, E-value 6e-104) while *Pt-trx* has the closest matches to thioredoxins of *Ascaris suum* (AAS78778.1) (68% identity, E-value 1e-31) and *O. volvulus* (AAN34969.1) (69% identity, E-value 3e-29). The coverage of the translated *Pt-trx* is 83% of that of a full-length *trx* sequence of *A. suum* (AY573598.1) (total score: 402). When compared with putative thioredoxins (partial sequences) of PPNs, *Pt-trx* have 74% coverage to that of *G. rostochiensis* (AWS05887.1) (total score: 259), 80% to *M. javanica* (CK427707.1) (total score: 603) and 66% to *H. schachtii* (CF101329.1) (total score: 661). A conserved domain search with putative Pt-TRX indicates that amino acids in the region 1 to 81 match protein family thioredoxin 8 (E-value 2e-22). In addition, the conserved structural motif, ‘thioredoxin-like fold’ and the WCPPC motif within the catalytic site that are present in most members of thioredoxin family, are also present. Pt-TRX also contains a consensus site for N-linked glycosylation, at N_26FTP_29 (http://prosite.expasy.org/scanprosite/) (Fig. 7.3, marked by grey arrow).

**Figure** 7.3 The translated sequence of the putative thioredoxin of *P. thornei*. The red box indicates the functional catalytic site of thioredoxin while the grey arrow indicates the N-glycosylation site.
The putative peroxiredoxin transcript of *P. thornei* also has a 'thioredoxin-like' fold motif. Amplified using primers designed from a putative peroxiredoxin contig sequence of the *P. thornei* transcriptome, *Pt-prx* shared 99-100% coverage with full-length peroxiredoxin amino acid sequences of *G. rostochiensis* (CAB48391.1) and *M. incognita* (ACZ67203.1). *Pt-PRX*, contains the conserved motif GGLG, but lacks the YF motif, possibly due to the incomplete sequence. Both the motifs are reported to be central in the stabilisation of its C-terminal structure, critical for sensitivity to H$_2$O$_2$. This sequence also contains three functional cysteine residues which is a common feature of Prxs, to form a catalytic triad (Fig. 7.4, red dot).

![Figure 7.4](image) Multiple alignment with selected full-length peroxiredoxin sequences of animal and plant-parasitic nematodes: *P. thornei* (*Pt-PRX*), *G. rostochiensis* (*Gr-PRX*) (CAB48391.1), *M. incognita* (*Mi-PRX*) (ACZ67203.1), *B. xylophilus* (*Bx-PRX2*) (ABW81468.1), *D. destructor* (*Dd-PRX*) (AFJ15099.1), *H. contortus* (*Hc-PRX*) (AAT28331.1), *A. suum* (*As-PRX*) (ERG83366.1), *C. elegans* (*Ce-PRDX*) (NP_001122604.1). Identical amino acids are shaded in black. The red dot indicates functional cysteine active site, and the H$_2$O$_2$ sensitive motif is boxed in blue.

Due to the lack of GST sequences of PPNs, alignment of sequences with putative GST of *P. thornei* was done with GST of animal parasitic nematodes, with the exception of *M. incognita* GST (Mi-GST-1). The putative *Pt-gst* has a domain containing the thioredoxin-like fold’ (IPR012336), a common structure of several disulfide proteins which consists of a three-layer $\alpha/\beta/\alpha$ core, according to analysis with Interproscan. The *Pt-GST* sequence also matches the GST N-terminal, from position 1-23 and GST C-
like terminal, from amino acid 34-59. Within the N-terminal, Pt-GST harbours the residues of G-site at positions 1, 2, 13, 14, 41 and 42 (Fig. 7.5, green inverted triangles). G-sites are important for the binding of GST to glutathione for the detoxification of genotoxic compounds of both endogenous and exogenous origin.

Figure 7.5 Multiple alignment of putative P. thornei GST sequence with selected full-length glutathione-S-transferase sequences of M. incognita (Mi-GST-1) (ABN64198), animal parasitic nematodes, H. contortus (Hc-GST) (AA81283), Ancylostoma duodenale (Ad-GST-2) (AGA61750), Heligmosomoides polygyrus (Hp-GST-2) (AAF36480), Oesophagostomum dentatum (Od-GST-2) (ACA30415) and free-living nematodes, C. elegans (Ce-GST-8 and -38) (NP_494884, CAB04293) and C. remanei (Cr-GST-30 and -7) (EFO91390, EFO91539). Identical amino acids are shaded in black. The green inverted triangles indicate residues of the G-site while the conserved regions are denoted by the red boxes.

BLASTP searches with Pt-GST revealed the closest similarities with GST of Nectator americanus (ETN84081.1) (55% identity, E-value 4e-16) and Haemonchus contortus (AAF81283.1) (58% identity, E-value 7e-16) while for Pt-GRX, the most similar matches from the BLASTP analysis were glutathione reductases of Onchocerca volvulus (CAA62130.1) (71% identity, E-value 7e-30) and Strongyloides ratti (CEF63210.1) (70% identity, E-value 8e-29). As the glutathione reductase sequences for PPNs to date are all partial (Fig. 7.6), the putative Pt-GRX was compared to the full-length glutathione reductase sequence of the animal parasitic nematode O. volvulus (Ov-GRX). Pt-grx identified in this study only covers 55% of Ov-grx total length of 1683 bp, with the highest identity between position 918 to 1296 bp.
(Fig. 7.6). Searches for conserved domains of Pt-GRX revealed that it has the FAD/NAD-linked reductase and the pyridine nucleotide-disulphide oxidoreductase dimerisation domains (http://www.ebi.ac.uk/interpro/). This finding coupled with the prediction made by GO term on Pt-GRX function as an oxidoreductase and a FAD linker (due to presence of a FAD binding site; Fig. 7.6) further confirm the identity of Pt-GRX as a glutathione reductase.

**Figure 7.6** TBLASTX analysis of deduced *P. thornei* grx sequence compared to homologous sequence of selected glutathione reductase sequences from animal and plant-parasitic nematodes: full length sequence of *O. volvulus* (CAA62130) and *A. ceylanicum* (AEY77128.1) and partial sequence of *B. malayi* (XP_001892443.1), *D. africanus* (FE925119.1), *M. javanica* (BI142733.1) and *R. similis* (EY191946.1). The red shading indicates conserved residues indirectly involved in FAD binding.

### 7.2.3 Temporal expression of fatty acid and retinoid binding proteins, glutathione reductase, glutathione-S-transferase, peroxiredoxin and thioredoxin of *P. thornei*

To assess temporal expression of putative genes encoding the antioxidants, *Pt-trx*, *Pt-prx*, *Pt-grx* and *Pt-gst*, as well as fatty acid and retinoid binding protein, *Pt-far*, semi-quantitative PCR was performed for different life stages of *P. thornei*. Gene specific primers against different genes were used together with 18s rRNA primers as a reference. PCR experiments showed that the *Pt-far* and *Pt-trx* mRNA was present in all life cycle stages tested (Fig. 7.7). In contrast, *Pt-grx*, *Pt-gst* and *Pt-prx* expression was only present in the more mature stages of *P. thornei*. When compared with the other genes, expression of thioredoxin was the highest in the egg and the juvenile stages. In the adult stage, the expression of *Pt-prx* was the highest, followed by *Pt-trx*. Expression of *Pt-grx* was the lowest in the adult
stage. *Pt-far* was expressed the most in the adult stage compared to the egg and juvenile stage. Similar observations were made for *Pt-gst* and *Pt-prx*.

![Figure 7.7 Expression of *Pt-trx*, *Pt-prx*, *Pt-gr*, *Pt-gst*, *Pt-far* and 18S rRNA in eggs, juveniles and adults of *P. thornei*.

### 7.2.4 Differential silencing effects of *Pt-far*, *Pt-grx*, *Pt-gst*, *Pt-prx* and *Pt-trx*

*In vitro* RNAi was performed on the putative transcripts encoding antioxidants and *far* of *P. thornei*, to evaluate the effect of silencing on their reproduction capability. After 16 h soaking with dsRNA of *Pt-far* (609 bp), *Pt-grx* (240 bp), *Pt-gst* (207 bp), *Pt-prx* (516 bp) and *Pt-trx* (606 bp), *P. thornei* were observed for changes in body shape and viability. The phenotype and activity of nematodes soaked in dsRNA of targeted genes did not differ from those of controls (without dsRNA and dsRNA of *gfp*). The reduction in expression of mRNA of *Pt-far*, *Pt-grx*, *Pt-gst*, *Pt-prx* and *Pt-trx* in nematodes treated with dsRNA were then quantified using qRT-PCR using expression of 18S rRNA as an endogenous reference for normalisation. *P. thornei* soaked in dsRNA of the five genes did show various degrees of silencing. The expression of *Pt-trx* in dsRNA-treated nematodes showed the highest degree of silencing in this study with 1278-fold and 296-fold reduction respectively compared to expression in nematodes fed with dsRNA of *gfp* and with no dsRNA. A similar trend of reduction in transcript abundance was found for nematodes treated with dsRNA of peroxiredoxin: higher degree of gene knockdown was observed relative to the nematodes soaked without dsRNA (64-fold) compared to those treated with dsRNA of *gfp* (18-fold). Similarly, RNAi of *Pt-grx* resulted in a high reduction of mRNA levels in nematodes when compared to no dsRNA (49-fold). The mRNA expression of glutathione reductase of *P. thornei* was also affected by *gfp* dsRNA treatment (reduction by 37-fold). Unlike nematodes soaked in dsRNA derived from *Pt-grx*, *Pt-prx* and *Pt-trx*, there was a comparatively low reduction of mRNA levels in nematodes.
exposed to Pt-gst dsRNA (6.6-fold and 1.3-fold). The expression of Pt-far in dsRNA-treated nematodes showed 22-fold and 9-fold reduction when compared with those fed with dsRNA of gfp and no dsRNA.

qPCR was also used to quantify the transcript abundance of other putative transcripts encoding antioxidants when only one of these transcripts was knocked down. When Pt-grx was silenced, there was an increase in mRNA abundance of Pt-prx and Pt-trx by 1.6 and 0.5-fold while Pt-gst was reduced by 3.9-fold. Conversely, knockdown of Pt-gst led to increased transcript accumulation of Pt-grx, Pt-prx and Pt-trx by 0.2-, 0.3- and 0.8-fold. Silencing of Pt-trx caused the reduction in mRNA abundance of Pt-grx, Pt-gst and Pt-prx in this study by 39-, 6- and 128-fold. Similar observations were made for Pt-prx but the fold decrease in expression for Pt-gr, Pt-gst and Pt-trx were higher (23-, 31- and 250-fold) (Fig. 7.8).

**Figure 7.8** Comparison of gene expression of Pt-grx, Pt-gst, Pt-prx and Pt-trx when only one of the genes was silenced. (A) Expression profile of Pt-gst, Pt-prx and Pt-trx when P. thornei was soaked in dsRNA of Pt-grx. (B) Expression profile of Pt-grx, Pt-prx and Pt-trx when P. thornei was soaked in dsRNA of Pt-gst. (C) Expression profile of Pt-grx, Pt-gst and Pt-prx when P. thornei was soaked in dsRNA of Pt-trx. (D) Expression profile of Pt-grx, Pt-gst and Pt-trx when P. thornei was soaked in dsRNA of Pt-prx. These graphs show that downregulation of one of the antioxidant genes impacted expression of other genes involved in detoxification of ROS.

### 7.2.5 Silencing of genes encoding FAR protein, as well as glutathione reductase, glutathione-S-transferase and peroxiredoxin reduces reproduction of P. thornei

Following soaking in dsRNA, mixed stage P. thornei were added to four replicates of mini carrot discs *in vitro* for each gene. After five weeks culture, nematodes were harvested from the discs using the mist apparatus and their numbers counted. Whilst there was no significant difference in the average number of nematodes extracted from discs infected with nematodes soaked in dsRNA of *gfp* and with
no dsRNA, on average, there was a 50% reduction in the number of nematodes retrieved from discs infected with *Pt-grx* dsRNA-treated nematodes, compared to that of nematodes soaked with no dsRNA (p<0.05) (Fig. 7.11). For GST dsRNA-treated nematodes, there was only a 38% reduction in number of *P. thornei* when harvested, five weeks after RNAi. Soaking in dsRNA derived from *Pt-prx* inhibited subsequent nematode reproduction on carrot discs after five weeks, with a 70% reduction in nematode numbers. Although *Pt-trx* dsRNA induced the highest degree of knockdown amongst the genes tested, this result did not translate to the ability of the dsRNA to completely retard the reproduction of *P. thornei*. In fact, for dsRNA of *Pt-trx* treated nematodes there was only a modest reduction in reproduction, with nematode numbers at 86% of those of control treatments (Fig. 7.9). In contrast, for treatments with dsRNA of *Pt-far*, although its transcript reduction measured by qRT-PCR was comparatively lower than that for *Pt-grx*, *Pt-prx* and *Pt-trx*, that treatment was sufficient to cause an 84% reduction in the number of nematodes extracted after 5 weeks of culture.

**Figure 7.9** Reduction in reproduction of *P. thornei* on carrot mini discs 5 weeks after treatment for 16 h with dsRNA of glutathione reductase (*Pt-grx*), glutathione-S-transferase (*Pt-gst*), peroxiredoxin (*Pt-prx*), thioredoxin (*Pt-trx*) and fatty acid and retinol binding protein (*Pt-far*), compared with treatment with dsRNA of gfp and with no dsRNA
7.3 Discussion

Previously, FAR have been studied intensely in animal parasitic nematodes (Kennedy et al., 1995, Basavaraju et al., 2003, Garofalo et al., 2003) but the study on FAR in PPNs has only been done on G. pallida (Prior et al. 2001). In recent years, studies on FAR in PPNs have intensified specifically on M. javanica, R. reniformis and R. similis (Cheng et al., 2013, Iberkleid et al., 2013, Zhang et al., 2015). In this study, a putative Pt-far transcript from P. thornei has been identified. The translated Pt-far shows features common to previously described nematode FAR proteins in its primary and secondary structures (Kennedy et al., 1997, Prior et al., 2001, Basavaraju et al., 2003, Garofalo et al., 2003, Cheng et al., 2013). The secondary structure of Pt-far is α-helix-rich with no prediction for β-extended structure. This α-helix-rich structure is an important feature that discriminates FAR from the similar lipid-binding proteins of vertebrates (such as the 20 kDa lipocalins, and the 14 kDa members of the FABP/P2/CRBP/CRABP family) (Banaszak et al., 1994, Flower, 1996, Kennedy et al., 1997, Garofalo et al., 2003).

Phylogenetic analysis showed that Pt-FAR was closely related to FARs present in other PPNs. Amongst FARs of PPNs, the putative Pt-FAR was found to be most closely related to the FAR of R. similis and the least related to FAR of A. besseyi. FAR proteins of different nematode are also observed to be grouped in the tree based on their trophic group (free-living vs parasitic nematodes), host (human, animals or plants) and parasitic strategy (migratory or sedentary). A study on filarial parasites showed that despite strong conservation in ligand binding properties of FAR proteins between different parasitic nematodes, these FARs appear to have different post-translational modifications which may relate to their mode of parasitism (Garofalo et al., 2002).

*In situ* hybridisation in Chapter 4 showed that Pt-far is expressed in the hypodermis of P. thornei. Expression of far in the hypodermis have been shown in G. pallida and M. javanica to suppress jasmonic acid (JA) responsive genes e.g. Pin2 and γ-thionin in host, which ultimately neutralises the plant defence system (Prior et al., 2001, Iberkleid et al., 2013). Whether this role is played by the Pt-far localised in the hypodermis of P. thornei, requires further investigation. Future work could incorporate immunolocalisation using polyclonal antiserum raised against Pt-FAR on host infected by nematodes to monitor the location where host and nematode FAR interacts and an overexpression of Pt-FAR in the host experiment to assess its effects of the transcription levels of genes involved in JA synthesis (Iberkleid et al., 2013).

The importance of far for P. thornei is further shown in the RNAi treatment of P. thornei with Pt-far dsRNA where silencing resulted in a decrease in reproduction when retrieved five weeks post...
infection. This is consistent with the report on A. besseyi (Cheng et al., 2013). Cheng and colleagues (2013) also found that reproduction of A. besseyi was significantly lower when exposure time to dsRNA was increased, with 48 h soaking inducing the strongest silencing. In this study, P. thornei was only soaked for 16 h in Pt-far dsRNA and although the degree of silencing is not very high (22.2-fold in relation to no dsRNA control), it strongly impacted reproduction of this nematode. This suggests that different exposure time to dsRNA are required to silence expression of the far gene in different nematodes. FAR represents an attractive candidate for silencing via transgenic plants to control P. thornei because, as for M. javanica, it is a protein which has a broad range of functions in nematodes, encompassing development, reproduction, and inhibition of plant defences (Cheng et al., 2013, Iberkleid et al., 2013, 2015).

Plant pathogens also have to mitigate the increased levels of damaging oxygen radicals, e.g. hydrogen peroxide, produced by plants during invasion by M. incognita and Heterodera avenae (Kuźniak and Urbanek, 2000, Melillo et al., 2006, Torres et al., 2006, Simonetti et al., 2009). In this study, transcripts encoding glutathione reductase, glutathione-S-transferase, peroxiredoxin and thioredoxin of P. thornei were identified. Gst of P. thornei is expressed in the juvenile and adult stages but not from the egg stage: this pattern of expression coupled with its localisation in the oesophageal gland cells (Chapter 4, Fig. 4.6B) suggests that it might have a role in parasitism. In addition, experiment to study knockdown of transcript encoding Pt-gst resulted in a 38% reduction in the number of nematodes retrieved from mini carrot discs five weeks after feeding with dsRNA of Pt-gst, indicating the importance of gst in the reproduction of P. thornei within the host. The secreted GST-1 of M. incognita was proposed to neutralise the hydroperoxides and derived aldehydes produced by the oxidative burst at the plasma membrane and cell wall surrounding migrating nematodes or during giant cell differentiation (Melillo et al., 2006, Dubreuil et al., 2007). More work has to be done to determine if Pt-gst has similar function. Although definitive functional studies on PPNs GSTs are lacking, it seems feasible that GSTs may play a role in detoxification of ROS in its interactions with host plants, as is the case for many animal parasitic nematodes.

The putative Pt-TRX isolated here contained a putative WCPPCR active site sequence, similar to those found in 16-kDa thioredoxins of B. malayi (Kunchithapautham et al., 2003). This motif is important for P. thornei because in other organisms it is associated with the catalysis of reducing and antioxidant activities (Kunchithapautham et al., 2003, Fomenko and Gladyshev, 2003). Since thioredoxins are ubiquitous and have critical functions in redox regulation and signalling (Arner and Holmgren, 2000), Pt-trx was expected to be expressed abundantly. The Pt-trx characterised in this study was expressed throughout the life cycle of P. thornei. Localisation of Pt-trx within the intestine, as seen in Chapter 4, showed that it could putatively be secreted. However, due to it being partial, it could not be determined
if Pt-TRX has a signal peptide. Nevertheless, thioredoxins from the full-length A. suum and B. malayi in which Pt-TRX shared the closest identity and coverage with, also did not have a signal peptide but were found to be present in the excretory-secretory extracts (Kunchithapautham et al., 2003).

The lack of peroxiredoxin and glutathione reductase gene expression in the egg stage of P. thornei is surprising as in silico analysis of prx and grx transcript representation in EST databanks showed that they are expressed in the egg stages. Nevertheless, a study on M. incognita reported that although present, the expression in eggs and pre-parasitic juveniles were much lower compared to that of the parasitic stages of nematodes, which could be an indication of the importance of peroxiredoxin in plant invasion (Dubreuil et al., 2011). The putative Pt-PRX identified in this study contained the GLGG motif, which form part of an ATP binding site required for the repair or retroreduction of 2-Cys Prx by sulfiredoxin to restore peroxidase activity and the regulation of signalling events (Jonsson et al., 2008). In addition, Prxs which contained this motif are believed to be ‘residential scavengers’ of low levels of H$_2$O$_2$ produced endogenously by aerobic metabolism (Bang et al., 2012). Biochemical studies could be done to determine if Pt-PRX does form an ATP binding site for the catalysis of H$_2$O$_2$ breakdown, like that of G. rostochiensis and B. xylophilus (Robertson et al., 2000, Li et al., 2011). Future experiments involving antioxidants of P. thornei could include a DNA-nicking assay to determine levels of oxygen free radicals before and after RNAi to determine P. thornei’s degree of oxidative stress resistance.

The functional significance of the putative thioredoxin, peroxiredoxin and glutathione reductase in P. thornei was established by RNAi and mini carrot discs infection experiments. Although no morphological changes were observed after soaking, the results showed clearly that in vitro RNAi induced by soaking nematodes with dsRNA effectively reduced the expression levels of Pt-prx, Pt-trx and Pt-grx of P. thornei. However, knockdown of genes putatively involved in detoxification of ROS yielded variable success of P. thornei in reproduction. In this study, strong silencing of a gene does not necessarily correlate with high reduction in reproduction rate, as in the case of Pt-trx. Silencing of Pt-prx resulted in the strongest effect with 70% of the dsRNA-fed nematodes unable to reproduce, followed by a 50% reduction in the number of nematodes fed with Pt-grx dsRNA. The mRNA abundance of other putative transcripts with possible role in removal of ROS was also assessed when only one of these transcripts was silenced. Knockdown of these genes showed that the impact made on other genes in the pathway involved in the detoxification of ROS could be negative/positive and slight/large dependent on the gene targeted. For example, silencing of Pt-grx led to a small increase in the expression of Pt-prx and Pt-trx while similar observation were made for Pt-grx, Pt-prx and Pt-trx when Pt-gst was silenced. Knockdown of Pt-grx also caused a small reduction in the expression of Pt-gst. On the other hand, significant reduction in the expression of genes encoding antioxidants were also observed when Pt-trx and Pt-prx was knockdown, respectively (Fig. 7.8). These study showed that there is much more to learn
about how the genes encoding glutathione reductase, glutathione-S-transferase, peroxiredoxin and thioredoxin work e.g. whether they might compensate for the loss of one by generating more transcripts, how the turnover rates of these proteins affect the efficacy of RNAi, et cetera. Both plant and animal parasitic nematodes possess antioxidants and secreted lipid binding proteins indicating that there are similarities in the ways in which they protect themselves against defence responses e.g. quick and transient burst of ROS or changes in plant hormone levels such as salicylic acid, jasmonic acid elicited by their hosts. Whilst the defence mechanism of animal and plant parasites might differ, some aspects of the studies on animal parasitic nematodes could provide insights for future functional studies of antioxidants of PPNs.
Chapter 8: Transthyretin-like proteins and galectin of *P. thornei*
8.0 Introduction

Although chemical nematicides have been used to control PPNs, their potentially toxic effects on the environment have led to many being banned or their use restricted (as discussed in Chapter 1). In addition, in some cases nematodes that can overcome host natural resistance are also arising (e.g. root knot nematodes which are not controlled by the Mi resistance gene in tomato). These two factors, and the need to intensify agricultural production, has resulted in an extensive search for other suitable control strategies. Silencing of target genes by RNA interference (RNAi) has emerged as one such potentially effective control method against phytoparasitic nematodes. Ideally, gene silencing requires the use of target genes that have to meet one or more of the following criteria: they should be plant nematode-specific, conserved and necessary for nematode survival and/or development, and ideally have broad-range effect against phytoparasitic nematodes of crop importance without causing side-effects in hosts. The types of target genes used can either be those required specifically for nematode parasitism in host plants (i.e. ‘effectors’), or more generally genes that are vital for some stage of the nematode development, growth or reproduction.

Galectin is one of the many proteins in phytoparasitic nematodes thought to be involved in parasitism. First observed in vertebrates, galectins are characterised by a unique affinity for β-galactoside sugar structures, which are the conserved amino acid residues present within a carbohydrate recognition domain (CRD) (Greenhalgh et al., 2000). Galectins can be classified into 3 main subgroups- prototype galectins (containing a single CRD), chimeric-type galectins (a CRD fused to a collagen-like repeating domain), and tandem repeat- type galectins (two CRDs fused in tandem) on the basis of their protein structure (Hirabayashi et al., 1992). Galectins of all nematodes identified so far have either been of the tandem repeat-type and/or proto-type (Newlands et al., 1999, Greenhalgh et al., 2000, Lee et al., 2011). In animal parasitic nematodes galectins appear to interact with glyco-conjugates on or around the cells and influence migration, chemotaxis, adhesion, proliferation, apoptosis and neurite elongation (Barondes et al., 1994, Puche et al., 1996, Hughes, 1997, Matsumoto et al., 1998). In addition, they have also been associated with regulation of the immune system of ruminants (Turner et al., 2008, Wang et al., 2014). In plant parasitic nematodes, galectin have been identified in M. incognita, R. similis, G. rostochiensis and B. xylophilus (Dubreuil et al., 2007, Lee et al., 2011, Haegeman et al., 2012), although its function in PPNs is still vague. For B. xylophilus, localisation of galectin at the median bulb and esophageal gland cells have led to suggestion that galectin might be involved in food perception and digestion (Lee et al., 2011) while in M. incognita, its elevated expression in J3 stages indicates that it may have a role in the sedentary phase of these nematodes (Dubreuil et al., 2007).
Another gene family which has not been characterised but has potential involvement in plant parasitism is the transthyretin-like (ttl) gene family. Identified as one of the largest conserved gene families in nematodes, ttl is characterised by proteins possessing a transthyretin-like domain (PF01060, IPR001534, DUF290). TTLs were first described in C. elegans (Sonnhammer and Durbin, 1997), and subsequently identified in the plant parasitic nematodes H. glycines (Hg-hsp-11) (Gao et al., 2003), M. incognita (McCarter et al., 2004), Xiphinema index (Xi-ttl-1, Xi-ttl-2) (Furlanetto et al., 2005), R. similis (Rs-ttl-1 to Rs-ttl-4) (Jacob et al., 2007) and also in animal parasitic nematodes, e.g. Ostertagi ostertagia (Saverwyns et al., 2008). Although ttl identified in H. glycines are identified from the gland cells and termed as hsp-11, it is highly similar to ttl of other phytoparasitic nematodes (Gao et al., 2003). TTLs are a set of proteins with sequence similarity to the transthyretins (TTR) and transthyretin-related proteins (TRP). Although they all possess the transthyretin domain (PF00576), different functions have been associated with them. TTRs, found only in extracellular fluid and blood serum of vertebrates, transport thyroid hormones and retinol (vitamin A) (Sonnhammer and Durbin, 1997, Hennebry et al., 2006). In contrast, TRPs are found in a wider range of organisms such as bacteria, fungi, plants, invertebrates and vertebrates (Eneqvist et al., 2003). TRPs in different organisms seem to have diverse roles in a wide range of metabolic pathways e.g. in mouse, they have an apparent function in the uricase reaction pathway as 5-hydroxyisourate hydrolase while in plants, they are reported to be involved in brassinosteroid signalling (Lee et al., 2006, Li, 2005). The role of TTL proteins in nematodes is relatively unknown, despite their relatively abundant presence. A number of functions have been hypothesised for TTLs, which includes transport of hormones (McElwee et al., 2004, Parkinson et al., 2004a), digestion or absorption of nutrients (Furlanetto et al. 2005) and in the nervous system (Jacob et al. 2007). The aims of this chapter were (i) to examine transcripts putatively encoding transthyretin-like protein and galectin in P. thornei, (ii) to explore the distribution of ttl genes in C. elegans and selected parasitic nematodes and (iii) to investigate the possible impact of RNA interference of ttl and galectin on reproduction of P. thornei.

### 8.1 Materials and methods

Nucleotide sequences of putative P. thornei transthyretin-like protein (Pt-ttl) and galectin (Pt-gal) were translated using the Translate program on the ExPASy molecular biology server (http://www.expasy.ch/). The DNA/RNA GC Content calculator (http://www.endmemo.com/bio/gc.php) was used to calculate GC contents while positions of introns/exons within the transthyretin-like protein...
genomic sequence were determined using Softberry FEX program (http://www.molquest.com/molquest.phtml?group=index&topic=gfind). Secondary structure prediction was performed with PredictProtein (Rost et al., 2004) while the disulphide bonding state of cysteines were predicted by DISULFIND (Ceroni et al., 2006) on the same server. Putative signal peptides and N-glycosylation sites were identified as described in Chapter 4. To identify possible domains in the putative protein sequence of the transthyretin-like protein and galectin, a domain search was done with InterProScan (Zdobnov and Apweiler, 2001). A blastp search was performed with Pt-ttl and Pt-gal to retrieve sequences of their closest homologues from GenBank. For TTL, protein sequences containing the PF01060 domain were obtained from NEMBASE and WormBase ParaSite (http://parasite.wormbase.org/index.html) and aligned with the ClustalW algorithm (Thompson et al. 1994) in BioEdit 7.0.5.3. (Hall, 1997-2013). Expression of Pt-ttl and Pt-gal transcripts at the egg, juvenile and adults stages were measured as in section 5.2.2 while in vitro RNAi was performed as in section 2.8.

8.2 Results

8.2.1 Analysis of P. thornei transthyretin-like proteins

Screening of the P. thornei transcriptome revealed 17 contigs and 83 singletons with matches to ttl. Following BLAST analysis, primers Pt-ttl-4F and Pt-ttl-4R (Table 2.1) were designed based on the ttl transcript from the P. thornei transcriptome which have the highest E-value to amplify the sequence in P. thornei using cDNAs generated from mixed stages of P. thornei. After PCR of the ttl transcript of P. thornei, an unintentionally amplified genomic DNA (gDNA) sequence of 678 bp (possibly due to incomplete DNase I activity) and a cDNA sequence of 465 bp were obtained. Comparing the genomic DNA to the cDNA revealed 4 short introns (49, 55, 45 and 57 bp) (Fig. 8.1), all of which have the consensus GT/AG splice site. The coding sequence has an overall GC percentage of 51%, a GC1 of 58%, GC2 of 70%, a GC3 of 57%, a GC4 of 45% and GC5 of 49%. The introns have a GC percentage of 45%.

![Figure 8.1 Comparison of Pt-ttl cDNA to its genomic structure. Positions and number of introns were indicated by the numbers.](image_url)
Although *in situ* hybridisation results showed that the *Pt-ttl* transcript was localised at the intestine (Fig. 4.5), this protein was predicted to have an N-terminal signal peptide for secretion with a cleavage site between amino acid 15 and 16 (Fig. 8.2). Analysis with WoLF PSORT further confirms that this protein could potentially be secreted extracellularly by *P. thornei*. Database searches identified five TTL proteins which showed high sequence similarity to Pt-TTL, with the highest similarity with transthyretin-like protein 4 precursor from *R. similis* (CAM84513.1) (75% identity, *E*-value 9E-74), followed by TTL of *N. americanus* (ETN77953.1) (63% identity, *E*-value 5E-62), *Oesophagostomum dentatum* (KHJ74882.1) (61% identity, *E*-value 1E-61) and TTL-46 of *A. suum* (ERG87132.1) (63% identity, *E*-value 5E-61).

Pt-TTL contained two conserved cysteine residues and the characteristic ‘TTL’ domain (PF01060, IPR001534, DUF290) with two conserved signature motifs (Fig. 8.2). The cysteine residues predicted to be involved in the formation of disulphide bridges (red star in Fig. 8.2), are conserved among the phytoparasitic nematode TTLs. Pt-TTL together with Rs-TTL-3 and -4 have 2 cysteines, whereas Hg-HSP11, Xi-TTL-1, Rs-TTL-1 and -2 have one additional cysteine pair. These covalent bridges could offer interchain stabilisation and can be essential for extracellular proteins (Thangudu et al., 2008). The secondary structure of the Pt-TTL protein was predicted to consist of an α-helix and ten β-strands, linked together by short loops (Fig. 8.3).
Figure 8.2 Alignment of the predicted amino acid sequence of the protein Pt-TTL and its closest homologues, *A. caninum* (EYC12488.1), *A. suum* (ERG87132.1), *N. americanus* (ETN77953.1), *C. elegans* (NP_001256514.1) and TTLs of other plant parasitic nematodes, *R. similis* TTL-1 to -4, *X. index* and *H. glycines* hypothetical protein. The two conserved signature motifs of the transthyretin-like protein domain are boxed in red while the green boxes indicated the signal peptide region. Conserved cysteine residues are indicated with a red star while the red inverted triangle indicates the signal peptide cleavage site for Pt-ttl.

Figure 8.3 Two-dimensional plot of secondary structure in the Pt-TTL model onto its sequence. Yellow arrows represent the beta-strand while the pink barrel represents the alpha helix.
TTL protein sequences from *C. elegans*, plant and animal parasitic nematodes were searched in NEMBASE and WormBase ParaSite databases to gain some insights on the difference between TTL sequences in nematode species. In total, 560 TTL sequences were found using the transthyretin-like domain search (PF01060). For this study, only *C. elegans* TTL sequences were used to represent free-living nematodes while animal parasitic nematodes were represented by sequences of *A. suum*, *N. americanus*, *H. contortus* and *O. ostertagi*. TTL sequences found in plant parasitic nematodes were from *B. mucronatus* (5), *B. xylophilus* (25), *D. africanus* (2), *G. pallida* (11), *G. rostochiensis* (20), *H. glycines* (3), *M. arenaria* (3), *M. chitwoodi* (6), *M. floridensis* (10), *M. hapla* (8), *M. incognita* (15), *M. javanica* (9), *M. paranensis* (4), *P. vulnus* (1), *R. similis* (4) and *X. index* (13). The transcript encoding putative *Pt-TTL* was added to the analysis. Generally, two transthyretin signature motifs are evident and the positions of these do mains in the TTLs are indicated in Fig. 8.2. Within the first signature motif, the sequences for each class and its selected members from *C. elegans*, plant- and animal- parasitic nematodes are shown in Fig. 8.4. To belong to class I, the sequence have to contain the amino acid stretch of GxLxC-GxxPAxN while class II has a segment of amino acids GxLxCxGxPA. Class III has the amino acids GxLxCxGx-PAxG, class IV G-xx-x-C and class V, the amino acids G-xx-x-CN. In this study, TTLs of most plant parasitic nematodes belonged in class I with 81 sequences. This was followed by class II (24), class IV (13), class V (12) and class III (9). The putative Pt-TTL was grouped in class I together with 14 TTL sequences of *G. rostochiensis*, 13 of *B. xylophilus*, 12 of *X. index*, seven of *G. pallida* and *M. javanica*, six of *M. incognita*, five of *M. floridensis*, four of *M. hapla* and *M. paranensis*, as well as two TTL sequences each of *B. mucronatus*, *M. arenaria*, *M. chitwoodi* and *R. similis* (Rs-TTL-3, Rs-TTL-4). Class II consisted of one TTL sequence of *H. glycines* as well as a Hg-HSP-11, which shared close sequence similarity to a TTL sequence, an *R. similis* Rs-TTL-1, seven TTL sequences of *B. xylophilus*, three of *G. rostochiensis*, two of *B. mucronatus* and one TTL sequence each of *G. pallida*, *M. paranensis*, *M. javanica*, *M. incognita*, *M. hapla*, *M. floridensis*, *M. chitwoodi*, *M. arenaria* and *D. africanus*. Nine TTLs of phytoparasitic nematodes: two of *G. pallida* and *B. xylophilus*, as well as one of *M. incognita*, *M. hapla*, *M. floridensis*, *H. glycines* and *B. mucronatus* are grouped in class III. Three TTL sequences of *G. rostochiensis* and *M. chitwoodi*, two TTLs of *M. incognita*, and one of *G. pallida*, *M. hapla*, *R. similis* (Rs-TTL-2), *P. vulnus* and *B. xylophilus* are classified in class IV. Class V comprised of one TTL sequence of *G. pallida*, two of *B. xylophilus* and *M. hapla*, three of *M. floridensis* and four of *M. incognita*. The second TTL signature motif was less conserved between sequences of different species (Fig. 8.2). Besides these two motifs, the TTL proteins were characterised by the presence of conserved cysteine residues, with the majority of proteins having four cysteines. However, in class I and class IV, some of the proteins including Pt-TTL were found to have only the first two conserved cysteines.
Figure 8.4 Overview of the five classes of TTL proteins from selected sequences of *P. thornei*, *P. vulnus*, *R. similis*, *X. index*, *H. glycines*, *G. pallida*, *G. rostochiensis*, *B. xylophilus*, *M. incognita*, *M. hapla*, *M. floridensis*, *M. javanica*, *M. chitwoodi*, *D. africanus*, *C. elegans*, *A. suum*, *H. contortus*, *O. ostertagi* and *N. americanus*. Gaps are indicated by a '-' and variable amino acids by an 'X'. Plant parasitic nematodes are highlighted in green, *C. elegans* in blue and animal parasitic nematodes in orange.
8.2.2. Analysis of transcript encoding galectin of P. thornei

Examination of 16 contigs and 27 singletons encoding putative galectin from the *P. thornei* transcriptome led to the identification of one contig which has a 99% match to an EST galectin sequence of *P. penetrans* (total score: 1632, *E*-value 4E-136). PCR of *P. thornei* cDNA using primers PtGalectinXhoI.XbaI and PtGalectinBamHI.KpnI (Table 2.1) designed from this contig sequence led to the amplification of a putative *Pt-gal* which contains an open reading frame (ORF) of 772 bp. This transcript encodes a predicted protein of 236 amino acids. This ORF contained a tandem repeat structure which is similar to that found in other nematode galectins (Fig. 8.5) (Hirabayashi et al., 1992, Klion and Donelson, 1994, Newton et al., 1997, Newlands et al., 1999). The mammalian S-type lectin consensus amino acid motifs HFNPRF and WG x E x R (Klion and Donelson, 1994) were fully conserved in the second domain but only partially in the first domain (HISVRY and WG x E x R) (Fig. 8.5). Although not completely conserved, the motifs in the first domain showed a high degree of conservation between galectins of *P. thornei* (*Pt*-GAL), *P. penetrans* (*Pp*-GAL), *B. xylophilus* (*Bx*-GAL), *M. incognita* (Mi-GAL), *H. glycines* (Hg-GAL), *C. elegans* (Ce-LEC-1) and *H. contortus* (Hco-GAL-4) but not with galectin of *G. rostochiensis* (Gr-GAL) (Fig. 8.5). When compared to the first domain of the CRD, the putative *Pt-GAL* was 92% similar to galectin of *P. penetrans*, *B. xylophilus*, *M. incognita*, *H. glycines* and *P. coffeae*, followed by 85% with Gp-GAL, 61% with galectin of *P. vulnus* and *D. africanus* and 54% with Gr-GAL and Rs-GAL. In contrast, comparison of second CRD showed that the putative *Pt-GAL* shared 100% similarity to that of *Pp*-GAL, *Bx*-GAL and Mi-GAL, 83% with Hg-GAL and 16% with Gr-GAL. Due to the comparatively short galectin sequences of *Pc*-GAL (188 aa), Gp-GAL (161 aa), *Pv*-GAL (164 aa) and Da-GAL (109 aa), they lacked the second domain and thus could not be compared.

BLASTP searches performed on the nr database with the putative *Pt-GAL* sequence as query revealed similarity to galectins from both free living and parasitic nematodes. The highest similarity was to galectin of *B. xylophilus* (ACZ13331.1) (74% identity, *E*-value 1E-130). Other closest matches to the transcript *Pt-GAL* in BLASTP analysis included galectin containing domain of *H. contortus* (CDJ93815.1) (70% identity, *E*-value 3E-124), *C. briggsae* LEC-1 (XP_002631053.1) (71% identity, *E*-value 1E-123), 32kDa beta-galactoside-binding lectin of *A. suum* (ERG81473.1) (69% identity, *E*-value 7E-122) and LEC-1 of *C. elegans* (NP_496801.2) (69% identity, *E*-value 4E-121). The putative *Pt-GAL* also shared a 68% sequence similarity with *Pc*-GAL (Sequence Read Archive:SRR090313.179912.2), 59% with *D. africanus* (CB278944), 58% with *Pp*-GAL (BQ538073), 55% with Mi-GAL (CF802668), 50% with Hg-GAL (CB278944), 20% with Gr-GAL (AW506176).
8.2.3 Temporal analysis of transcripts encoding transthyretin-like protein and galectin of *P. thornei*

To examine the expression of transcripts encoding galectin and transthyretin-like of *P. thornei*, a semi-quantitative PCR was undertaken on the egg, J2 and adult stages. Gene specific primers were used for amplification of *Pt-gal* and *Pt-ttl* while 18s rRNA primers was used as control PCR. *Pt-gal* was expressed the most in the J2 stage, followed by adult stage but detectable expression was not observed in the eggs. Similar observations were made for *Pt-ttl* but *Pt-ttl* is expressed in eggs although at a very low level (Fig. 8.6). After 25 cycles, the expression of *Pt-ttl* is higher than the expression of *Pt-gal* at all life stages of *P. thornei*.
8.2.4. In vitro RNAi of transcripts encoding galectin and transthyretin-like protein of *P. thornei*

To evaluate the effects of silencing genes which putatively encode galectin and transthyretin-like protein, 1000 mixed stages *P. thornei* were fed with 2 mg/mL dsRNA of *Pt-gal* or *Pt-ttl* (with and without introns) in a basic soaking solution with 50 mM octopamine for 16 h as in section 2.8.2. DsRNA of galectin used was 772 bp while dsRNAs of *Pt-ttl* generated were 468 bp (exons only) and 678 bp (with introns) respectively. There were no differences in sigmoidal body shape and wavy movement of nematodes soaked in target gene dsRNAs compare to control nematodes fed with dsRNA of *gfp* and no dsRNA.

Approximately 800 *P. thornei* from each treatments were used for RNA extraction and the cDNA synthesised from these nematodes were used for qRT-PCR. qRT-PCR was used to measure the reduction in mRNA expression of *Pt-gal* and *Pt-ttl* in nematodes treated with dsRNA with 18S rRNA used as an endogenous reference for normalisation. Reduction in transcript accumulation was calculated using the Comparative Ct method ($\Delta \Delta Ct$) with reference to expression of the gene in nematodes fed with dsRNA of *gfp* and those soaked with no dsRNA. After 16 h treatment in dsRNA, there was a significant decrease in gene expression in *P. thornei* soaked in dsRNA of transthyretin-like (with or without introns). The expression of *Pt-ttl* in dsRNA-treated nematodes showed 831-fold (±0.93) (without introns) and 879-fold (±0.25) (with introns) reduction compared to expression in nematodes fed with dsRNA of *gfp* and with no dsRNA, respectively. A similar trend of reduction in transcript abundance was found for nematodes treated with dsRNA of *ttl* with introns: higher degree of gene knockdown was observed relative to the nematodes soaked without dsRNA (1017-fold) compared to those treated with dsRNA of *gfp* (962-fold). This decrease in transcript accumulation of *ttl* (with and without introns) also correlated with a reduced rate of replication of *P. thornei* on mini carrot discs when J2s were washed and transferred to the discs after soaking, and the nematodes subsequently

![Figure 8.6 Expression of *Pt-ttl*, *Pt-gal* and 18S rRNA in eggs, juveniles and adults of *P. thornei*.](image)
extracted and counted five weeks after *in vitro* RNAi treatment. However, although gene expression in nematodes exposed to dsRNA of *ttl* containing both exons and introns resulted in higher fold knockdown than that of nematodes exposed to dsRNA of *ttl* containing only the exons, this does not translate to a lower number of *P. thornei* retrieved five weeks after transfer to mini carrot discs (Fig. 8.7). The average number of nematodes retrieved from Pt-ttl (with introns) dsRNA treatment (n=94) was almost double of that of the treatment with Pt-ttl (without introns) dsRNA (n=52) over the five weeks culture period (Fig. 8.7). As can be seen in Fig. 8.7, the reproduction of Pt-gal dsRNA-fed nematodes did not differ statistically from those of controls (p<0.05), and this correlated with the minimal reduction in transcript of Pt-gal recorded with qRT-PCR (0.69-fold compared to those treated with no dsRNA and 1.8-fold (±1.22) reduction compared with those treated with dsRNA of *gfp*).

**Figure 8.7** Reduction in reproduction of *P. thornei* on carrot mini discs after a 5 week culture period, following treatment with dsRNA of transthyretin-like protein with introns (Pt-ttl), Pt-ttl without introns (Pt-ttl-e) and galectin (Pt-gal), compared with treatment with dsRNA of *gfp* and with no dsRNA
8.3 Discussion

Transcriptomic studies of several nematodes have revealed more than 4000 protein families encoded exclusively by nematode genes (Parkinson et al., 2004a). One of the largest gene families is the transthyretin-like protein family. In the present study, a partial putative ttl transcript of *P. thornei* (*Pt-ttl*) was identified from the transcriptome sequencing and amplified from cDNA of mixed stages of *P. thornei*. Using the transthyretin-like domain (PF01060), protein search of NEMBASE and WormBase ParaSite databases identified 560 TTL sequences of plant parasitic nematodes (Parkinson et al., 2004b). Analyses of the sequence data for *C. elegans*, plant-and animal-parasitic nematodes showed that they could be grouped into five classes of TTLs, using amino acid sequences in the first of the two TTL signature domains. Most TTL sequences of the parasitic species belong to class I, whilst only 25% of *C. elegans* TTLs were grouped in this class. In contrast, TTLs of *C. elegans* were represented the most in class III with less representation of the parasite datasets. It is suggested that classification of TTLs of most parasitic nematodes in class I rather than other classes could be due to duplication events which arose during the evolution of the parasitic nematodes (Saverwyns et al., 2008). It could also imply that class I of TTLs is important in parasite biology. To date, the biological significance of different TTL classes remains unclear.

Furlanetto et al. (2005) suggested that TTL proteins of PPNs could play a role in parasitism because of the presence of a signal peptide for secretion and the observation that the Xi-ttl-2 gene was expressed in the pharyngeal gland cell region of *X. index*. However, there is no experimental evidence to support this hypothesis. The putative Pt-TTL also contained a signal peptide but was expressed in the hypodermis of *P. thornei* as shown in Chapter 4. The putative Pt-TTL also contained a signal peptide but was localised in the hypodermis of *P. thornei* as shown in Chapter 4. The putative Pt-TTL could have a role in host-parasite interactions as some genes of PPNs e.g. glutathione peroxidase of *G. rostochiensis* and peroxiredoxin of *M. incognita*, expressed in the hypodermis, encode proteins secreted on the parasite surface that are in direct contact with host cells during nematode invasion (Jones et al., 2004b, Dubreuil et al., 2011).

Alternatively, the *Pt-ttl* transcript could actually be localised in the ventral cord (which is part of the hypodermis), similar to that of the *ttl* gene R13A5.6 of *C. elegans* and *Rs-ttl-2* of *R. similis* (Jacob et al., 2007). As with R13A5.6 and *Rs-ttl-2*, no aberrant phenotypes were observed following RNAi of *Pt-ttl*, which led to suggestions that ttlS have putative functions in the nervous system, given that the neural system of *C. elegans* is recalcitrant to RNAi (Jacob et al., 2007). However, silencing of *Pt-ttl* resulted in reduced reproduction, thus challenging this proposed function in *P. thornei*. Nevertheless, it
has been shown that some nervous system-related genes, e.g. *flp* genes in PPNs such as *G. pallida* and *M. incognita* are also susceptible to RNAi despite the contrary in *C. elegans*, suggesting that Pt-ttl could still have a role in the nervous system (Kimber et al., 2007, Dalzell et al., 2009, Atkinson et al., 2013). Since TTLs are a multi-gene family, it is expected that not all TTLs would have identical expression sites and functions even in the same species. For example, some *ttls* of *R. similis* have specific localisation in the ventral cord (*Rs-ttl-2*) and vulval region (*Rs-ttl-4*) while others have unspecific expression sites (*Rs-ttl-3, Rs-ttl-4*) (Jacob et al., 2007). To provide new insights in the biological role of TTLs in nematodes, in addition to identifying more members and applying combinatorial RNAi within the family, more work should be done to obtain the spatio-temporal profiles of most if not all the members of the TTL family in nematodes.

Another multigene family in nematodes is galectins, which are characterised by conserved sequence motifs in the carbohydrate recognition domain (CRDs) and their affinity for β-galactoside sugars (Barondes et al., 1994). All known galectins can be categorised into three types, depending on their molecular architecture, i.e. prototype, chimera-type, and tandem repeat-type. Like the galectins of all PPNs identified so far, the putative Pt-GAL, has a tandem repeat structure. It was proposed that this type of galectin, which have two CRDs joined by a linker peptide, could have a role as a ‘heterobifunctional cross-linker’, which enable them to cross-link a broad range and combinations of glycol-conjugates (Arata et al., 1997). This suggestion was backed by the observation that the two CRDs of rat galectin-4 show significantly different sugar binding properties (Arata et al., 1997). Given that tandem repeat-type galectins are commonly present in nematodes (protostomes) and mammals (deuterostomes), this indicates that these cross-linking molecules are of significance (Arata et al., 1997).

Nevertheless, the function of galectins in PPNs has not been studied in detail. Galectin of *M. incognita*, *Mi-gal*, was reported to be expressed in the intestinal region of *M. incognita* (Dubreuil et al., 2007). Although the putative *Pt-gal* transcript studied here was also found to be localised in the intestine (Fig. 4.6), more work has to be done to determine if *Pt-gal* is involved in digestion. However, the lack of transcription in the egg stages of *P. thornei* suggests that its main role is in more mature stages of the parasite’s life cycle.

In this study, treatment of *P. thornei* J2s with dsRNA of *Pt-gal* did not significantly reduce transcript abundance or reproduction of *P. thornei*, possibly because it belongs to a multigene family. It is interesting because RNAi was successfully applied to *P. thornei* with dsRNA corresponding to *Pt-ttl*, another member of a multigene family. Since the amount and length of dsRNA as well as incubation time were similar, differences in silencing efficacy of *Pt-ttl* and *Pt-gal* can be attributed to these
aspects: amount of target genes within the organism and the protein turnover rate. Further studies should examine whether parasitic nematodes have a repertoire of other types of galectins, i.e. proto- or chimeric-types, to what level the family members are conserved, and if there are novel sequences that could be specific for parasitism. Ligand isolation studies with affinity chromatography from both hosts and PPNs will enhance the understanding of the biological function of galectins in the nematodes, and possibly in host-parasite interactions.
Chapter 9: General discussion
The overall aim of the work described in this thesis was to determine whether *Pratylenchus* spp. were amenable to RNAi technology as a means for their control. Specific aims included optimising conditions to induce uptake of exogenously supplied dsRNA to initiate *in vitro* RNAi, identifying potential target genes for their control using NGS, including various classes of genes required for parasitism and suppression of host defences, determining the levels of silencing conferred by feeding dsRNA, identifying some genes which encode potentially secreted proteins using *in situ* hybridisation, and determining the reduction in nematode reproduction after down-regulation of some of the target genes, to identify those potentially most effective for control. All of these aims were successfully achieved to greater or lesser extents, and the data generated in this work has provided substantial new knowledge on the molecular basis of RLN-host plant interactions. Since RLNs do not form long term feeding sites, the results also indicate which genes are involved in movement through plant tissues (i.e. they are common to migratory and sedentary endoparasites), and genes encoding those effectors absent (or at least not found) in RLNs, but which are present in the endoparasites (i.e. their absence in RLNs provides additional support for specific function in feeding site formation). These aspects are discussed in this Chapter in more detail.

Since the discovery of RNAi, treatment of cells with dsRNA (*in vitro* RNAi) has been an invaluable tool for functional analysis of genes in many systems, and especially for nematodes (including free-living and parasitic nematodes) (Fire et al., 1998a, Urwin et al., 2002, Landmann et al., 2012). However, when this project was initiated there was very little molecular information available for root lesion nematodes, and so, in relation to RNAi there was a need to establish and optimise a system to make use of RNAi technology to apply it to these nematodes: this was achieved first for *in vitro* RNAi by ‘soaking’. In *Chapter 3*, effects of treating *P. thornei* and *P. zeae* with dsRNA was investigated to induce gene silencing, RNAi, as a potential strategy for their control. Mixed stage populations of nematodes of *P. thornei* and *P. zeae* were induced to ingest dsRNA when incubated in a basic soaking solution (M9 buffer, 0.05 % gelatine, 3 mM spermidine) in the presence of the neurostimulant octopamine. Incubation for up to 16 hours in basic soaking solutions containing 10-50 mM octopamine, 0.1-1.0 mg/mL FITC, and 0.5-6 mM spermidine did not affect vitality. Spermidine phosphate salt hexahydrate rather than spermidine or spermidine trihydrochloride improved uptake of FITC fluorescence by nematodes, and also resulted in more effective gene silencing. Silencing *pat-10* and *unc-87* genes of *P. thornei* and *P. zeae* resulted in paralysis and uncoordinated movements in both species, although to a higher degree in *P. thornei*. There was also a greater reduction of transcript expression of both genes in *P. thornei* indicating that it may be more susceptible to RNAi than *P. zeae*. *P. thornei* treated with dsRNA of *pat-10* and *unc-87* failed to establish and replicate normally in carrot mini discs, and there was respectively an 81 % and 77 % reduction in numbers of nematodes extracted.
after 5 weeks culture following dsRNA treatment. The results show clearly that RLNs are amenable to
gene silencing, and that in planta delivery of dsRNA to target genes in both species of nematode should
confer host resistance. Indeed, hairy roots generated with constructs containing Ptpat-10 led to a
significant reduction in P. thornei reproduction. Also of significance is that dsRNA constructs of either
nematode species elicited RNAi effects in both species indicating a possible cross-species control of
nematodes via RNAi.

9.1 Identification and characterisation of cell wall
degrading enzymes in root lesion nematodes

Understanding the mechanisms by which phytoparasitic nematodes can invade their hosts is
important and necessary for their control. Products secreted from these pests from different secretory
organs in the nematodes, such as the oesophageal, intestine, amphidial and rectal glands and the
hypodermis, have been recognised to play an important role in host-pathogen relationships and
consequently have been subjected to detailed study. Different approaches have been used to
identify and characterise these components, e.g. by PCR using degenerate primers, generation and
analysis of cDNA libraries, suppressive subtractive hybridisation and sequence signal trapping (Wang et
al., 2001a, Huang et al., 2004), but these are in general either technically challenging, time consuming
or costly. The generation of ESTs libraries represents an invaluable source for gene discovery, and in
this case, candidate parasitism genes. The introduction of next generation sequencing (NGS) has
unlocked a myriad of opportunities for the discovery of known and novel genes in both sedentary and
migratory nematodes (Abad et al., 2008, Opperman et al., 2008, Haegeman et al., 2011, Nicol et al.,
2012).

One of the most important groups of genes associated with plant parasitism by PPNs are the
cell wall degrading proteins (CWMPs). From the genome of M. incognita, 81 CWMPs have been
identified (e.g. arabinase, β-1,4-endoglucanases, pectate lyases, polygalacturonases and xylanases)
(Abad et al., 2008). However, comparative studies of the transcriptomes of P. coffeae, P. thornei and P.
zeae undertaken here (Chapter 4) identified fewer CWMPs – 43 in P. coffeae, 27 in P. thornei and 56 in
P. zeae. It has been proposed that migratory endoparasitism evolved from migratory ectoparasitic
ancestors, in the polyphyletic Pratylenchidae while Meloidogyne spp. appear to have evolved from
migratory endoparasitic nematodes (Bert et al., 2008, Holtermann et al., 2009). It is possible that root-
knot nematodes have acquired an additional arsenal of parasitism genes during evolution from
migratory endoparasites. In addition, differences in the number of parasitism genes acquired could also
result from the fact that root lesion nematodes migrate intracellularly in their host while root-knot nematodes move intercellularly. For example, *P. coffeae* contained transcripts with similarity to arabinogalactan endo-1,4-β-galactosidase found only in cyst nematodes (*H. schachtii* and *G. pallida*), which also migrate intracellularly (Vanholme et al., 2004, Haegeman et al., 2011). This gene is not present in *Meloidogyne* spp. genomes. Comparative analysis between the three *Pratylenchus* spp. also revealed that within the same genus, there can be variation in the number of parasitism genes. A similar trend was observed for *Meloidogyne* spp., for example, in *M. incognita*, 21 cellulases were found (Abad et al., 2008) while only four were identified from *M. hapla* (Opperman et al., 2008). As discussed in the previous chapter, it would be interesting to see if the differences in the number of parasitism genes in *Pratylenchus* spp. is affected by their collection from different locations and hosts in different parts of the world. Nevertheless, it was perhaps surprising that only a small proportion of the transcriptome consisted of CWMPs although they are vital for successful plant parasitism. A possible explanation for this is that proteins corresponding to the parasitism genes are relatively stable in a broad range of abiotic conditions i.e. pH, temperature (Gao et al., 2004a). Therefore, even with fewer copies of these genes, they are effective in plant invasion. It may also be possible that nematodes can ‘sense’ which type of plant cell wall it is dealing with, and they might adjust their repertoire of CWMPs based on the cell wall type perceived.

As has been found for other PPNs, the cellulases identified in this study from *P. thornei* (*Pt-eng*) belongs to the glycosyl hydrolase 5 family (Chapter 5). Although the *Pt-eng* sequence obtained was only partial, the sequence similarity search revealed that this sequence encodes a cellulase with both a catalytic domain and a carbohydrate binding module (CBM). The presence of the CBM in most endoglucanases of nematodes indicates that this domain is important for its functioning. In bacteria, the CBM, along with other subunits, is contained in a multi-enzyme high molecular weight complex called a ‘cellulosome’. Cellulosomes in bacteria are thought to be involved in facilitating the anchoring of the CWMPs to the plant cell wall (Bayer et al., 2004, Doi and Kosugi, 2004). It is worth exploring if the CBM in nematodes has a role similar to that in bacteria.

Because of the complexity of plant cell walls, *Pratylenchus* spp. also need other CWMPs to overcome the physical barrier presented by host plant cells walls: these include pectate lyases and polygalacturonases. Unlike cellulases, which cleave cellulose polymers, both pectate lyase and polygalacturonase are used by the nematodes to degrade the pectic components of host cell walls. A family of at least three pectate lyases was found in *Meloidogyne* spp. but there was no experimental evidence to show the same for *Pratylenchus* spp. Herron et al. (2000) suggested that plant pathogens with wide host ranges possess numerous isozymes of pectate lyases with complementary catalytic properties, which bind to differently composed oligogalacturonate units of the host cell walls. Hence, it
is possible that RLNs, which are polyphagous, also have multiple isozymes of pectate lyase. In this study, a partial sequence of polygalacturonase was also amplified from *P. thornei*; its sequence matched the GHF28 family. Although lacking experimental biochemical evidence, *Pt-pg* could be an exo-polygalacturonase based on its similarities with other exo-acting polygalacturonase. To confirm if *Pt-pg* is indeed an exo-polygalacturonase, its enzymatic properties could be investigated, for example, by measuring the release of reducing groups from polygalacturonate (Somogyi, 1952). If galacturonic acid is the only degradation product detected during enzyme activity assays, the identity of *Pt-pg* as an exo-PG could then be determined.

**9.2 Identification of cysteine proteases and venom allergen-like protein of *P. thornei***

Unlike CWMPs, there are groups of genes which are present in abundance in the *Pratylenchus* transcriptomes. One such group are the cysteine proteases. Cysteine proteases can be divided in 10 subfamilies, depending on their primary sequence and substrate compatibility (B, C, F, H, K, L, O, S, W, Z) (Berti and Storer, 1995, Barrett and Rawlings, 1996). In Chapter 6, two cathepsins from *P. thornei*, *Pt-ctpL* and *Pt-ctpZ* were studied. Like those found in other PPNs (Urwin et al., 1997a, Neveu et al., 2003a, Shingles et al., 2007), *Pt-ctpL* which was found in the intestinal region, could have a role in digestion. However, expression of *Pt-ctpL* was only seen in the infective stages of *P. thornei*, indicating that this gene might a role more directly related to parasitism. In support of this, *in vitro* RNAi undertaken against this gene led to a reduction in the replication rate of *P. thornei*.

In *N. americanus* and *H. contortus*, cysteine proteases are reported to have roles in the degradation of host haemoglobin (Ranjit et al., 2009, Rhoads and Fetterer, 1995). It is possible that *Pratylenchus* cysteine proteases have similar functions to those in the Strongylid nematodes in that they may be involved in suppressing the immune response of the host by degrading immunity components of host cells. It will be interesting to find out whether the cathepsin L of *P. thornei* interacts with specific components of the host, and if so, whether how this might subsequently affect the host. Unlike cathepsin L, *Pt-ctpZ* is expressed in the cuticle/hypodermis layer of *P. thornei*. Since there is little information on cathepsin Z of phytoparasitic nematodes, it is difficult to postulate a function. The only other publication for a nematode cathepsin Z is in *C. elegans* (Hashmi et al., 2004, Lustigman et al., 2004). It is suggested that *Ce-ctpz*, which is also expressed in the cuticle, may have a role in moulting. Before conclusions can be made on a role for *Pt-ctpZ* in moulting, future experiments need to be done, for example by the use of scanning/transmission electron microscope to document
any changes in cuticle structure at different days post infection after RNAi treatment against cathepsin Z of *P. thornei*.

Cysteine proteases could play different roles in plant parasitism by PPNs. A recent report suggested that apoplastic expression of the cysteine protease Rcr3pim of *S. pimpinellifolium* could enhance the virulence of *G. rostochiensis*, when interacting with venom allergen-like protein of the nematodes (Lozano-Torres et al., 2014). This finding is a major achievement as the function of venom allergen proteins in nematodes was previously unknown. *Pt-vap* identified in this study is most similar to that found in *G. rostochiensis* and other cyst nematodes. It is possible that *vap* of *P. thornei* could function in a similar manner to that of *Gr-vap-1*. It would be interesting to determine if *P. thornei* also interacts with a gene similar gene to *Rcr3pim* in its hosts which could similarly increase its virulence. Based on the *in vitro* RNAi data and spatio-temporal expression studies undertaken here there is good evidence to propose that *Pt-vap* is involved in plant invasion. The localisation of *Pt-vap* in the oesophageal gland cells and its expression only in the infective stages of *P. thornei*, coupled with an almost complete inhibition of reproduction of *P. thornei* observed five weeks after administration of dsRNA of *Pt-vap*, suggested that this gene must be regarded as a ‘parasitism’ gene.

9.3 Identification and characterisation of transcripts putatively encoding antioxidants and fatty acid and retinoid binding protein in *P. thornei*

The *Pratylenchus* transcriptomes also contains many transcripts encoding antioxidants, such as glutathione peroxidase, glutathione reductase (gr), glutathione-S-transferase (gst), peroxiredoxin (prx), superoxide dismutase and thioredoxin (trx). Their abundance in the transcriptomes indicates that the nematodes are constantly bombarded with environmental stresses, and so more copies and high expression of genes involved in minimising such stresses are required to neutralise such oxidative stresses. In Chapter 7, the focus was on *Pt-gr*, *Pt-gst*, *Pt-prx* and *Pt-trx* of *P. thornei*. Not all the proteins involved in synthesis of antioxidants were predicted to be secreted, although *in situ* hybridisation of *Pt-gst* and *Pt-trx* showed expression in secretory organs. That *Pt-gst* hybridised to the oesophageal gland cells was not surprising because this gene was shown to be expressed at the same locations in *M. incognita* (Dubreuil et al., 2007). *In vitro* RNAi of the genes with potential functions in detoxification of ROS led to various degrees of inhibitory effects on *P. thornei* reproduction on mini carrot discs. Feeding dsRNA of all such genes resulted in a significant reduction in the numbers of *P. thornei* harvested after the RNAi treatment, except for nematodes fed with dsRNA derived from *Pt-trx*. 174
A possible explanation for this is that there might be other thioredoxins in *P. thornei* which could compensate for the silenced *Pt-trx*. Alternatively, *Pt-trx* could have a high transcript turnover rate, and so silencing of *Pt-trx* did not greatly affect reproduction of *P. thornei*. The *in vitro* RNAi experiments undertaken here also demonstrated that when one of the genes encoding antioxidant was knocked down, the effects on other antioxidising genes could be different. For example, knockdown of *Pt-gr* was also accompanied by decreased *Pt-gst* expression, but this also led to an increased transcript accumulation of *Pt-prx* and *Pt-trx*. More work (i.e. enzymatic assays) must be done to confirm if silencing of one of the genes encoding antioxidants is sufficient to impair the detoxification ability of the nematodes in its hosts. It would also be of interest to examine whether there is a compensatory mechanism in the nematodes which counteracts the absence of a gene expression after RNAi. As in animal parasitic nematodes, future experiments involving the role of antioxidant genes of *P. thornei* could include a DNA-nicking assay to determine levels of ROS before and after RNAi treatment to determine the extent of resistance of *P. thornei* to oxidative stress.

In addition to reactive oxygen species, *Pratylenchus* could also potentially suppresses host immune systems by interfering with plant hormone production (i.e. jasmonic acid) using the fatty acid and retinol binding proteins. Similar to the *far* genes of other phytoparasitic nematode, the *Pt-far* identified in this study contained a signal peptide and is expressed in a secretory organ. FAR proteins in nematodes have been shown to inhibit the lipoxygenase-mediated metabolism of unsaturated linoleic and linolenic acids of host plants, which subsequently inhibits the synthesis of jasmonate and suppress host defences (Prior et al., 2001, Haegeman et al., 2011, Iberkleid et al., 2013). *In vitro* RNAi of *Pt-far* resulted in decreased establishment and reproduction of *P. thornei*, suggesting that without FAR proteins, the jasmonate pathway could function without inhibition, and so the host could detect nematode infestation and mobilise its defences against nematode attack. Alternatively, down-regulation of *far* could have impaired the ability of the nematodes to bind lipids and retinols, thereby depriving them of these essential metabolites, and which subsequently would result in reduced reproduction/development. It will be interesting to see which of these possible functions is actually performed by *far* of the nematodes. It could also be that FAR proteins evolved from internal lipid transport proteins of free-living nematodes to become secretory/surface products with extracellular functions.
9.4 Identification and characterisation of transthyretin-like protein and galectin of *P. thornei*

Galectins are genes with potential to be involved in plant invasion. Although these proteins can be divided into three subgroups, only two of these have been found in nematodes - tandem repeat-type and/or proto-type (Newlands et al., 1999, Greenhalgh et al., 2000, Lee et al., 2011). Similarly, the *Pt-gal* identified in this study is a tandem repeat galectin, characterised by a flexible linker domain between two carbohydrate recognition domains (CRDs) (Chapter 8). Typically, tandem repeat galectins are required for crosslinking of glycoprotein receptors and subsequent cellular signalling. Several studies have reported that tandem repeat galectins are effective in triggering many cell responses, including cell death (Stillman et al., 2006, Lu et al., 2007). In APNs the linker regions in these galectins increases signalling potency, by allowing intermolecular interaction of CRDs, thus promoting higher-order multi-mer formation (Nagae et al., 2006, Stowell et al., 2008, Earl et al., 2011). The question again arises on whether linkers in phytoparasitic galectins have similar roles to those in APNs. Although galectin has been proposed to be involved in parasitism in APNs, the work undertaken here for *Pt-gal* did not demonstrated any potential involvement of *P. thornei* in plant parasitism since *in vitro* RNAi did not significantly reduce their replication rate. One possibility is that *P. thornei* also possesses multi-gene families of galectins, some may play a role in parasitism whilst others could have housekeeping functions. Further studies could examine whether parasitic nematodes have other types of galectins, i.e. proto- or chimeric-types, the level of conservation between different galectin family members, and if there are novel sequences that could be specific for parasitism.

Another gene family which is broadly represented in the *Pratylenchus* transcriptomes are the transthyretin-like proteins. In this study, a *ttl* gene from *P. thornei* most similar to *R. similis ttl-4* was identified. Phylogenetic study of TTL sequences of *P. thornei* with those of *C. elegans*, other phytoparasitic nematodes and animal parasitic nematodes showed that most parasitic nematodes were grouped into class I while most *C. elegans* TTL are grouped in class III. These differences might be the result of additional duplication events which occurred during the evolution of PPNs (Saverwyns et al., 2008). It could also indicate that class I of TTLs are important in parasite biology. More research is required to understand why TTLs from most PPNs are grouped in class I.

Several functions have been postulated for TTL proteins of nematodes, including transport of lipophilic constituents or hormones and potential roles in the nervous system (McElwee et al., 2004, Hansen et al., 2005, Jacob et al., 2007). However, expression of *Pt-ttl* in the hypodermis region coupled with reduced reproduction rate after *in vitro* RNAi treatment probably rules out the possibility of *Pt-ttl*
being involved in functioning of the nervous system as proposed for *R. similis* (Jacob et al., 2007). Further work has to be done to determine if *Pt-ttl* is really involved in the transport of hormones and lipophilic compounds or if it is involved in other functions, although the reduction in replication of treated nematodes when transferred to carrot discs indicates that *Pt-ttl* performs a vital function. To provide new insights in the biological role of TTLs in nematodes, in addition to identifying more members of the family and applying combinatorial RNAi to the family, more work should also be done to obtain the spatio-temporal profiles of most if not all the members of the TTL family in nematodes.

### 9.5 Conclusion

In the research undertaken for this thesis, many new genes in *P. thornei* have been identified for which there was no experimental data available previously. The generation of holistic transcriptome data is indeed beneficial for discovery of genes at much lower cost than has been possible before. However, care must be taken in assigning functions to sequences solely on their similarities. In addition, there are some limitations to identification of novel parasitism genes from *Pratylenchus* spp. as illustrated by *M. incognita* (Abad et al., 2008). For example, novel CWMPs which were not discovered from the transcriptome (e.g. arabinase) were identified when the genome of *M. incognita* was sequenced. In fact, the recently completed genome of *P. coffeae* (Burke et al., 2015) revealed similarities with potential sedentary endoparasitic nematode-specific effector molecules identified in *M. incognita* (Huang et al., 2003), which were not found in the comparison with *P. coffeae* transcriptome. Nevertheless, the *Pratylenchus* transcriptomes have provided a very valuable avenue for comparing the differences between migratory and sedentary endoparasites which offers valuable insights into similarities and differences in gene content in relation to lifestyle. For *P. thornei* and *P. zeae*, this work represents the first steps towards functional characterisation of a number of new genes. This has been achieved by down-regulating the expression of such genes by soaking the nematodes in dsRNA and examining the effects on infection and reproduction. The result clearly show that the silencing effect can vary depending on a number of factors. These include: the choice of target gene, whether it is part of a multi-gene family, the specific sequence chosen and also the experimental details and treatments of the experiment itself. These factors indicate that soaking nematodes in dsRNA has some limitations, for example in the fact that a short soaking treatment is followed by a long culture period, in which some recovery from the effects of soaking can occur. A more effective approach is to ensure that the nematode is exposed continuously to the dsRNA by making transgenic plants producing dsRNA (Gheysen and Vanholme, 2007). Although time did not permit such a study of
transgenic plants for all genes studied here by soaking in dsRNA, the generation of transgenic carrot hairy roots carrying the Ptpat-10 constructs was shown to be effective in controlling P. thornei, a result which complements those for sedentary phytoparasitic nematodes (e.g. Yadav et al., 2006, Huang et al., 2006, Patel et al., 2010). In future experiments it would be interesting to challenge additional plant species transformed with RNAi constructs with different Pratylenchus spp, to check that RNAi based resistance is transportable to different crops. In the longer term, this could lead to the development of transgenic crops with inherent genetic resistance to multiple species of migratory nematodes.

In relation to acceptance of RNAi technology as a transgenic trait, companies in the USA are advancing some products through the regulatory system, and although there have been scare stories about siRNAs affecting humans, these have been strongly refuted, for example by Petrick et al. (2013), who conclude that ‘food’ and feed derived from these crops utilising RNA-based mechanisms is therefore expected to be as safe as food and feed derived through conventional plant breeding’. The work undertaken in this thesis provides a strong basis for further study on the control of root lesion nematodes using gene silencing technology: in time this strategy could well be deployed commercially.
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