Comparative and Functional Analysis of the Spliceosome Units of the Cyst and Root Lesion Nematodes

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Declaration

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

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Abstract

The root lesion nematode Pratylenchus thornei and the cyst nematode Heterodera schachtii belong to the top three plant parasitic nematode groups with regards to scientific and economic importance. RNA interference (RNAi) technology is a potential strategy for delivering transgenic resistance for nematode management in agriculture. The natural cellular mechanism of gene silencing, RNAi, is a sequence specific mRNA degradation process induced by the presence of double stranded RNA (dsRNA). The aim of this research was to investigate whether genes involved in cis and trans-splicing of mRNA in plant parasitic nematodes present suitable targets for nematode control using RNAi. In plant parasitic nematodes, RNAi was induced either through ingestion of dsRNA of genes involved in splicing with the help of a neurostimulant or via in planta delivery. Comparative analysis with Caenorhabditis elegans identified 55 spliceosome genes in the root lesion nematodes P. thornei, P. zeae and P. coffeae and 44 genes in the cyst nematodes H. glycines and H. schachtii. Identification of spliced leader (SL) RNA specific sut-1, sut-2, sna-1 and sna-2 genes suggests the occurrence of SL trans-splicing of mRNA in these nematodes. In vitro RNAi induced by soaking mixed stages of P. thornei and second stage juveniles of H. schachtii in 1 mg/mL dsRNA for selected spliceosome genes for 16 hours in some cases affected nematode movement causing paralysis, ‘knots’ and loss of direction, compared to those fed with dsRNA of green fluorescent protein (gfp) or controls not exposed to dsRNA. When dsRNA treated nematodes were transferred to carrot mini discs or Arabidopsis thaliana plants, after 5 weeks compared to control treatments there was a 55-99% reduction in the number of RNAi-treated P. thornei cultured on the carrot mini discs, and a 9-81% reduction in the number of H. schachtii cysts which developed on wild type A. thaliana, demonstrating that silencing of specific target genes reduced nematode survival and reproduction for both species. Reduction in transcript levels of some genes was also evident when analysed by semi-quantitative PCR. When carrot hairy roots were generated
which expressed sequences of dsRNA for SL specific genes of *P. thornei*, there was a wide variation in the number of nematodes extracted per gram root, between different transgenic events. *H. schachtii* cyst development over 5 weeks was reduced by 3-100% on transgenic *A. thaliana* plants transformed to express dsRNA of target 8 genes. The results suggest that some of the target genes studied are potential candidates for further development of nematode resistance in crop plants, and could contribute to the on-going search for delivering sustainable control of these and related nematodes using RNAi technology.
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CHAPTER 1

General Introduction and Literature Review

1.0. General introduction

Plant parasitic nematodes are one of the major groups of plant pests of both temperate and tropical agriculture. Even though most parts of vascular plants can be infected by at least one plant parasitic nematode species, the most economically important impact on agriculture is caused by species that parasitize roots. It has been argued by Fuller et al., (2008) that transgenic resistance reduces risks to the environment and to human health when compared with other chemical and biological nematode control methods. An exciting and upcoming strategy for transgenic resistance is the use of RNAi technology, which limits the transcript level of a particular gene or genes by activating a sequence-specific mRNA degradation process, triggered by dsRNA. Expression of a dsRNA homologous to a gene that is essential for the parasite and its ingestion by the nematode is required for in planta control of plant parasitic nematodes (Agrawal et al., 2003).

The research in this thesis was designed to identify spliceosome genes in the plant parasitic nematodes Pratylenchus thornei (a root lesion nematode) and Heterodera schachtii (a cyst nematode) and to study the effects of silencing selected genes in vitro and in planta via RNAi. The spliceosome is a ribonucleoprotein that mediates mRNA splicing in nematodes. The chosen targets were validated by RNAi, using two approaches. These were: ‘soaking’ and ‘in planta’ delivery of dsRNA; the first can provide a more rapid indication of whether a silenced gene can affect a nematode process, whereas the latter provides the ‘gold standard’ proof-of-concept. The overall aim of the research was to provide identity and evidence of genes that can be used to develop novel ‘synthetic resistance’ to plant parasitic nematodes. When
implemented, this could help contribute to reducing the 7-15% losses (Tan et al, 2013) for most crops caused by these parasites, and help contribute to sustainable food production in the future.

1.1. Literature review

1.1.1 Nematodes

Nematodes constitute the phylum Nematoda. They are the most abundant multi-cellular animals on earth. Nematodes have successfully adapted to almost every ecosystem: they are ubiquitous in fresh water, marine and terrestrial environments. They are called round worms mainly due to their cross sectional shape. A nematode is often thread like, cylindrical, generally fusiform or more rarely sac-like (e.g. female *Tetrameres* and *Meloidogyne*). Plant nematodes are about fifty microns in diameter, but nematodes can vary in length from microscopic (females of *Aphelenchoides bicaudatus* which are only up to 0.47 mm long) to several meters in length (more than 8 m long for female *Placentonema gigantissima* discovered in the placenta of the sperm whale) (Lee, 2002). Nematodes have digestive, nervous, excretory and reproductive systems. They lack a discrete circulatory or respiratory system, relying on diffusion through the cuticle instead. Most nematode species have separate male and female individuals (sexual dimorphism) and reproduction is usually sexual. Some nematodes are hermaphroditic and keep their self-fertilized eggs inside the uterus until they hatch. *C. elegans* and *Caenorhabditis briggsae* exhibit androdioecy. Their populations consist of separate male and hermaphrodite individuals. The plant infecting genus *Meloidogyne* exhibits different modes of reproduction, including sexual reproduction, facultative sexuality, meiotic and mitotic parthenogenesis. In some species males are rare (e.g. *Pratylenchus*). The typical life cycle of all nematodes consists of an egg, four juvenile stages (J1, J2, J3, J4) and a fifth, adult stage. They all molt four times during development. The reproductive structures become fully developed and functional in the adult stage. After each juvenile stage, a molt occurs and the
cuticle is shed allowing the nematode to increase in size. The generation time depends on the species. Nematodes can be free-living or parasitic on insects, humans, animals and plants (Chitwood & Chitwood, 1974; Lee, 2002).

1.1.2. *Caenorhabditis elegans*, a free living ‘model’ nematode

*C. elegans* is a free living, soil-inhabiting, microbivorous nematode found in many parts of the world and it is the most thoroughly studied nematode as a result of its choice as a ‘model’ nematode by Sydney Brenner in 1965 and extensive research that has been done on it (Chitwood & Chitwood, 1974). *C. elegans* adults are 1 mm long and have a life cycle of 3 days at 25°C. The worm exists as a self-fertilizing hermaphrodite and males occur at 0.2% frequency. Their small size, quick generation time, survival of long-term cryopreservation, transparency, invariant somatic cell number and the ability to reduce gene expression using RNAi are advantages of *C. elegans* as a model to study eukaryotic biology (Corsi *et al.*, 2015).

*C. elegans* was the first multi-cellular eukaryote to have its ~100 Mb genome sequenced (*C. elegans* Sequencing Consortium, 1998). *C. elegans* has five autosomal chromosomes and the X chromosome. Major genes in developmental and cell biological processes and features of gene regulation (transcription, translation, chromatin remodeling, post-transcriptional modifications) have been studied in *C. elegans* (Corsi *et al.*, 2015). The knowledge generated by extensive research is available in on line resources such as WormBase (www.wormbase.org), WormBook (www.wormbook.org) and others listed in table 1 in Corsi *et al.*, 2015.

1.1.3. Plant parasitic nematodes

Nematodes that are parasitic on plants are among the five most important groups of plant pathogens/pests with regards to economic loss caused in agriculture and horticulture. The
others are viruses, fungi, bacteria and insects. Root knot nematodes of the *Meloidogyne* spp.,
cyst nematodes of the *Heterodera* and *Globodera* spp. and root lesion nematodes of the
*Pratylenchus* spp. are among the top three from ten most significant nematode pests of crop
and horticultural plants worldwide. The other seven nematodes are *Radopholus similis*
(burrowing nematode), *Ditylenchus dipsaci*, *Bursaphelenchus xylophilus* (Pine wilt nematode),
*Rotylenchulus reniformis* (Reniform nematode), *Xiphinema index*, *Nacobbus aberrans* and
*Aphelenchoides besseyi* (Jones *et al.*, 2013).

### 1.1.3.1. Root knot nematodes

Root knot nematodes parasitize almost every species of vascular plants. The most important
species are the tropical *Meloidogyne incognita*, *Meloidogyne javanica* and *Meloidogyne
arenaria* and the more temperate *Meloidogyne hapla*. They induce galls (knots) on the roots of
their host plant. They are sedentary endoparasites. The second stage juvenile, which is the
only infective stage, invades roots soon after hatching and establishes a permanent feeding
site within the host root. Females stay at that site for the rest of their lives. The adult male
leaves the root and fertilizes the female, which lays over 300 eggs into a gelatinous egg mass
(Lee, 2002; Jones *et al.*, 2013).

### 1.1.3.2. Cyst nematodes

The most damaging cyst nematode species are soybean cyst nematode (*H. glycines*), potato
cyst nematodes (*Globodera pallida* and *G. rostochiensis*) and cereal cyst nematodes (including
*H. avenae* and *H. filipjevi*) (Jones *et al.*, 2013). Eggs of cyst nematodes are protected within a
tanned cyst and may remain dormant in soil for many years. Under favourable conditions the
eggs hatch and J2s migrate towards a host root. Hatching of many cyst nematode species
occurs in response to root exudates from a suitable host plant (Lilley *et al.*, 2005a). J2s
penetrate the root epidermis using repeated thrusts of their stylet mostly in the root
elongation zone, just behind the root tip and where lateral roots emerge (Wyss & Zunke, 1986). They migrate intracellularly, migrating through the cortical tissues towards the vascular cylinder. Cortical cell walls are disrupted by stylet thrusts that produce perforations aided by enzymes secreted by the nematode (Wyss & Grundler, 1992).

After invasion, a single cell is penetrated by the nematode stylet. Secretions from the dorsal pharyngeal gland of the nematode are secreted through the stylet into this initial feeding cell inducing the formation of a syncytium. Formation of the syncytium involves reprogramming of root cell development. Cell walls of adjacent cells are dissolved and incorporated into the syncytium to form a large, multinucleate feeding structure. The syncytium is a metabolically active structure with dense granular cytoplasm and proliferating mitochondria, endoplasmic reticulum and free ribosomes. Endoreduplication of DNA leads to enlarged nuclei and nucleoli (Jones & Northcote, 1972; Niebel et al., 1996). A number of small vacuoles are formed from the large central vacuole. Cell walls adjacent to xylem vessels develop ingrowths increasing the surface area through which solute uptake occurs (Jones & Northcote, 1972). A syncytium acts as if it were a single feeding cell from which the nematode obtains nutrients. A feeding tube that is considered to form from stylet secretions develops from the stylet tip each time it is inserted for feeding (Lilley et al., 2005a). The feeding tube acts both as a pressure regulator and a molecular sieve. In H. schachtii it excludes dextrans of 40 kDa but not of 20 kDa (Böckenhoff & Grundler, 1994) and proteins of 23 kDa but not of 11 kDa (Urwin et al., 1998).

After the syncytium is initiated, J2s undergo three molts and reach the adult stage. J3 and J4 stages each last for 3-4 days at 25°C. Cyst nematodes are sexually dimorphic: adult males leave the root (De Boer et al., 1999). Females of some cyst nematode species are known to release sex pheromones to attract males. The rear part of the adult female enlarges to form a spherical (Globodera spp.) or lemon (Heterodera spp.)-shaped saccate structure and the vulva
ruptures from the root before fertilisation. After fertilisation the cuticle of the female becomes tanned by a polyphenol oxidase and a tough, leather-like cyst is formed. In *Globodera* spp. the cyst contains and protects up to 500 eggs. Species such as *H. glycines* deposit some of the eggs through the vulva into a gelatinous matrix. Most species complete the life cycle in about 30 days although this is influenced by temperature. The corn cyst nematode (*H. zeae*) completes its life cycle in 15–18 days and the potato cyst nematode may take up to 3 months to complete its life cycle (Lilley *et al*., 2005a).

1.1.3.3. Root lesion nematodes

There are more than 60 species of root lesion nematodes (*Pratylenchus* spp.) which are distributed worldwide. The most important of these are *P. penetrans*, *P. thornei*, *P. neglectus*, *P. zeae*, *P. vulnus* and *P. coffeae* (Jones *et al*., 2013). The necrotic lesions they cause on host roots have given the common name for these nematodes (Lesion Nematodes, 1999). They are migratory endoparasites that enter the host plant root and migrate from cell-to-cell in the root tissues. They do not become sedentary in the roots, as do the cyst or root knot nematodes, but feed from individual host cells and move on. Root lesion nematodes are attracted to the root hair producing region of roots, the zone of elongation and to the root tips (Lesion Nematodes, 1999). They usually penetrate the root behind the root elongation zone. Younger nematodes (e.g. J2, J3) may feed from the root surface ectoparasitically for a short period with more advance stages entering the root. Penetration of the root through the epidermis is supported by the mechanical action of the stylet and probably by secretion of cell wall modifying enzymes secreted from esophageal glands. Within the roots, the nematode feeds on cortical cells, creating cavities as tissue is destroyed (Lesion Nematodes, 1999).

They do not normally enter the vascular cylinder, as do cyst and root knot nematodes. The nematodes migrate to healthy cortex areas or can exit the root and re-enter the soil or another
root when conditions in an infected root becomes unfavourable for feeding and reproduction. Female nematodes lay eggs singly or in small groups in the host root or in the soil near the root surface. J2s emerge from the eggs within 1 - 3 weeks, depending on the soil temperature. J2s undergo three more molts to become adults. *P. penetrans* and *P. alleni* reproduce sexually, whereas males are rare or absent in *P. hexincisus, P. neglectus* and *P. scribneri*, and eggs then develop parthenogenically (Lesion Nematodes, 1999).

All motile life stages after hatching from the egg are infective. However, root lesion nematodes can remain dormant as eggs, juveniles or adults in host roots or soil to survive adverse conditions. The time taken to complete the life cycle depends on the species and the soil temperature (Jones & Fosu-Nyarko, 2014). Nematodes feeding on root cells can cause disintegration of cortical tissue and shedding of the epidermis. Initially small, light to dark brown lesions on roots form and then expand and merge giving the roots a discoloured appearance. Lesion nematodes are known to interact with fungi such as *Verticillium, Fusarium* (Thompson *et al.*, 2008) and *Trichoderma viride* and this secondary infection causes disease complexes that may kill plants (Lesion Nematodes, 1999).

### 1.2. Control of plant parasitic nematodes

Plant-nematode parasitism is one of the most destructive and hard-to-control biotic stresses of crops, and worldwide plant parasitic nematodes cause an estimated US$125 billion crop loss annually (Chitwood, 2003). Stunted growth, wilting, chlorosis and poor yield that results from the interruption of water transport and diversion of nutrients to the nematode have a considerable impact on crop yield. Symptoms developed by nematode infections are commonly confused with nutritional or other disease problems (Atkinson *et al.*, 2003; Bakhetia *et al.*, 2005; Lilley *et al.*, 2005a).
Root lesion nematodes can be managed with agricultural practices, but cannot be eradicated. Low populations can be maintained by using rotations and resistant crops. Controlling weeds reduces build-up and carry-over of nematodes. Adequate nutrition will enable crops to tolerate nematode infection better (Collins, 2016). Once an area is infested with \textit{H. schachtii}, the nematode can be managed but cannot be eradicated. Pre-planting soil treatment with nematicides is not fully effective because the protective cysts are tough. Therefore, avoiding planting infested seedlings and practising good hygiene are important. Controlling weeds and rotation with non-host crops such as legumes, corn, cereal, onion or potato can reduce population build-up. Seedlings are more prone to severe damage than older plants. Therefore, planting crops when soil temperature is low as nematodes are less active and reproduce more slowly can reduce damage (Collins, 2016).

Chemical control of plant parasitic nematodes by nematicides is restricted by health and environmental constraints and may not be cost effective for the farmers. Crop rotation may be effective for disease management for species with a narrow host range, but it is less effective against species with a broad host range: in addition cyst nematodes such as potato cyst nematodes can survive in soil for many years, perhaps up to thirty years under favourable soil conditions in the absence of host plants. Crop rotation is also impractical for growers of specialist crops and may impose losses when alternative non-host crops provide low yields to the growers (Bakhetia \textit{et al.}, 2005; Fuller \textit{et al.}, 2008). Tomato, potato and soybean with some resistance against several \textit{Meloidogyne} species, \textit{G. rostochiensis} and \textit{H. glycines} respectively have been developed (Atkinson \textit{et al.}, 2003).

Nematode resistance in plants may occur either naturally, and such resistance may be transferred to crop cultivars from wild relatives or breeding lines through conventional breeding, or be engineered through molecular techniques. Durable and broad spectrum
resistance to plant nematodes can be achieved by transgenic expression of natural resistance genes in the host or genes that target and disrupt the nematode or genes that cause feeding site attenuation. These approaches reduce risks to the environment and to human health when compared with other chemical and biological control methods (Fuller et al., 2008). RNAi technology has become an exciting and upcoming strategy for transgenic resistance for nematode management in agriculture, since the induction of RNAi in C. elegans by injecting dsRNA, by Fire et al., (1998) (Sharma et al., 2013). Double stranded RNA can also be delivered to plant parasitic nematodes through a host plant that is engineered to produce dsRNA or its small interfering RNA, siRNA, a strategy termed ‘host-induced gene silencing’ (HIGS) (Gheysen & Vanholme, 2007).

1.3. RNA interference (RNAi) as a strategy for transgenic resistance for nematode control

1.3.1. RNA interference (RNAi)

RNAi is a gene regulatory mechanism that limits the transcript level of a gene or genes by activating a sequence specific mRNA degradation process. It has also been called ‘post-transcriptional gene silencing’ (PTGS) in plants, quelling in fungi and is also the mechanism underlying ‘virus induced gene silencing’ (VIGS). The inducer of the RNAi mechanism is dsRNA. Target mRNA is degraded in a homology dependent manner and the mechanism requires a set of proteins which are similar in structure and function amongst most organisms. RNAi was initially observed in plants and C. elegans, but has now been described in almost all eukaryotic organisms including protozoans, animals (vertebrates and invertebrates), fungi and algae (Agrawal et al., 2003; Meister & Tuschl, 2004).

An attempt, by R. Jorgensen and others in 1990, to up-regulate the activity of a gene for chalcone synthase (chsA), an enzyme involved in the production of anthocyanin pigments to
make petunia flowers more purple, resulted in some of the transgenic petunia plants carrying the chsA coding region under the control of a 35S promoter losing the activity of both the endogene and transgene, with many of the flowers being variegated. They termed the phenomenon ‘co-suppression’ (Napoli et al., 1990). Later it was discovered that the expression of the transgene led to the formation of RNA duplexes as there were self-complementary regions between the mRNAs of the endogene and transgene which initiated Post Transcriptional Gene Silencing (PTGS) (Metzlaff et al., 1997). A similar phenomenon was also observed in fungi. In an attempt to increase the production of an orange pigment made by the gene al1 of the fungus Neurospora crassa, a fungal strain containing a wild type al1 gene (orange phenotype) was transformed with a plasmid containing a segment of the al1’ gene. A few transformants showed albino phenotypes, in which expression of the native al1 mRNA was highly reduced. The level of unspliced al1’ mRNA was similar to that of the wild type strain, indicating that quelling and not the rate of transcription affected the level of mature mRNA in a homology dependent manner (Cogoni et al., 1996; Cogoni & Macino 1997).

In C. elegans PTGS was induced by Fire et al., (1998) who injected purified dsRNA directly into either the gonad or body cavity region of an adult worm. The dsRNA corresponded to a 742 nucleotide segment of unc-22, a gene that encodes an abundant but non-essential myofilament protein. The decrease in unc-22 activity produced a twitching phenotype. The worms showed weak twitching whereas the progeny showed strong twitching (Fire et al., 1998). RNAi was later demonstrated in C. elegans by soaking the worms in a solution containing dsRNAs or by feeding the worms with Escherichia coli that expressed dsRNAs. The silencing effect was observed to spread from the gut of the worm to the rest of the body and through the germ line to several generations (Timmons & Fire, 1998).
PTGS is known to occur during virus infection of plant genomes. RNA viruses make abundant dsRNA during intracellular replication of their genomes and therefore elicit cellular PTGS activity. PTGS can also occur with DNA viruses. Viruses can be the source, the target, or both the source and the target of silencing (English et al., 1996; Fagard & Vaucheret, 2000). Earlier, it had been found that plants could be protected from a severe virus by prior infection with a mild strain of a closely related virus. Based on this observation plants were transformed to express virus-derived transgenes (e.g. coat protein), however, this gave protection against the challenge viruses even when no transgene protein was produced (Lindbo & Dougherty, 1992). This observation suggested that RNAi in plants operated at the RNA rather than protein level.

1.3.2. Mechanism of RNAi

RNAi is triggered by dsRNA of different lengths and origins. These dsRNAs are processed into short RNA duplexes of 21 - 28 nucleotides in length, which recognise and cleave complementary single stranded RNAs, such as messenger or viral RNAs. In living cells, dsRNA may be produced by RNA dependent RNA polymerization as in the case of viral infections. DsRNA may also be produced by hybridization of overlapping transcripts from repetitive sequences such as transgenes or transposons which are processed into siRNAs or repeat-associated short interfering RNAs (rasiRNAs), which usually direct mRNA degradation. Furthermore, endogenous transcripts that contain complementary 20-50 bp inverted repeats can fold back to form dsRNA hairpins which are processed into micro RNAs (miRNAs) that may also direct mRNA degradation (Meister & Tuschl, 2004). Artificial introduction of long dsRNAs or siRNAs has also been used to inactivate gene expression, in cultured cells (Elbashir et al., 2001) and in living organisms (Fire et al., 1998; Tan et al., 2013).

DsRNA is recognized by dsRNA specific RNase III type endonucleases called Dicer and Drosha, which cleave the molecule into a series of 21–28 bp duplexes that carry two nucleotide
overhangs at their 3’ ends, which are called siRNAs. This cleavage is ATP dependant. These proteins contain catalytic RNase III and dsRNA binding domains (dsRBDs). Drosha is specifically required for processing miRNA precursors. Some organisms have more than one Dicer which processes dsRNA from different sources (Meister & Tuschi, 2004). In C. elegans, only one Dicer (DCR-1) has been identified that functions together with the dsRBD protein RDE-4 during RNAi (Tabara et al., 2002). Drosophila melanogaster (fruit fly) has Dicer-1 (DCR-1) to process miRNA precursors and Dicer-2 (DCR-2) to process long dsRNA (Lee et al., 2004). In the plant Arabidopsis thaliana, four Dicer-like proteins (DCL1 to DCL4) have been identified. DCL1, along with two more proteins HEN1 and dsRBD protein HYL1, process miRNA precursors. DCL2 is required for the production of siRNAs from plant viruses. DCL3 with HEN1 is involved in the production of rasiRNAs. DCL4 processes endogenous trans-acting siRNA (tasiRNA) (Gasciolli et al., 2005). SiRNAs can be transmitted systemically in C. elegans by interaction with proteins such as SID (transmembrane channel protein, product of sid-1 gene) (Winston et al., 2002).

The siRNAs form a complex with the RNA Induced Silencing Complex (RISC) (Fig. 1.1.). Assembly of RISC is ATP dependent. Argonaute proteins, which are the catalytically active proteins in RISC, cleave target mRNA in target RNA-siRNA hybrids. Different organisms have different numbers of Argonaute proteins: for example one in Schizosaccharomyces pombe, twenty seven in C. elegans, ten in A. thaliana, five in D. melanogaster and eight in humans have been identified (Höck & Meister, 2008). Other proteins in various RISCs have been identified, and in D. melanogaster RISC includes the Vasa intronic gene product (VIG), fragile X related protein (dFXR), and the tudor staphylococcal nuclease domain containing protein (Tudor-SN). The mRNA cleavage reaction guided by RISC is not ATP dependent (Agrawal et al., 2003; Meister & Tuschi, 2004).
The siRNA duplex is unwound to give single stranded siRNA leading to activation of the RISC which is also an ATP dependent step. The activated RISC searches for homologous mRNA transcripts by base pairing and cleaves the mRNA from the 3’ end of the siRNA leading to mRNA degradation (Meister & Tuschl, 2004). In C. elegans, single stranded siRNA (ss siRNA) molecules derived from dissociated dsRNA released from the activated RISC act as primers on complementary mRNA to amplify new strands leading to the production of new dsRNA molecules that become substrates for Dicer. This amplification is carried out by a RNA dependent RNA polymerase (RdRp) which is a product of the gene ego-1 (Bakhetia et al., 2005). The stepwise detailed mechanism of RNAi, its related processes and the subcellular locations of the RNAi processes are still being explored (Agrawal et al., 2003).
**1.3.3. Functional analysis of nematode genes by RNAi, *in vitro* and *in planta***

RNAi triggered by the presence of dsRNA complementary to the target mRNA sequence was demonstrated in 1998 for the free living nematode *C. elegans*. The genome of this model nematode has now been sequenced completely and annotated in detail, making it one of the best understood of all multicellular organisms. This has provided an exciting new resource to study plant parasitic nematodes, including genes involved in plant parasitism and genes vital for their survival. Targeted knockdown of genes by RNAi using dsRNA molecules corresponding to the gene of interest has proved to be a powerful approach for studying gene function in *C. elegans* (Fire *et al.*, 1998). In *C. elegans*, RNAi is usually achieved by their feeding on bacteria expressing dsRNA (Kamath *et al.*, 2001), by oral uptake of dsRNA from solution.
Effective use of RNAi technology to control plant parasitic nematodes in crop plants requires *in planta* expression of a dsRNA homologous to a target gene that is essential for the parasite’s survival, and its ingestion by the nematode. *C. elegans* and most probably other nematodes have a mildly alkaline gut lumen that does not digest nucleic acids rapidly (Bakhetia et al. 2005).

RNAi of plant parasitic nematode genes was first reported by Urwin et al., 2002. Genes encoding a cysteine proteinase and C-type lectin of cyst nematodes were targeted by soaking nematodes in a dsRNA solution with the neurochemical octopamine to stimulate ingestion. Cyst nematodes do not normally ingest liquid or food until a feeding site is developed within the host plant root. Delivery of the dsRNA to J2 cyst nematodes has been achieved by exposure to the neuroactive compound octopamine which induces pharyngeal pumping. Uptake of dsRNA caused a reduction in target gene transcript abundance and induced phenotypic effects for both *G. pallida* and *H. glycines* (Urwin et al., 2002). An aminopeptidase gene (Lilley et al., 2005b) and two parasitism genes of *H. glycines* (Sukno et al., 2007) have also been successfully targeted by RNAi using this method.

Knockdown of a β-1,4-endoglucanase reduced the ability of *G. rostochiensis* J2 nematodes to invade roots and a protein secreted from the amphids was shown to be essential for host location (Chen et al., 2005). Silencing *pat-10* (troponin C) and *unc-87* (calponin) genes of root lesion nematodes *P. thornei* and *P. zeae*, *in vitro*, resulted in paralysis and uncoordinated movements. There was a 77-81% reduction in *P. thornei* reproduction on carrot mini discs over a 5 week period after treatment with dsRNA of *pat-10* and *unc-87*. DsRNA derived from either nematode species silenced the corresponding gene in both species (Tan et al., 2013). Nematodes infecting transgenic soybean plants expressing a hairpin construct targeting the major sperm protein gene reduced egg production of soybean cyst nematode *H. glycines*.
(Steeves et al., 2006). Tobacco plants expressing dsRNA hairpin constructs for two
*Meloidogyne* genes encoding a splicing factor and a component of a chromatin remodelling
complex were reported to be resistant to *M. incognita* (Yadav et al., 2006). The gene 16D10
encodes a peptide that is secreted from the subventral esophageal gland cells of *M. incognita.*
*In vivo* delivery of 16D10 dsRNA from *Arabidopsis* to *M. incognita* suppressed their subsequent
development when inoculated onto *Arabidopsis* roots (Huang et al., 2006).

Since dsRNA itself does not produce a functional protein, non-target effects of expression of
dsRNA to a nematode target gene in a plant is expected to be minimal. The expression of
dsRNA in plants can be targeted at nematodes by using promoters that are preferentially
active in root cells or specifically in feeding cells. Non-target species that also feed on a plant
expressing dsRNA to a nematode gene can be protected by using dsRNA that has no
homologous sequences / genes in those or any other organisms: in this way off-target effects
can be minimised (Lilley et al., 2004; Bakhetia et al., 2005). In this research, RNAi technology
has been used for functional analysis of spliceosome genes in plant parasitic nematodes, as a
potential method for their control.

### 1.4. The spliceosome of plant parasitic nematodes

The spliceosome of nematodes is a complex of proteins and RNA that mediates mRNA splicing.

#### 1.4.1. Splicing of eukaryotic mRNA

The immediate product of transcription of eukaryotic protein-coding genes is the
primary/precursor mRNA transcript (pre-mRNA). In eukaryotic pre-mRNA, coding sequences
are interrupted by non-coding sequences. During pre-mRNA processing, it undergoes three
major modifications. A 7-methylguanosine cap is added at the 5′end of the pre-mRNA
molecule followed by the addition of a series of adenosine ribonucleotides at the 3′end, and
introns are removed and exons are joined in a sequential manner. All these modifications occur in the nucleus of eukaryotic cells. The process of removal of introns from eukaryotic pre-mRNA transcripts and joining (splicing) of exons in the correct order is called mRNA splicing. After mRNA splicing, together with other necessary modifications, the pre-mRNA molecule becomes a mature mRNA molecule that is transported to the cytoplasm from the nucleus through nuclear pores and translated by ribosomes in the cytoplasm. Precise excision of introns from pre-mRNA is important for all eukaryotic cells to produce required gene products to carry out all its metabolic reactions accurately; in certain cases alternative splicing can also occur to generate different gene products from the same pre-mRNA (Proudfoot et al., 2002).

1.4.2. *Cis, trans* and spliced leader (SL) *trans*-splicing of eukaryotic mRNA

The process of splicing exons in a single pre-mRNA transcript is called *cis*-splicing. The process of splicing exons from two different pre-mRNA transcripts is called *trans*-splicing. Both *cis* and *trans*-splicing mechanisms are catalysed by a large multi component complex known as the spliceosome. There are two main types of spliceosomal *trans*-splicing, *genic* *trans*-splicing and SL *trans*-splicing. In genic *trans*-splicing (also known as alternative *trans*-splicing) exons from two different pre-mRNA transcripts are joined together at splice sites. The exons may originate from different pre-mRNA transcripts of the same gene, from transcripts of different genes or intergenic regions or transcripts from different chromosomes. Genic *trans*-splicing greatly expands the diversity of RNA transcripts and therefore the proteome complexity. In SL *trans*-splicing an exon is donated from a non-coding RNA molecule (SL RNA) to the 5’ end of a large number of different pre-mRNA transcripts. The length of the SL RNA and exon vary in size amongst organisms. SL *trans*-splicing produces a variety of mature mRNA molecules containing a common sequence at the 5’ end of transcripts (Lasda & Blumenthal, 2011).
SL RNA molecules have similarities to the U snRNA molecules that form the cis-spliceosome. They form snRNPs that are bound by Sm proteins, carry a hypermethylated trimethylguanosine cap at the 5’ end and are predicted to form a number of intra-molecular base paired stem loop structures. The differences between SL RNA and spliceosomal snRNA are: SL RNA contains a donor 5’ splice site that, together with the cap, marks the boundaries of the SL exon, SL snRNAs are consumed during the splicing reaction when the exon is separated from the SL RNA while spliceosomal snRNA remains intact and can be recycled for multiple rounds of splicing (Blumenthal, 2004; Lasda & Blumenthal, 2011).

The number of genes SL trans-spliced varies among organisms: it ranges from 1% in *Trichinella spiralis* (Pettitt et al., 2008) to 100% in trypanosomes (Ambrósio et al., 2009; Günzl., 2010). In *C. elegans* 70% (Allen et al., 2011) of genes and 80-90% of *Ascaris* sp. (Maroney et al., 1995) genes are trans-spliced. Genes of plants, fungi, insects, most protists and vertebrates are not known to be SL trans-spliced (Lasda & Blumenthal, 2011).

**1.4.3. Cis and trans-splicing mechanisms**

The mechanism of cis-splicing involves two trans-esterification reactions (Fig 1.2.). The first is a nucleophilic attack from the 2’ OH group of the adenosine (branch point) located in the intron, on the 5’ phosphate of the first nucleotide (guanosine) of the introns. This reaction forms a lariat shaped intron intermediate and a free 5’ exon. The lariat contains the branch point adenosine, with a 2’-5’ linkage to the first nucleotide of the intron. The second reaction is a nucleophilic attack from the 3’ OH group of the last nucleotide of the free 5’ exon on the 5’ phosphate of the first nucleotide of the second exon. As a result of the two reactions the two exons ligate and the intron is released (Fig. 1.2.a) (Garcia-Blanco, 2003).
Both \textit{cis} and \textit{trans}-splicing use most of the same spliceosome components and RNA sequence signals. Both SL and genic \textit{trans}-splicing require the presence of a 5' splice site on one RNA molecule, a branch point, a polypyrimidine tract and a 3' splice site on the other RNA molecule (Lasda & Blumenthal, 2011). The sequences of these sites differ slightly among organisms (Schwartz \textit{et al}., 2008). Within one organism \textit{cis} and \textit{trans}-splicing sites appear to be identical. There are splice sites that can be used in either a \textit{trans} or a \textit{cis}-reaction. \textit{Trans}-splicing appears to have arisen through evolution of \textit{cis}-splicing (Blumenthal, 2004; Lasda & Blumenthal, 2011).

The possibility of converting a non-\textit{trans}-spliced \textit{C. elegans} gene into a \textit{trans}-spliced gene by placing an intron without only the 5' splice site, into the 5' untranslated region (Conrad \textit{et al}., 1991) and conversion of a \textit{trans}-spliced \textit{C. elegans} gene into one that is not, by providing an upstream splice donor site (Conrad \textit{et al}., 1993) have also been reported.

\begin{figure}[h]
\begin{center}
\includegraphics[width=\textwidth]{fig12.png}
\end{center}
\caption{Fig. 1.2. The two \textit{trans}-esterification reactions in (a) \textit{cis}-splicing and (b) \textit{trans}-splicing of nuclear pre-mRNA. ss-splice site; BP-branch point adenosine; Py-polypyrimidine tract (Garcia-Blanco, 2003)}
\end{figure}

The SL \textit{trans}-splicing mechanism involves two successive reactions analogous to the two reactions in \textit{cis}-splicing. Pre-mRNA transcripts with \textit{trans}-splice sites contain a region of RNA
which is called the ‘outron’ at the 5’ end. As for introns, the outron has a similar GC content upstream of the \textit{trans}-splice site, the branch point adenosine and a polypyrimidine tract associated with the 3’ splice site. In the first reaction of \textit{trans}-splicing, the 2’ OH group of the branch point adenosine on the outron attacks the 5’ phosphate of the first nucleotide of an intron in a second mRNA molecule. As a result a cleavage occurs at the 5’ splice site in the SL RNA releasing the SL with a 3’ hydroxyl terminus. Due to this cleavage a 2’-5’ phosphodiester bond is formed between the 5’end of the SL intron and the branch point adenosine upstream of the \textit{trans}-splice acceptor site. In the second step, the 3’ OH on the free SL exon attacks the 3’ splice site phosphate, creating the \textit{trans}-spliced product and releasing the Y shaped intron product. Afterwards, the 2’-5’ linkage in the Y intron is hydrolyzed by a debranching enzyme and produces two linear introns (Fig. 1.2.b) (Nilsen, 1993; Garcia-Blanco, 2003; Blumenthal, 2005).

\textbf{1.4.4. \textit{Cis} and \textit{trans}-spliceosomes}

The spliceosome is composed of small nuclear ribonucleoproteins (snRNPs) and many different proteins. A snRNP is composed of small nuclear RNAs (snRNAs) and additional proteins. SnRNAs are 56-217 nucleotides long (Maniatis & Reed, 1987) and are transcribed by RNA polymerase II (U1, U2, U4, U5 \textit{snRNA}) or III enzymes (U6 \textit{snRNA}) (Waibel & Filipowicz, 1990; Connelly \textit{et al}., 1994). Spliceosomal snRNPs and numerous other cofactors recognise splice sites on pre-mRNA and catalyse two \textit{trans}-esterification reactions resulting in the formation of spliced mRNA (Nilsen, 1993; Lasda & Blumenthal, 2011).

Assembly of the \textit{cis}-spliceosome on pre-mRNAs includes the interaction of five U snRNPs (U1, U2, U4, U5, U6) and a large number of protein factors (Guthrie, 1991; Ruby & Abelson, 1991; Wahl \textit{et al}., 2009). U1 snRNP recognizes the pre-mRNA by base pairing between the 5’ end of U1 snRNA and the 5’ splice site. Then U2 snRNP binds to the pre-mRNA at the branch site by
base pairing between U2 snRNA and pre-mRNA. Subsequently U4, U5, and U6 join the pre-
splicing complex as a triple snRNP with U4 and U6 base paired to each other. Besides base
pairing with U4 snRNA, U6 snRNA base pairs with U2 snRNA, an interaction that correctly
position the triple snRNP U4-U5-U6 in the spliceosome. This base pairing involves bases near
the 5' end of U2 snRNA and a region near the 3' end of U6 snRNA. Recognition and
juxtaposition of the splice donor and acceptor sites may lead to reorganization within the
spliceosome, after which the initiation of the two sequential trans-esterification reactions
occur (Hausner et al., 1990; Nilsen, 1993; Wahl et al., 2009).

Both cis- and trans-splicing require some common components. Using a permeabilized cell
system and RNase H targeted degradation, it has been demonstrated that U2, U4, and U6
snRNAs are required for trypanosome trans-splicing (Tschudi & Ullu, 1990). The same U
snRNAs are required for Ascaris lumbricoides (an animal parasitic nematode) trans-splicing
(Hannon et al., 1991). The U1 snRNP that defines the 5' splice site through RNA base pairing
interaction in cis-splicing was found to be unnecessary for trans-splicing in C. elegans and A.
lumbricoides (Hannon et al., 1991).

Without the participation of U1 SL RNA, the 5’ trans-splice site and the 3’ trans-splice sites are
brought to close proximity by SL RNA interacting with sequences on U6 snRNA (Hannon et al.,
1992). This may also be achieved by a preformed complex containing SL, U4, U5, U6 snRNPs
binding at the 3’ splice site (Maroney et al., 1996), by SL snRNP specific proteins interacting
with pre-mRNA branch point binding proteins (Denker et al., 2002) or by primary mRNA
sequence elements base pairing with SL snRNP as U1 substitutes (Lasda et al., 2010). Analysis
of affinity purified trans-spliceosomes from splicing extracts has shown that U5 snRNP is an
essential component of trans-spliceosomes in A. lumbricoides. U5 is found in the form of a
tetra-snRNP, containing U4, U5, U6 and SL RNAs which may be the trans-spliceosomal
counterpart of the U4-U6-U5 tri-snRNP in cis-spliceosome. U5 snRNA makes contacts with the 5’ splice site in the SL RNA and with the free SL exon intermediate (Maroney et al., 1996).

1.4.5. SL-trans splicing in nematodes

SL trans-splicing is found throughout the phylum Nematoda. It has been extensively studied in the free living nematode C. elegans and the animal parasitic nematode A. lumbricoides. Some nematodes have a single class of SL RNA for all trans-splicing. However, C. elegans and related nematodes have two classes of SL RNAs (SL1 and SL2) that are used differentially. Nematodes with these two classes trans-splice SL1 at outrons and use the SL2 class specifically for downstream operonic genes (intercistronic regions). In C. elegans SL2 has sequence variants SL3-SL12. There are other organisms including nematodes with multiple classes of SL RNAs. SL1 RNA is more abundant than SL2 RNA. Both SL RNAs contain a 5’ 7-methylguanosine cap, a 22 nucleotide SL exon and a Sm binding site. The RNAs are similar in length (SL1 RNA 95 nt, SL2 RNAs 107 -114 nt) (Spieth et al., 1993; Blumenthal, 2005; Allen et al., 2011). All SL RNAs form almost similar secondary structures consisting of three stem loops separated by short single stranded regions (Fig. 1.3.) (Nilsen, 1993).

The single stranded region separating stem loops II and III is conserved in all nematode SL RNAs and contains the sequence AAUUUUUGGAA. This region corresponds to the consensus Sm binding sequence found in U snRNAs that are necessary co-factors for cis-splicing. The Sm binding sequence in U snRNAs mediate assembly of U snRNAs into ribonucleoprotein particles (U snRNPs) by promoting the association of a common set of core proteins with epitopes recognised by antibodies with Sm specificity. SL RNA is transcribed by RNA polymerase II (Thomas et al., 1988; Van Doren & Hirsh, 1988; Bruzik et al, 1988; Nilsen et al., 1989; Maroney et al., 1990; Nilsen, 1993).
Fig. 1.3. *C. elegans* and *Ascaris* SL RNA sequences and their predicted secondary structures (TMG - 5’ 7-methylguanosine cap; horizontal line - Sm protein binding sites; arrow - 5’ *trans*-splice site) (Lasda & Blumenthal, 2011).

1.4.6. Importance of SL *trans*-splicing in organisms

Many of the organisms that carry out SL *trans*-splicing have operons. Operons are groups of tandemly arranged genes transcribed from a single upstream promoter. These organisms use SL *trans*-splicing to process polycistronic RNA transcripts from operons into mature, monocistronic mRNAs (Fig. 1.4). Individual messages are then transported from the nucleus and translated as separate mRNAs. These operons differ from the type of operons typically found in bacteria, and those sometimes in plants and flies, in which the polycistronic mRNA is the mature, translated form. SL *trans*-splicing adds a 5’ cap to downstream operon genes. Eukaryotic pre-mRNAs are capped at the 5’ end by capping machinery mediated by polymerases. This provides protection from the exonucleolytic degradation to the 5’ end of the first gene, but genes downstream within a polycistronic cluster do not have this type of capping mechanism. When mRNAs are separated by *trans*-splicing, each of them receives a 5’ cap. SL *trans*-splicing possibly prevents transcription termination within operons. The
The formation of the 3’ end of mRNA includes a cleavage of the RNA at the end of the message and addition of a polyadenosine (poly A) tail. The poly A tail protects the RNA upstream of the cleavage site from exonucleolytic degradation. The downstream RNA, is unprotected and may quickly degraded in the 5’ to 3’ direction. This can be the reason for release of RNA polymerase from the DNA template and transcription termination in non-operon genes. SL trans-splicing to the 5’ end of downstream operon genes adds a 5’ cap that may interfere with exonucleolytic degradation allowing transcription to continue through the rest of the operon. As outrons in C. elegans are typically long (~300 nt) they may contain out-of-frame AUGs or unfavourable untranslated region (UTR) sequences. Pre-mRNA 3’ trans-splice sites are generally located close to (within 10 nt upstream of) the start codon AUG. Trans-splicing replaces all upstream AUGs and other outron sequences with the SL exon, ensuring that only the correct start codon is used. There may be other functions of SL trans-splicing, possibly relating to the regulation or localisation of mature trans-spliced mRNAs (Blumenthal, 2004, 2005).

**Fig. 1.4.** Schematic representation of trans-splicing in nematodes, based on findings in C. elegans (Blaxter and Liu, 1996)
1.4.7. Targeting SL trans-splicing / trans-spliceosome for selective control of plant parasitic nematodes

SL trans-splicing has been reported in nematodes, euglenozoans, dinoflagellates, sponges, cnidarians, ctenophores, flatworms, crustaceans, chaetognaths, tunicates, and rotifers. It has not been detected in other eukaryotes, such as fungi, plants and vertebrates. Many of the organisms that perform SL trans-splicing, such as trypanosomes (euglenozoans), flatworms and nematodes are pathogenic to humans, animals and plants. Drugs that target components of the trans-spliceosome or disrupt the downstream effects of SL trans-splicing may be very effective against these parasites, whilst causing little harm to hosts (Stover et al., 2006).

In the free-living nematode C. elegans, three trans-spliceosome specific genes sna-1, sna-2, and sut-1 are known. SL1 snRNP contains a complex of sna-2 (SL75p) and sna-1 (SL21p). SL2 snRNP does not contain these proteins. However, SL75p (sna-2) and SL26p (sut-1), a paralog of SL21p, are components of another Sm snRNP that contains the snRNA, Sm Y. These proteins are involved in SL trans-splicing as SL1 RNA/Sm/SL75p/SL21p and Sm Y/Sm/SL75p/SL26p complexes. The Sm Y snRNP has the potential to interact transiently with the SL2 snRNP and bring the SL75p/SL26p complex into a splicing reaction. SL2 snRNP is involved in the formation of 3’ ends of upstream genes of operons interacting with CstF (Cleavage stimulation factor, mRNA 3’ end forming protein) and SL2 trans-splicing of downstream genes of C. elegans operons (Maroney et al., 1990; MacMorris et al., 2007). Such genes, if present in plant parasitic nematodes, would be good RNAi targets, that should reduce or prevent off target effects, and when silenced, all or most of the genes employing the SL trans-splicing strategy for expression should be affected, depending on which genes are SL trans-spliced.
1.5. Aim of the research

The overall aim of this research was to study the effects of silencing selected spliceosome genes in the plant parasitic nematodes *P. thornei* (a root lesion nematode) and *H. schachtii* (beet cyst nematode), *in vitro* and *in planta* via RNAi, as a potential method for their control.

1.6. Specific objectives of the research

1. To identify spliceosome genes of *P. thornei* and *H. schachtii* based on sequence homology to *C. elegans* genes and RNAi data.

2. To functionally characterise spliceosome genes of *P. thornei* and *H. schachtii* via RNAi.

3. To study the effects of silencing selected genes of both nematodes using transgenic plants as a source of dsRNA.
CHAPTER 2

General Materials and Methods

2.1. Maintaining cultures of *P. thornei* and *H. schachtii*

2.1.1. Maintaining *P. thornei* cultures in vitro

The *P. thornei* population was originated from a single female isolated from an infected wheat plant in Western Australia (Nicol et al., 2015). *P. thornei* cultures were maintained *in vitro* on sterile carrot discs placed in sterile plastic containers. Whole carrots (*Daucus carota*) of Nantes type Stefano variety were purchased from the Mercer Mooney wholesale market in Canning Vale, Perth, Western Australia and stored at 4°C until needed. Carrots were surface sterilised by first soaking in 2% bleach (sodium hypochlorite; 125 g/L chlorine) for 30 minutes on a shaker at 50 rpm. They were then transferred to a tub with sterile water and left to stand for 5 minutes. The carrots were then peeled and transferred to a tub with sterile water and left to stand for 5 minutes. The large carrots were then cut into 3-4 cm thick pieces, flamed lightly using a Bunsen burner, and placed in sealed sterile plastic containers (7 cm diameter and 8 cm height, Fig. 2.1). The carrot discs were kept at 21°C for 2 weeks after which callusing carrot discs were infected with mixed stage *P. thornei* and incubated at 21°C for 5 weeks.

![Fig. 2.1. A sterile container with a callusing carrot disc that was used to maintain *P. thornei* cultures in vitro.](image)

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2.1.1.1. Extraction of \textit{P. thornei} from carrot discs

Mixed stage \textit{P. thornei} were collected from 5-8 week old carrot discs using a mist apparatus. Carrot discs were cut into small pieces with a sharp sterile scalpel and sprayed with a fine mist of water for 10 seconds every 10 minutes as described by Tan \textit{et al.}, (2013). Nematodes were collected in capped plastic tubes at 2-day intervals and kept in a \textit{15°C} incubator in water until used.

2.1.1.2. Surface sterilisation of \textit{P. thornei}

Nematodes were surface sterilised and used for infection of new carrot discs or \textit{in vitro} RNAi experiments, or for infection of carrot hairy roots \textit{in vitro}, within a week after collection from the mist apparatus. To surface sterilise \textit{P. thornei}, for \textit{in vitro} RNAi experiments or infection of new carrot discs, they were washed with 1% hibitane (4% chlorhexidine, Chlorhex C, Murdoch University Veterinary Hospital) for 15 minutes and subsequently washed 5 times with sterile water. After each washing step the nematodes were collected by centrifugation at 1,200 g for 2 minutes. When the nematodes were used to infect carrot hairy roots \textit{in vitro}, they were first washed with 1% hibitane for 15 minutes and five times with sterile water. Then they were suspended in 1% streptomycin sulphate (Tan \textit{et al.}, 2013) and 25 mg/L cycloheximide (Yamashita, 1997) for 16 hours and then washed 5 times with sterile water. The nematodes were treated with streptomycin sulphate to inhibit growth of bacteria and cycloheximide to inhibit growth of fungi. Surface sterilised nematodes were suspended in sterile water and stored in a \textit{15°C} incubator until used. Immediately after surface sterilisation and after 16 hours (overnight) the nematodes were observed under a dissecting microscope to check the viability (vitality) and suitability for further experiments.
2.1.2. Maintaining *H. schachtii* cultures in vitro

*H. schachtii* was originally obtained from mature female cysts from a freshly harvested cabbage and broccoli farm north of Perth, Western Australia (Fosu-Nyarko *et al.*, 2016). *H. schachtii* cultures were maintained on roots of white mustard (*Sinapis alba*) *in vitro*. White mustard seeds were surface sterilised: a 500 µL packed volume of seeds was added to 1 mL of 100% ethanol and washed by inversion 8-10 times, allowed to stand for 1 minute after which ethanol was replaced with 5 mL of bleach [3% (w/v) available chlorine] and inverted occasionally for 15 minutes. The seeds were then washed 5 times with sterile water and transferred into 15 cm diameter Petri dishes containing modified Knop medium (Table 2.1.) using sterile forceps. Petri dishes with five seeds each, were sealed with parafilm and kept at 25±2°C at 16/8 light/dark cycles. After 2 weeks roots of germinated seedlings were infected with surface sterilised second stage juveniles (J2s) of *H. schachtii*. These J2s were obtained from cysts grown on roots of cabbage (*B. oleracea* var. *capitata*) and broccoli (*Brassica oleracea* var. *botrytis*) plants in soil in the glasshouse.

To make 1 L of the modified Knop medium, 10 mL from four solutions A, B, C, D (Table 2.1.), 7.341 mg of Ethylenediaminetetraacetate ferric sodium (FeNaEDTA), 10 g sucrose and 7 g of Daishin agar were used. Before adding agar, pH of the medium was adjusted to 6.4. The medium was sterilised in an autoclave (BMM-Weston sterilizer) in a media cycle (121°C, 15 minutes).
Table 2.1. Composition of modified Knop medium (Sijmons et al., 1991)

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Components</th>
<th>100X (g/L)</th>
<th>For 1L (1X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O</td>
<td>30.0029</td>
<td>10 mL</td>
</tr>
<tr>
<td>B</td>
<td>MgSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>5.0033</td>
<td>10 mL</td>
</tr>
<tr>
<td></td>
<td>CuSO\textsubscript{4}.5H\textsubscript{2}O</td>
<td>0.0015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MnSO\textsubscript{4}.H\textsubscript{2}O</td>
<td>0.0304</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZnSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>0.0040</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CoCl\textsubscript{2}.6H\textsubscript{2}O</td>
<td>0.0006</td>
<td>10 mL</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>14.9100</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
<td>6.8045</td>
<td>10 mL</td>
</tr>
<tr>
<td></td>
<td>H\textsubscript{3}BO\textsubscript{3}</td>
<td>0.0556</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O</td>
<td>0.0015</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>FeNaEDTA</td>
<td>-</td>
<td>7.34 mg</td>
</tr>
</tbody>
</table>

2.1.2.1. Surface sterilisation of *H. schachtii* cysts and eggs

*H. schachtii* cysts were picked from infected roots of cabbage and broccoli onto a 250 µm sieve and washed six times with sterile water by flushing water through the sieve. The cysts were then washed with absolute ethanol for 2 minutes followed by another wash with sterile water (6 times). Afterwards the cysts were soaked in 1.25% bleach for 8 minutes or until cysts were bleached (turned white). The cysts were finally washed with sterile water (6 times) and transferred to a sterile glass petri dish using a clean filter tip. Eggs were squeezed out of the cysts with sterile fine tipped forceps and collected on a 28 µm sieve using a pipette and washed 4 times with sterile water. The eggs were soaked in 0.1% mercuric chloride for 4 minutes, washed 10 times with sterile water and transferred into 5 mL Falcon tubes using a filter tip and the eggs were allowed to settle to the bottom of the tube for 30 minutes. After this some water was decanted leaving the eggs in a minimal volume (approximately 1 mL). Then 2 week old white mustard roots were infected with a known number of eggs. After infection, plates were kept open in a laminar flow bench for 15 minutes to dry out excess water after which the lids were closed, sealed with parafilm and kept at 25±2°C with 18/6 light/dark cycles. Plates were partially covered with strips of white paper to reduce light intensity and to facilitate nematode infection. The plates were observed immediately after inoculation using a dissecting microscope, and again after 2 days to confirm infection. After 2-
3 weeks white cysts, approximately 1 mm long, were observed and these began to tan (turn brown) by the fourth week (Fig. 2.2a,b). Cysts collected from the sterile cultures were used to make more cultures in vitro and to infect host plants in soil in the glasshouse (Fig. 2.2c).

Fig. 2.2. In vitro H. schachtii cultures (a) 2-3 week old white cyst of H. schachtii (x40) (b) Tanned (brown) 8 week old H. schachtii cysts (x40) (c) H. schachtii culture maintained on white mustard roots. Photographs (a) and (b) were taken using a camera (Nikon coolpix (UR-E11), MDC lens) through a stereomicroscope.

2.1.3. Maintaining H. schachtii cultures in soil

H. schachtii cultures were maintained on roots of broccoli and cabbage in soil. Broccoli and cabbage seeds were sown in soil in trays (7 x 8 wells), 1-2 seeds per well in a glasshouse at 25±2°C. Soil composition is given in Table 2.2. After 2 weeks, 6-8 seedlings were transferred into soil in tubs (width 100 cm, length 220 cm, depth 70 cm). The plants were infected with J2s of H. schachtii 3 weeks after transfer, when the root system was well developed. The J2s were obtained from cysts cultured on white mustard in vitro. To hatch J2s, cysts were crushed with fine tipped forceps and incubated in 5 mL of 3.14 mM zinc chloride (ZnCl₂) in the dark (wrapped in aluminium foil) for 2 days at 25±2°C. Previously watered broccoli and cabbage seedlings were inoculated with about 200 J2s. Inoculated plants were not watered for 5 days. Thereafter plants were watered lightly and when required. Mature dark brown cysts could be seen on roots and soil 4-5 weeks after infection. These cultures were maintained in a healthy
state by fertilising the plants as required, spraying with Confidor to control aphids where necessary, and adding new soil to tubs when the roots were exposed.

Table 2.2. Composition of soil used to grow broccoli and cabbage plants for *H. schachtii* culture in soil.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potting mix (for general plant use)</td>
<td></td>
</tr>
<tr>
<td>Murdoch mix</td>
<td>4 buckets (40 L)</td>
</tr>
<tr>
<td>Dolomite</td>
<td>1 measure (20 g)</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>1 measure (12 g)</td>
</tr>
<tr>
<td>Growers blue (fertiliser)</td>
<td>4 measures (40 g)</td>
</tr>
<tr>
<td>Osmocote (fertiliser)</td>
<td>4 measures (40 g)</td>
</tr>
<tr>
<td>Water</td>
<td>½ bucket (5 L)</td>
</tr>
<tr>
<td>Murdoch mix (from Richgro)</td>
<td></td>
</tr>
<tr>
<td>Composted pine bark</td>
<td>2 parts</td>
</tr>
<tr>
<td>Coarse river sand</td>
<td>2 parts</td>
</tr>
<tr>
<td>Coco peat</td>
<td>1 part</td>
</tr>
</tbody>
</table>

2.2. RNA and DNA extractions

2.2.1. RNA extraction from nematodes using TRIzol reagent

Nematodes collected in a 1.5 mL eppendorf tube were centrifuged at 10,000 g for two minutes, after which the supernatant was removed and the pelleted nematodes flash frozen in liquid nitrogen. Two clean stainless steel beads (3 mm diameter, Qiagen) were added to the tube and the pellet was macerated using a Qiagen TissueLyzer at a frequency of 25 strokes per second for 2 minutes. Immediately afterwards 800 μL diluted TRIzol LS (Life Technologies; 600 μL TRIzol LS diluted with 200 μL water) was added, mixed well by vortexing and incubated at room temperature for 5 minutes. Then 200 μL of chloroform was added, shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. The mixture was then centrifuged at 4°C at 12,000 g (or maximum speed) for 15 minutes to separate the phases. The three phases that were separated were the colourless aqueous phase on top, containing the RNA, which was about 60% TRIzol volume, the interphase, which contained debris, and the
bottom organic layer which contained proteins and lipids. The aqueous upper phase with nucleic acids was transferred into a new 1.5 mL eppendorf tube. Then 1/20 volume of 5 M sodium chloride (NaCl) and 2 volumes of ice-cold absolute ethanol was added and mixed well. The solution was kept overnight at -20°C or 4 hours at -80°C, and then centrifuged at 4°C at 12,000 g for 30 minutes. The supernatant was removed, 1 mL of ice-cold 80% ethanol was added and the RNA pellet was washed by pipetting. The solution was then centrifuged at 4°C at 12,000 g for 10 minutes. The RNA pellet was air dried by laying the tube on the bench for 15 minutes. RNA was resuspended in an appropriate amount of nuclease free water (≤50 µL).

2.2.1.1. DNaseI treatment of RNA and cleaning of RNA

DNaseI treatment of RNA was performed using the Qiagen’s RNase free DNase set according to the instructions given in the QIAGEN RNeasy Handbook for DNaseI treatment. In a 1.5 mL eppendorf tube, ≤87.5 µL RNA solution, 10 µL buffer RDD and 2.5 µL DNaseI stock solution were mixed well. The final volume was made to 100 µL with nuclease free water. The reactions were incubated at room temperature (25±2°C) for 10-15 minutes. After this 100 µL of nuclease free water was added to each reaction and mixed well followed by 200 µL of 25:24:1 phenol:chloroform:isoamyl alcohol (Tris buffered pH 8.0), mixed well and left at room temperature for 3 minutes. The tube was then centrifuged at 4°C at 12,000 g for 15 minutes. The upper aqueous layer was carefully transferred into a 1.5 mL eppendorf tube and then 1/20 volume of 5 M NaCl and 2 volumes of cold 100% ethanol were added, mixed well and incubated overnight at -20°C. Afterwards the tube was centrifuged at 4°C at 12,000 g for 30 minutes. The supernatant was carefully removed and the pellet was washed with 80% ethanol. It was then centrifuged at 4°C at 12,000 g for 10 minutes, and washed with 80% ethanol once again, and finally air dried by laying the tube on its side on the bench for 15 minutes. RNA was resuspended in an appropriate amount of RNase free water (≤50 µL).
2.2.2. RNA extraction from nematodes using ARCTURUS PicoPure RNA Isolation Kit

The ARCTURUS PicoPure RNA Isolation kit (Life Technologies) was used to extract RNA from small numbers (750-1000) of nematodes. The nematodes were either lysed using the Tissuelyzer or worm lysis buffer (100 mM sodium chloride, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 100 mM Tris-HCl pH 8.5, 1% sodium dodecyl sulphate, 100 µg/mL Proteinase K, 1% β-mercaptoethanol).

The Extraction Buffer (50 µL) was added to the nematodes in 1.5 mL eppendorf tubes. Two clean stainless steel beads (3 mm diameter, Qiagen) were added to a tube and the pellet was macerated using the Qiagen TissueLyzer at a frequency of 25 strokes per second for 2 minutes. The tube was then briefly centrifuged using a mini centrifuge (Force Mini, Fisher Biotech) to collect the contents at the bottom of the tube and the homogenate was incubated at 42°C for 30 minutes. Then 50 µL of 70% ethanol was added to the cell extract and mixed by pipetting up and down. This solution was then transferred to the RNA purification column filter membrane preconditioned with the Conditioning Buffer. DNaseI treatment and RNA washing steps were performed according to the manufacturer’s instructions.

When the worm lysis buffer was used for nematode lysis, the nematodes were suspended in 50 µL of the buffer, frozen at -80°C for 1 hour, thawed to room temperature and incubated at 55°C for 30 minutes. The lysed nematode solution was then centrifuged for 3 minutes at 16,000 g and the supernatant was then added to 50 µL of Extraction Buffer and incubated at 42°C for 30 minutes. The other steps were performed as described above. RNA was eluted in 11 µL of nuclease free water.
2.2.3. DNA extraction from carrot hairy roots

DNA was extracted from about 4 cm long carrot hairy roots. Each root was placed in a 2 mL centrifuge tube, frozen in liquid nitrogen and crushed with three clean stainless steel beads (3 mm diameter, Qiagen) using the Qiagen TissueLyzer at a frequency of 25 strokes per second for 3 minutes. Immediately afterwards 500 µL of 10% cetyltrimethylammonium bromide (CTAB) buffer was added, mixed well by vortexing and incubated at 65°C for 45 minutes.

Components of the CTAB buffer were 1.4 M sodium chloride (NaCl), 20 mM EDTA, (pH 8), 0.1 M Tris-HCl (pH 8), 2% CTAB, 1% polyvinylpyrrolidone (PVP) and 1% β-mercaptoethanol, which was added immediately before use.

After incubation, the CTAB-plant extract mixture was centrifuged at 12,000 g for 5 minutes. The supernatant was then transferred to a new 2 mL tube, an equal volume of chloroform:isoamyl alcohol (24:1) added, mixed well by inversion and centrifuged at 12,000 g for 2 minutes. The upper aqueous phase was transferred to a new 1.5 mL eppendorf tube, to which 1/10 volume of 7.5 M ammonium acetate and 2 volumes of ice-cold absolute ethanol were added, inverted several times and kept at -20°C for 16 hours to precipitate DNA. The solution was centrifuged at 12,000 g for 20 minutes and the supernatant removed. The DNA pellet was then washed with 500 µL ice cold 70% ethanol and centrifuged at maximum speed for 10 minutes. Washing with 70% ethanol was done twice. The supernatant was removed, the DNA pellet dried for about 15 minutes and resuspended in 50 µL of nuclease free water. RNA and DNA were quantified using a NanoDrop (ND-1000 spectrophotometer, Thermo Fisher Scientific).

2.3. Reverse transcription of total RNA

Complimentary DNA (cDNA) was obtained from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions.
This kit uses random primers to initiate first strand cDNA synthesis from all species of RNA molecules. After reverse transcription, 1/10 dilutions of cDNA was mostly used for PCRs.

2.4. Polymerase chain reactions (PCRs)

PCRs were done to amplify spliceosome genes from nematode cDNA, to identify transformed bacteria (colony screening) and to quantify target mRNA after RNA interference treatments. PCRs were done with MyTaq DNA Polymerase (Bioline) in thermal cyclers (Applied Biosystems 2720 or Veriti 96 wells). Quantitative real time PCRs (qRT PCRs) were performed using SYBR Green RT-PCR reagents (Applied Biosystems) in a Corbett Rotor-Gene Quantitative Thermal Cycler.

2.4.1. Primers used for PCRs

The primers used to amplify genes in this study were designed manually from available sequences. Self-complimentarity, annealing temperature (55-65°C) and GC content (30-50%) were checked using Oligo Calc:Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html). To obtain reverse compliments of sequences, the Nucleic Acid Sequence Massager program (www.attotron.com/cybertory/analysis/seqMassager.htm) was used. Primers for qRT PCR were designed with Primer3 (v.0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) with expected amplicon size of 90-120 bp. All primers were synthesised by Integrated DNA Technologies (IDT), USA (www.idtdna.com).
2.5. Separation and visualisation of DNA or RNA by agarose gel electrophoresis

Agarose (Fisher Biotec) gels of varying concentrations (1%, 1.5% or 2%) were used to separate DNA or RNA depending on the size of fragments. Agarose gels were prepared with 1X TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.0) and SYBR Safe DNA gel stain (Invitrogen) according to the manufacturer’s instructions. Gels were allowed to set for about 30-45 minutes at room temperature (25±2°C). After gel electrophoresis, DNA or RNA was visualised under a UV transilluminator (Fisher Biotec) with 100 bp (100-3000 bp, Axygen Biosciences) or 1 Kb (300-10,000 bp, Axygen Biosciences) DNA markers as standards.

2.5.1. Elution of DNA from agarose gels

DNA was eluted from agarose gels using a SV Gel and PCR Clean-Up System (Promega) according to the manufacturer’s instructions.

2.6. DNA sequencing

DNA sequencing reactions were carried out according to instructions given in “Sequencing guide for beginners” provided by the sequencing facility of the WA State Agricultural Biotechnology Centre (SABC). Sequencing reactions (1/16 dilutions) were prepared by mixing 1.75 µL 5X sequencing buffer, 0.5 µL Big Dye terminator mix, 3.2 pmoles of primer, appropriate amount of template DNA and nuclease free water to a total reaction volume of 10 µL. Sequencing reactions were performed in a thermal cycler at 96°C for 2 minutes, 25 cycles of 96°C for 10 seconds, 50-60°C for 5 seconds, 60°C for 4 minutes. Ethanol precipitation of the product was carried out according to the instructions given in the sequencing guide. Sequences were viewed and edited using FinchTV 1.4 software and annotated using the BLAST suite of programs available at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
2.7. Cloning of amplicons

2.7.1. Cloning using pGEM-T easy vector

Amplicons were ligated to the pGEM-T easy vector (3000 bp, Fig. 2.3.) according to the technical manual provided by the manufacturer (Promega). The vector contained T7 and SP6 RNA polymerase promoters flanking a multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allowed identification of recombinants by blue-white bacterial colony screening on indicator plates [Luria-Bertani (LB) agar supplemented with 100 mg/L ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)]. Inserts could be sequenced using the SP6 promoter primer, T7 promoter primer, M13 forward or M13 reverse primer (Promega pGEM-T and pGEM-T Easy Vector Systems, Technical manual). Plasmids with the pGEM-T vector backbone were multiplied using competent *Escherichia coli* JM109 cells.

![Fig. 2.3. pGEM-T easy vector map (Promega pGEM-T and pGEM-T Easy Vector Systems, Technical manual)](image)

2.7.2. Cloning using pDoubler vector

The vector pDoubler (2617 bp, Fig. 2.4.) was used to clone genes and to obtain template DNA for *in vitro* transcription. The partial sequence of the *lev-11* gene (566 bp) of *H. schachtii* in
pDoubler allows confirmation of successful digests using restriction enzymes. To ligate a DNA fragment to this vector, both insert and vector were first digested with the desired restriction enzymes, mostly KpnI and XhoI, resulting in the release of the lev-11 fragment from pDoubler, and later ligated as described in section 2.8. To obtain template DNA for in vitro transcription, a gene cloned into pDoubler and flanked by T7 RNA polymerase promoters, was digested with either EcoRI or NotI restriction enzymes. Successful ligations of genes to this vector were confirmed by restriction enzyme digestions and PCRs.

Fig. 2.4. pDoubler vector map (constructed by Dr. John Fosu-Nyarko)

2.7.3. Generation of hairpin expression cassettes using pCLEaver-NosA vector

The vector pCLEaver-NosA (4024 bp, Fig. 2.5.) was used to generate hairpin expression cassettes for trans-spliceosome ESTs. Sense DNA (5’-3’) template was obtained by digesting pDoubler carrying the ESTs with KpnI and XhoI restriction enzymes, whereas the antisense (3’-5’) DNA template was obtained by digesting with BamHI and XbaI restriction enzymes. After ligating sense and antisense templates to pCLEaver-NosA, the expression cassette was digested from the vector with NotI restriction enzyme. The vector was multiplied using E. coli JM109 selected with kanamycin (25 mg/L).
2.7.4. Generation of binary vectors with hairpin expression cassettes for hairy root transformation

Hairpin expression cassettes were inserted at the NotI restriction enzyme site in the T-DNA region of the binary vector pART27-LacZ to generate vectors for hairy root transformation (Gleave, 1992, 11667 bp, Fig. 2.6). Expression of hairpin RNA was driven by the CaMV 35S promoter whereas the neomycin phosphotransferase (NPTII) gene that confers resistance to the antibiotic kanamycin, which was also in the T-DNA region, was driven by a Nos promoter. Spectinomycin (100 mg/L) was used as the selection marker for *E. coli* JM109 and *Agrobacterium rhizogenes* K599 after plasmid transformation, whereas kanamycin (25 mg/L) was used to select carrot hairy roots (induced by *A. rhizogenes* K599).
2.7.4.1. Dephosphorylation of pART27-LacZ vector after digesting with NotI

To prevent self ligation of pART27-LacZ vector after digestion with NotI, the 5’ phosphate group was removed using Antarctic phosphatase (New England Biolabs Inc.) according to the manufacturer’s instructions.

2.8. Ligation of DNA fragments to vectors

T4 DNA ligase (Promega or New England Biolabs Inc.) or Quick T4 DNA ligase (New England Biolabs Inc.), was used to ligate DNA to vectors. Ligation reactions were performed according to the manufacturer’s instructions. A vector:insert DNA molar ratio of 1:3 was used in ligations. The amount of insert DNA that was used in the ligation reactions with 25 ng of vector was calculated using the formula below as given in Promega pGEM-T and pGEM-T Easy Vector Systems, technical manual.

\[
\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio}
\]
2.9. Transformation of bacterial cells with plasmid DNA

2.9.1. Transformation of *Escherichia coli* JM109

All plasmids used in the study were multiplied with *E. coli* JM109 cells. To do this *E. coli* JM109 chemically competent cells (rubidium chloride treated) stored at -80°C were thawed on ice and 25 μL aliquoted into a sterile 1.5 mL eppendorf tube. Ligation reactions (5 μL) or 1-100 ng of plasmid DNA was added to the cells, mixed gently by flicking the tube and kept on ice for 30 minutes. Cells were shocked at 42°C for 50 seconds in a water bath and immediately transferred to ice for 2 minutes. After this 700 μL of sterile LB broth was added to the cells which were then incubated for 2 hours on a shaker at 225 rpm at 37°C. Afterwards 350 μL of the cell culture was spread on LB agar supplemented with an appropriate antibiotic in a 9 cm diameter petri dish. Petri dishes were inverted and kept at 37°C for 16-20 hours in the dark.

2.9.2. Transformation of *Agrobacterium rhizogenes* K599

Chemically competent *A. rhizogenes* K599 cells (calcium chloride treated) were used to generate hairy roots. After thawing frozen cells, 10 μL was transformed with approximately 1 μg of plasmid DNA (pART27-LacZ carrying hairpin expression cassettes). Competent *A. rhizogenes* K599 and plasmid DNA was mixed in a 1.5 mL eppendorf tube by gently flicking the tube and chilled on ice for 10 minutes. The cells were then frozen in liquid nitrogen for 5 minutes followed by incubation at 37°C in a water bath for 5 minutes. LB broth (700 μL) was added to the tube and incubated at 28°C for 4 hours on a shaker (RATEK-Platform mixer, Rowe Scientific) at 225 rpm. Then 250 μL of bacterial culture was spread on LB agar medium supplemented with 25 mg/L rifampicin and 100 mg/L spectinomycin in 9 cm petri dishes. Petri dishes were kept inverted for 1-3 days at 28°C in the dark for colony formation.
2.9.3. Selection of transformed bacterial colonies by PCR (colony screening)

Transformed colonies were picked with a 10 µL pipette tip and resuspended in 15-25 µL of sterile water in a PCR tube by vortexing. Five microliters of the bacterial suspension was used in a PCR. A PCR reaction (20 µL) was made from 4 µL of 5X MyTaq reaction buffer, 0.05 µL MyTaq DNA polymerase (Bioline), 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 5 µL bacterial suspension, 6 µL cresol red dye and 2.95 µL of PCR water. The PCR cycle was 95°C for 1 minute, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 72°C for 10 minutes. Primers used for colony screening when the bacteria were transformed with plasmids with the following vector backbones, pGEM-T, pDoubler, pCLEaVer-NosA and pART27-LacZ, and sizes of the expected amplicons are given in Table 2.3.

Table 2.3. Primers used for bacterial colony screening, by PCR, after transformation with pGEM-T, pDoubler, pCLEaVer-NosA and pART27-LacZ vectors and expected amplicon sizes.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Primers used for bacterial colony screening by PCR</th>
<th>Sequence (5’-3’)</th>
<th>Size of the amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>T7 forward</td>
<td>TAATACGACTCCTAGATAGGG</td>
<td>140 bp + gene size</td>
</tr>
<tr>
<td></td>
<td>SP6 reverse</td>
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<td></td>
</tr>
<tr>
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<td>359 bp + gene size</td>
</tr>
<tr>
<td></td>
<td>M13 reverse</td>
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<td>pCLEaVer-NosA</td>
<td>SIntron forward</td>
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</tr>
<tr>
<td></td>
<td>S33S reverse</td>
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<tr>
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<td></td>
<td>ASIntron reverse</td>
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<tr>
<td>pART27-LacZ</td>
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<td>35SART-R reverse</td>
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</table>

2.10. Bacterial culture conditions

Liquid cultures of *E. coli* JM109 and *A. rhizogenes* K599 were made with LB broth supplemented with appropriate antibiotics in McCartney's bottles or 50 mL sterile, capped plastic tubes. To make 1L of the LB medium, 10g of tryptone, 10g of sodium chloride and 5g of yeast extract were used. The pH was usually 7.0. If semi solid LB medium was required 12-15
44 g/L agar was added after adjusting the pH. The medium was sterilised in an autoclave in a media cycle (121°C, 15 minutes). *E. coli* JM109 was cultured at 37°C whereas *A. rhizogenes* K599 was incubated at 28°C.

### 2.11. Plasmid DNA extraction

Plasmid DNA was extracted from bacterial cultures using the Wizard Plus SV Minipreps DNA Purification System (Promega) or QIAprep spin mini prep kit (Qiagen) according to the manufacturer’s instructions.

### 2.12. Synthesis of double stranded RNA (dsRNA)

Double stranded RNA was synthesised using HiScribe T7 *In Vitro* Transcription Kit or T7 high yield RNA synthesis kit (New England Biolabs Inc.), according to the manufacturer’s instructions. If the genes were cloned with pGEM-T easy vector, template DNA for *in vitro* transcription was generated by PCR using gene specific primers that contained the T7 RNA polymerase promoter sequence (5’TAATACGACTCACTATAGGG3’) at 5’ ends. If the genes were cloned with the pDoubler vector, the template DNA was obtained by digesting the gene flanked by T7 RNA polymerase promoter sequences using EcoRI or NotI restriction enzymes. After *in vitro* transcription, DNaseI treatment was carried out to digest residual template DNA. Afterwards dsRNA was cleaned up as described in 2.2.1.1. After dsRNA precipitation, the pellet was dried and resuspended in 42 µL of nuclease free water. One microliter from a 1/10 dilution was used to quantify dsRNA using the NanoDrop (ND-1000 Spectrophotometer, Thermo Scientific). The integrity of dsRNA was also checked by running 9 µL of the dilution with cresol red loading dye on a 2% agarose gel for 1 hour at 60 V.
CHAPTER 3

In silico Identification of Putative Transcripts of Spliceosome Units of three Root Lesion Nematodes (Pratylenchus spp) and Cyst Nematodes (Heterodera spp)

3.1. Introduction

The spliceosome is a dynamic complex of proteins and RNA that catalyses pre-mRNA splicing: it enables an important post transcriptional modification during which non-coding RNA is removed before mRNA translation in organisms. Spliceosome proteins and other factors have been well characterised in humans and yeast (Jurica & Moore, 2003; Wahl et al., 2009; Günzl, 2010). Based on those systems, proteins involved in pre-mRNA splicing in the trypanosomatid Trypanosoma brucei, for which all mRNAs are known to be SL trans-spliced, but for which there are only rare reports of existence of cis-splicing, have also been characterised (Ambrósio et al., 2009; Günzl, 2010). In nematodes, nuclear pre-mRNAs are processed both by cis and SL trans-splicing, catalysed respectively by cis and trans-spliceosomes. Some components are shared by both spliceosomes, while some components such as SL RNA, SUT-1, SNA-1 and SNA-2 proteins are specific for the C. elegans trans-spliceosome (Hannon et al., 1991; Maroney et al., 1996; Furuyama & Bruzik, 2006; MacMorris et al., 2007).

In C. elegans about 70% of genes are reported to be SL trans-spliced (Allen et al., 2011). Among these are three actin genes, ubiquitin, heat shock protein (hsp70a) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH Ia) (Bektesh et al., 1988). The first cDNA library of the root lesion nematode Pratylenchus penetrans was generated from mixed life stages using SL1 primers from which 1,928 expressed sequence tags (EST) involved in important biological
processes such as protein metabolism, nucleic acid metabolism, nucleic acid binding, nucleotide binding and encode ribosomal proteins were obtained (Mitrev et al., 2004). These results appear to indicate that the genes were SL trans-spliced making SL trans-splicing an important mRNA processing mechanism for these nematodes.

SL trans-splicing is reported to occur only in some lower eukaryotes (Table 1 in Lasda & Blumenthal, 2011), and if this is the case and the genes involved in this process are restricted to these organisms, they would be good RNAi targets for controlling plant parasitic nematodes via host induced gene silencing. Using such genes as targets for control would minimise or even eliminate possible off-target effects in other organisms that may feed on transgenic RNAi crops. The objective of this chapter was therefore to identify putative transcripts of cis and trans-spliceosome genes in root lesion nematodes (P. thornei, P. zeae, P. coffeae) and cyst nematodes (H. glycines, H. schachtii) based on sequence homology to C. elegans spliceosome genes. Target genes for RNAi studies were selected based on their presumed essential roles and RNAi data of these genes reported for C. elegans. Selected genes of P. thornei and H. schachtii used for RNAi studies were amplified and confirmed by DNA sequencing.

3.2. Materials and methods

3.2.1. Identification of C. elegans orthologues of spliceosome genes for root lesion and cyst nematodes

C. elegans spliceosome gene sequences were obtained from WormBase (www.wormbase.org). WormBase was searched for genes, gene classes or proteins using orthologues of spliceosome genes or proteins characterised for humans, yeast (Jurica & Moore, 2003; Wahl et al., 2009) and the trypanosomatid T. brucei (Ambrósio et al., 2009; Günzl, 2010). Genes that encode trans-spliceosome specific proteins were obtained from information available for the animal parasitic nematode A. lumbricoides (Denker et al., 2002) and the free living nematode C.
_elegans_ (MacMorris et al., 2007). RNAi phenotypes of _C. elegans_ spliceosome genes were also obtained from WormBase. Putative transcripts of spliceosome genes were also identified from mixed life stage transcriptomes of _P. thornei_ (Nicol et al., 2012), _P. zeae_ (Fosu-Nyarko et al., 2015), and _P. coffeae_ (Haegeman et al., 2011) using _C. elegans_ orthologues. The reads of each of the transcriptomes were assembled with NextGEne v2.1. Similar transcripts for cyst nematodes were identified from EST databases of _H. glycines_ (taxid:51029) and _H. schachtii_ (taxid:97005) at the National Centre for Biotechnology Information (NCBI) Genbank (http://www.ncbi.nlm.nih.gov/) as well as from the _H. schachtii_ J2 stage transcriptome (Fosu-Nyarko et al., 2016). The BLAST search was done with Tblastn at an e-value cut off of 1E-5.

3.2.2. Characterisation of putative transcripts of _P. thornei_ and _H. schachtii_

Selected putative transcripts of spliceosome units of _P. thornei_ and _H. schachtii_ used for further studies were characterised by PCR, sequencing and annotation using publicly available sequences. Total RNA was extracted from about 40,000 mixed stages of _P. thornei_ using a QIAGEN RNeasy Mini kit according to the manufacturer’s instructions, and the RNA was reverse-transcribed as described in section 2.3. Primers were designed manually to amplify 250-750 bp fragments using best matching transcripts to characterised spliceosome units. To select a _P. thornei_ transcript for PCR amplification, all transcripts putatively encoding a spliceosome unit of the three root lesion nematode species were compared with the transcripts of _C. elegans_ orthologues. The _P. thornei_ transcript with the highest maximum score was selected and primers designed to amplify from a cDNA of a mixed stages of the nematode. Transcripts for _SUT-1, SUT-2_ and _SNA-2_ were amplified from 380 ng of cDNA and 10 pmol each of primer pairs. Transcripts for _PRP-4, PRP-8, PRP-17, PRPF-4, UAF-1, UAF-2, T08A11.2, SFA-1_ and _SWP-1_ were amplified using a touchdown PCR cycling profile (annealing at 60-55°C followed by annealing at 55°C for 40 cycles, denaturing and extension temperatures are as indicated in section 2.9.3.). Cleaned-up amplicons were sequenced using 3.2 pmol of the forward primer
and 20 ng of PCR products as described in section 2.6. Sequences were edited and confirmed by aligning with that of the original transcripts and further annotated using BLAST and the nucleotide collection (nr/nt) or non-redundant protein sequences (nr) in NCBI using 1E-5 as the cut off e-value. Plasmid DNA clones of partial transcripts for SUT-1, SNA-1, PRP-4, PRP-8, PRP-17A, PRP-17B, UAF-1, UAF-2 and PRP-21 for H. schachtii were obtained from Dr. John Fosu-Nyarko (SABC, Murdoch University) who had previously amplified, sequenced and cloned the amplicons.

3.3. Results

3.3.1. Spliceosome genes of C. elegans

Fifty-six genes that encode spliceosome proteins in the C. elegans genome were identified in silico from WormBase. Amongst them, sna-1, sna-2, sut-1 and sut-2 genes (sna - snRNA associated; sut-suppressor of Tau pathology) code for proteins that are specific for the trans-spliceosome (Table 3.1). The other 52 genes code for proteins that may be involved in both cis and trans-splicing in C. elegans and included Sm (small nuclear ribonucleoprotein) proteins, Lsm (Sm like) proteins, U1 specific proteins, U2 specific proteins, U4/U6 specific proteins, U5 specific proteins, SR (serine/arginine) proteins, DExD/H type RNA dependent ATPases/helicases and splicing factors. Table 3.1 shows classification of the genes into ten functional groups.
sequences of any of the three root lesion nematodes. Putative transcripts match \( P. \) \( \text{zeae} \) sequences of the three root lesion nematodes. Specifically, putative transcripts of \( P. \) \( \text{zeae} \) orthologues were identified from sequences of the three root lesion nematodes. In total, putative transcripts matching 55 \( P. \) \( \text{thornei} \), \( P. \) \( \text{zeae} \) and \( P. \) \( \text{coffeae} \) matched 51, 45 and 52 \( P. \) \( \text{elegans} \) orthologues respectively. Putative transcripts matching the SL snRNP specific genes \( \text{sna}-2 \), \( \text{sut}-1 \) and \( \text{sut}-2 \) were identified in all three root lesion nematodes. No putative transcript was identified for the \( \text{sna}-1 \) gene from sequences of any of the root lesion nematodes (Table 3.2).

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<tr>
<th>Protein type</th>
<th>Genes (Protein and/or WormBase transcript ID)</th>
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<tbody>
<tr>
<td>Sm proteins</td>
<td>( \text{snr}-2 ) (SmB, W08E3.1), ( \text{snr}-3 ) (SmD1, T28D9.10), ( \text{snr}-4 ) (SmD2, C52E4.3), ( \text{snr}-1 ) (SmD3, Y116A8C.42), ( \text{snr}-6 ) (SmE, Y49E10.15), ( \text{snr}-5 ) (SmF, ZK652.1), ( \text{snr}-7 ) (SmG, Y71F9B.4)</td>
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<tr>
<td>Lsm proteins</td>
<td>( \text{lsm}-1 ) (LSm1, F40F8.9), ( \text{lsm}-2 ) (LSm2, T10G3.6), ( \text{lsm}-3 ) (LSm3, Y62E10A.12), ( \text{lsm}-4 ) (LSm4, F32A5.7), ( \text{lsm}-5 ) (LSm5, F28F8.3), ( \text{lsm}-6 ) (LSm6, Y71G12B.14), ( \text{lsm}-7 ) (LSm7, ZK593.7), ( \text{lsm}-8 ) (LSm8, Y73B6BL.32)</td>
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<tr>
<td>SL specific</td>
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<td>U1 specific</td>
<td>( \text{F08B4.7} ) (U1-C, F08B4.7), ( \text{rnp}-2 ) (U1-A, K08D10.4), ( \text{rnp}-7 ) (U1-70kD, K04G7.10)</td>
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<td>U2 specific</td>
<td>( \text{rnp}-3 ) (U2-B, K08D10.3), ( \text{mog}-2 ) (U2-A', H20J04.8), ( \text{prp}-21 ) (SF3a120, W07E6.4), ( \text{t08a11.2} ) (SF3b/Hsh155, T08A11.2), ( \text{sap}-49 ) (SF3b/Hsh49, C08B11.5)</td>
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<td>U4/U6 specific</td>
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<td>U5 specific</td>
<td>( \text{prp}-8 ) (Prp-8, C50C3.6), ( \text{snrp}-200 ) (U5-200kD, Y46G5A.4), ( \text{prp}-6 ) (U5-102kD, Y59A8B.6)</td>
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<tr>
<td>SR proteins</td>
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<td>DExD/H type RNA dependent ATPases/Helicases</td>
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<td>Splicing factors</td>
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### Table 3.1. Cis and trans-spliceosome genes in \( C. \) \( \text{elegans} \) (WormBase)

3.3.2. Putative transcripts of spliceosome units of \( P. \) \( \text{thornei} \), \( P. \) \( \text{zeae} \) and \( P. \) \( \text{coffeae} \)

In total, putative transcripts matching 55 \( C. \) \( \text{elegans} \) spliceosome genes were identified from sequences of the three root lesion nematodes. Specifically, putative transcripts of \( P. \) \( \text{thornei} \), \( P. \) \( \text{zeae} \) and \( P. \) \( \text{coffeae} \) matched 51, 45 and 52 \( C. \) \( \text{elegans} \) orthologues respectively. Putative transcripts matching the SL snRNP specific genes \( \text{sna}-2 \), \( \text{sut}-1 \) and \( \text{sut}-2 \) were identified in all three root lesion nematodes. No putative transcript was identified for the \( \text{sna}-1 \) gene from sequences of any of the root lesion nematodes (Table 3.2).
Amongst the 15 genes that code for core snRNP proteins (Sm, Lsm) in *C. elegans*, putative transcripts encoding four Sm proteins and three Lsm proteins were identified in all three transcriptomes (Table 3.2). In addition, putative transcripts encoding three U1 snRNP specific proteins, two U2 snRNP specific proteins, three U5 snRNP specific proteins, two U4/U6 specific proteins, three U4/U6.U5 tri snRNP complex components, ten splicing factors, four SR proteins and three DExD/H type RNA dependent ATPases/helicases were also found in the three transcriptomes (Table 3.2).

Putative transcripts of the core snRNP gene *lsm-1* and SR protein coding gene *rsp-7* were only found in the *P. coffeae* transcriptome. In both the *P. thornei* and *P. coffeae* transcriptomes, putative transcripts encoding three Lsm proteins (*LSM-2*, *LSM-6*, *LSM-8*), two U2 snRNP-specific proteins (*MOG-2*, *SAP-49*) and three SR proteins (*RSP-3*, *RSP-5*, *RSP-6*) were present.

Putative transcripts were found for Sm protein coding *snr-3*, Lsm protein coding *lsm-4* and U2 specific protein coding *rnp-3* in transcriptomes of *P. thornei* and *P. zeae*. Putative transcripts of Sm protein coding genes *snr-1* and *snr-2* were identified in *P. zeae* and *P. coffeae* transcriptomes. The numbers of contigs and singletons of putative spliceosome transcripts found in transcriptomes of each of the root lesion nematode species are given in Table 3.2. The highest number of transcripts was found for *prp-8* in the three species (Table 3.2.).
Table 3.2: Putative spliceosome transcripts found in the three root lesion nematodes (RLN) and the numbers of contigs and singletons found in each species (nf—not found, cont-contig, singl-singleton, Pt-P. thornei, Pz-P. zeae, Pc-P. coffeae).

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<th>C. elegans sequence ID</th>
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<th>P. zeae</th>
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</tr>
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51
3.3.3. Putative ESTs of spliceosome genes of *H. glycines* and *H. schachtii*

The comparative sequence analysis of ESTs of *H. glycines* and *H. schachtii* with sequences of *C. elegans* identified 44 ESTs of the plant parasitic nematodes putatively encoding spliceosome genes: 38 and seven ESTs respectively for *H. glycines* and *H. schachtii* (Table 3.4.). In addition, putative ESTs for *H. schachtii* for seventeen spliceosome proteins were identified from the *H. schachtii* transcriptome (Fosu-Nyanko et al., 2016; Table 3.3, row 46).

Putative transcripts that encode nineteen spliceosome proteins, including a DExD/H type RNA dependent ATPase/helicase (MOG-4), three Sm proteins (SNR-2, SNR-3, SNR-5), one Lsm protein (LSM-6), one U2 specific protein (PRP-21), one U4/U6 specific protein (PRP-3), three U5 specific proteins (SNRP-200, PRP-8, PRP-6), four SR proteins (RSP-1, RSP-4, RSP-6, RSP-8) and five splicing factors (PRP-31, PRP-17, PRP-38, RNP-6, UAF-1) were identified in both species (Table 3.3, rows 1-19, 39-42, 45, 46).

Partial putative transcripts for nineteen genes (Table 3.3, rows 20-38) were identified only in *H. glycines* while sut-1, t08a11.2, Ism-4, mog-1 and mog-5 transcripts were identified only in *H. schachtii* (Table 3.3., rows 43, 44, 46). The number of putative spliceosome transcripts found in each root lesion and cyst nematode species and the putative transcripts that were not found are given in Table 3.4.
**Table 3.3.** *H. glycines* and *H. schachtii* ESTs putatively encoding orthologues of *C. elegans* spliceosome units.

<table>
<thead>
<tr>
<th>Row no.</th>
<th>C. elegans gene</th>
<th>C. elegans sequence ID</th>
<th>NCBI Accession of ESTs</th>
<th>Total score (Tblastn)</th>
<th>% identities</th>
<th>E-value</th>
</tr>
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<tbody>
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<td>CA940094.1</td>
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**H. schachtii**

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<th>NCBI Accession of ESTs</th>
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<th>% identities</th>
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<td>5.00E-74</td>
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*H. schachtii transcriptome, Fusu-Nyarko et al., 2016.*
Table 3.4. The number of spliceosome proteins in each root lesion and cyst nematode species for which putative transcripts were identified from available sequences

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<th>Nematode species</th>
<th>Root lesion nematodes</th>
<th>Cyst nematodes</th>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</table>

3.3.4. Amplification of putative transcripts for spliceosome units of P. thornei and H. schachtii

Primers were designed to amplify putative transcripts for 12 spliceosome genes from cDNA of mixed stages of P. thornei for further functional characterisation. These transcripts were selected because presumed functions of these genes in splicing and/or functional RNAi data available for C. elegans suggest RNAi will impact negatively on growth and development. The selected genes and primers used for PCR amplification for both P. thornei and H. schachtii are provided in Table 3.5. For H. schachtii where no EST of a particular gene was identified from available sequences, primers were designed based on sequences on the related H. glycines (Table 3.5). Each of the primers successfully amplified products with the expected sizes.
Table 3.5. Primers used to amplify putative spliceosome transcripts of *P. thornei* and *H. schachtii*.

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<tr>
<th>Nematode and gene</th>
<th>NCBI accession /sequence ID and length</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
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R-GAGCAAGATTAGTGCACAGCA | 698 |
| *P. thornei* sut-2 | contig00491 (1152 b) | F-GAACAGCGAAACAAAGTG  
R-GCTTTCTTCTCCTCCTCAT | 588 |
| *P. thornei* sna-2 | contig04173 (456 b) | F-CTACATGAGCGGACAAGAG  
R-GCTTCTTCTCCTCCTCAT | 431 |
| *P. thornei* prp-4 | JL862432.1 (1778 b) | F-CTTTATACGAGGTAATGAGCGAC  
R-TGGGAGCCGAGCTTGGCATG | 443 |
| *P. thornei* prp-8 | JL860741.1 (1542 b) | F-TCGAAGATGGTGACGAGAAGGT  
R-TGCTTTCTTCTCCTCCTCAT | 588 |
| *P. thornei* prp-17 | JL860068.1 (530 b) | F-CGACGCGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 347 |
| *P. thornei* prp-4 | JL861428.1 (1479 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 707 |
| *P. thornei* t08a11.2 | JL864899.1 (1776 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 482 |
| *H. glycines* sut-1 | CD750664.1 (554 b) | F-GTGATGGTGACGAGAAGGT  
R-TGGGAGCCGAGCTTGGCATG | 347 |
| *H. glycines* sna-1 | CB281686.1 (612 b) | F-GCCAGATGGTGACGAGAAGGT  
R-TGGGAGCCGAGCTTGGCATG | 430 |
| *H. glycines* prp-4 | JL862401.1 (1296 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 362 |
| *H. glycines* prp-8 | JL862781.1 (827 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 430 |
| *H. glycines* prp-17a | CB281686.1 (612 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 362 |
| *H. glycines* prp-17b | CB281686.1 (612 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 430 |
| *H. glycines* prp-21 | CB825077.1 (523 b) | F-GCCGTTACGACGAAACAGAC  
R-CTTCACTCAGGCAATCATTGCGA | 522 |
3.3.5. Sequence analysis of amplified putative transcripts for spliceosome units of *P. thornei* and *H. schachtii*

Sequences of all the amplicons obtained for *P. thornei* spliceosome units were 94% to 99% identical to the ESTs from the transcriptome based on which primers were designed. BLASTX analysis of these sequences with those of other nematodes including species of the free-living *Caenorhabditis*, animal and plant parasitic nematodes indicate such genes are common among nematodes. Mostly, the *P. thornei* sequences were more identical to those of parasitic nematodes than sequences of the free-living nematodes. For example, the sequence of the amplicon putatively encoding *P. thornei* SUT-1 was 99% identical to that of the transcript used to design primers. BLASTX analysis of this EST matched sequences of a spliced leader 30kDa protein of the human parasitic nematode *Brugia malayi* (total score 55.1 and e-value 3e-6), and spliced leader 30kDa proteins of *Haemonchus contortus*, *Ascaris suum* and hypothetical proteins of other nematodes including *Loa loa*, *Wucheraria bancrofti* and *Strongyloides ratti* (Fig. 3.1a). For the 345 bp amplicon putatively encoding *P. thornei* SNA-2 (98% identity, 9e-179 e-value and 75% query coverage to the original transcript) the translated amino acid was partially identical to proteins of a number of animal parasitic nematodes namely a hypothetical protein of *W. bancrofti* (total score 53.5, e-value 1e-5) and a 175 kDa protein of *B. malayi* and *A. suum*, a spliced leader protein of *L. loa*, but with a slightly lower identity to the snRNA associated protein of *C. elegans* and SNA-2 of *Caenorhabditis remanei* and *Caenorhabditis briggsae*. For the putative transcript for *P. thornei* SUT-2, apart from the *C. elegans* query sequence used for BLAST analysis that led to its identification, sequences of the amplicon did not have significant similarities with other sequences in databases available at NCBI.
Fig. 3.1. Representation of partial alignments of sequences of *P. thornei* amplicons putatively encoding three spliceosome-specific genes. (A) Alignment of *P. thornei* SUT-1 (query) and *B. malayi* spliced leader 30kDa protein (subject). (B) Alignments of *P. thornei* Sna-2 (query) with (a) hypothetical protein WUBG_04637 of *W. bancrofti* (subject) and (C) Alignment of snRNA associated protein of *C. elegans* (subject) with *P. thornei* sna-2.

Sequences of the other nine transcripts putatively encoding *P. thornei* PRP-4, PRP-8, PRP-17, PRPF-4, UAF-1, UAF-2, T08A11.2, SFA-1 AND SWP-1 had 92-99% identities (and e-values between 4e-75 and 5e-176) with not only the original transcripts but also matched several such sequences for the cyst nematodes *H. glycines* and *H. schachtii* and root knot nematodes.
such as *M. incognita* and *M. hapla* (Fig. 3.2). Details of alignment results of sequences of the *P. thornei* with other nematodes are provided as Appendix 1.

Similarly, sequences of amplicons of *H. schachtii* used in further RNAi studies, initially identified via comparative analysis of transcriptome of the pre-infective J2s and ESTs of other nematodes and published by Fosu-Nyarko et al (2016), were identical to the original transcripts. These were confirmed by further analysis and annotation using databases at NCBI.

| pre-mRNA splicing factor [Heterodera glycines] | gb|AAD43464.1| Length: 570 | Number of Matches: 1 |
|-----------------------------------------------|----------------|--------------|---------------------|
| Score                                        | Expect Method | Identities | Positives | Gaps | Frame |
| 132 bits(331)                                 | 4e-34( ) Compositional matrix adjust. | 64/80(80%) | 71/80(88%) | 1/80(1%) | -2 |
| Features:                                     | Query 241 | 5GVEAAGKQSLFESVXYTGQKRRVNYDASIDGTYGPMARFEKEATVARPDPE | 62 |
|                                              | Objct 136 | VGQVQVQAQKAGSLFE-SVXYTGQKRRVNYDASIDGTYGPMARFEDEKTARPDPE | 194 |
|                                              | Query 61 | LOKEMDEIVRKKLKSRRGR | 2 |
|                                              | Objct 195 | LOKEMDEIVRKKLKSRRGR | 214 |

**Fig. 3.2.** Partial alignment of EST putatively encoding *P. thornei* PRP-17 (query) with *H. glycines* pre-mRNA splicing factor (subject).

### 3.4. Discussion

Pre-mRNA *cis* and *trans*-splicing are two important mRNA processing mechanisms that occur during gene expression in many organisms including nematodes. These processes require a number of proteins that function together and are collectively referred to as *cis* and *trans*-spliceosomes. In this chapter, comparative analysis with the free living *C. elegans* spliceosome revealed putative transcripts of three root lesion nematodes and two cyst nematodes that are similar to those of 55 and 44 *C. elegans* spliceosome units respectively. The presence of some transcripts in one or more transcriptomes or EST databases and not in the other may reflect differences in coverage and available sequence data in each of the databases.
Putative identification of transcripts for the SL specific genes, sna-1, sna-2, sut-1, sut-2, in sequences of the root lesion and cyst nematodes is a strong indication that SL trans-splicing of mRNA is a feature of plant parasitic nematodes, since these four proteins are important components of the SL snRNP of trans-spliceosomes which mobilise the SL RNA to the SL trans-splicing reaction and mediate operon processing (Denker et al., 2002; MacMorris et al., 2007).

Based on the important functions of the identified genes for which putative transcripts were found (Table 3.2, Sanford & Bruzik, 1999; Jurica & Moore, 2003; Mitreva et al., 2004; Wahl et al., 2009; Gündl, 2010) in cis and trans-spliceosomes, as emphasised earlier, these genes could be good targets for using RNAi as a mechanism to control plant parasitic nematodes. Evidence for this also comes from the first demonstration of in planta RNAi of a splicing factor of M. incognita and a recent study in which RNAi of the H. glycines Ichinohe prp-17 gene, which also encodes a mRNA splicing factor, can result in significant reduction in reproduction of H. glycines in a compatible host (Li et al., 2010). When infective J2s were inoculated onto transgenic soybean plants engineered to produce dsRNA from a 289 bp long transcript, potential ingestion of processed siRNAs resulted in significant reductions in cysts/g root tissue (53%), eggs/g root tissue (79%) and eggs/cyst (55%) five weeks after nematode inoculation (Li et al., 2010). Klink et al., (2009) have suggested that genes involved in different aspects of mRNA metabolism, such as ribosomal proteins and an SR gene involved in mRNA splicing, are promising target genes for H. glycines biocontrol.

Blastx analysis of fragments amplified from P. thornei transcripts putatively encoding SUT-1, SUT-2, SNA-2, PRP-8, PRP-17, T08A11.2, UAF-2, SWP-1 AND SFA-1 indicate that they are present mostly in nematodes and their sequences are more similar to those of animal parasitic nematodes such as W. bancrofti, B. malayi, H. contortus and the plant parasitic nematode H. glycines than to the free living C. elegans (section 3.3.4.). This finding suggests that interfering
the expression of these genes by RNAi will minimise off-target effects during plant mediated RNAi. Similarities found in free-living nematodes such as *Caenorhabditis* species and animal parasitic nematodes may not impose off target effects in *in planta* gene silencing since neither group feeds from live plants.
5.1. Introduction

Plant-derived RNAi of plant parasitic nematode genes that are vital for survival, is a strategy of interest for nematode management aimed at reducing nematode populations and infectivity of important host plants (Sindhu et al., 2009). Agrobacterium rhizogenes-induced hairy roots provide a convenient system for studying gene functions without going through the long process of producing stable transgenic plants (Yang et al., 2013). A. rhizogenes-induced roots grow rapidly, are genetically stable, lack geotropism and are high-branching on phyto-hormone free medium (Hu & Du, 2006). Hairy roots have significant applications in plant genetic engineering: they have been employed for functional analysis of genes, production of secondary metabolites and production of therapeutic proteins (Hu & Du, 2006). In some cases it is possible to regenerate whole plants from hairy roots (Hu & Du, 2006).

Hairy root systems have been used to study the interactions of plants with parasitic nematodes. Examples include Meloidogyne incognita and Rotylenchulus reniformis and resistant cotton (Wubben et al., 2009), Heterodera glycines and soybean expressing tandem inverted repeats of four H. glycines genes (Klink et al., 2009), M. incognita and tomato expressing dsRNA of two M. incognita genes (Charlton et al., 2010), M. incognita and grapevine expressing hairpin RNA of 16D10 effector gene (Yang et al., 2013) and Pratylenchus penetrans and soybean expressing dsRNA of two locomotion related genes of P. penetrans, pat-10 and unc-87 (Vieira et al., 2015).
Whole carrot discs and calli are often used to maintain in vitro cultures of migratory endoparasitic nematodes such as *P. vulnus* (Moody et al., 1973), *P. thornei* (Tan et al., 2013), *P. agilis*, *P. brachyurus*, *P. scribneri*, *Radopholus citrophilus* and *R. similis* (Reise et al., 1987). *Pratylenchus* spp. can complete their life cycle and induce the same root tip hypertrophy symptoms that are characteristic of the infection in the soil when they are co-cultured on carrot hairy roots (Flores et al., 1999). In an attempt to determine the impact of arbuscular mycorrhizal fungus *Glomus intraradices* on the reproduction of *R. similis*, a major root burrowing nematode infecting various economically important crops, in a dixenic culture system, the life cycles of both the fungus and the nematode were completed in the presence of each other with Ri T-DNA transformed carrot roots as host (Elsen et al., 2001).

The objective of this chapter was to use *A. rhizogenes* K599 to generate carrot hairy roots harbouring hairpin RNAs (hpRNAs) corresponding to sequences of the spliced leader-associated genes *sut-1*, *sut-2* and *sna-2* of *P. thornei*. These hairy roots were then used to assess the effect of knocking down these important genes involved in the effective trans-splicing of most *P. thornei* genes on reproduction of the nematode. This chapter provides first evidence of *in planta* RNAi analysis of spliced leader-associated genes of *P. thornei*.

### 5.2. Materials and methods

#### 5.2.1. Generation of hairpin vectors for *sut-1*, *sut-2* and *sna-2* genes of *P. thornei*

The genes *sut-1*, *sut-2* and *sna-2* were previously ligated to the vector pDoubler. The partial sense and antisense sequences of the genes were digested and ligated sequentially to the vector pCLEaver-NosA (Fig. 2.5.) to obtain the hairpin expression cassettes. The hairpin cassettes were then cloned into the binary vector pART27 (Fig. 2.6.) for plant transformations.
5.2.1.1. Ligation of DNA sequences to vectors

Plasmid DNA - pDoubler carrying DNA fragments of sut-1, sut-2 and sna-2, was extracted from E. coli JM109 cultures and each was digested with KpnI and XhoI and then with BamHI and XbaI enzymes to obtain the 5′-3′ and 3′-5′ fragments respectively. The fragments were ligated sequentially to pCLeaver-NosA vector using T4 DNA ligase. The ligated fragments were used to transform E. coli JM109 and the transformants selected and screened by PCR (sections 2.9.1. and 2.9.3.). Ligation of the sense and antisense DNA fragments to pCLeaver-NosA was confirmed by PCR respectively using the primer pairs SIntron and S35S and ASIntron and ASNosA as described section 2.9.3. To assess successful ligations of the DNA fragments to the vector, the resulting plasmid DNAs were digested with KpnI/XhoI and BamHI/XbaI in separate reactions to release the respective inserts from the vector.

5.2.1.2. Ligation of hairpin cassettes of sut-1, sut-2 and sna-2 genes to binary vector pART27

Plasmids confirmed by digestion to have both the sense and antisense fragments of target genes ligated to pCLeaver-NosA were digested with NotI to release the hairpin cassette (3S promoter, sense sequence, intron, antisense sequence and terminator); this was done so the fragment could be ligated to NotI-linearised and dephosphorylated binary vector pART27. About 20 ng of hairpin cassette DNA for each target gene was ligated to 22 ng of pART27 using Quick T4 DNA ligase (NEB). The reactions were incubated at room temperature for 5 minutes and chilled on ice. About 50 µL of competent E. coli JM109 were transformed with 5 µL each of the ligation reactions. Transformed colonies were screened by PCR (Fig. 5.1) with primer pair SP6 and 3SART-R as described in section 2.9.3.
Fig. 5.1. Agarose gel photographs showing amplicons obtained after colony PCR with SP6 and 35SART-R primers for (a) pAsut-1 and pAsna-2 (b) pAsut-2.

Successful ligation of hairpin cassettes to pART27 was confirmed by two double digestion reactions as described above using the enzyme pair KpnI/XhoI and the other with BamHI/XbaI, as well as by capillary sequencing of the resulting plasmids to confirm the orientation of inserts. The sense strand of each gene was sequenced and analysed using 3.2 pmol of SIntron and ASIntron primers in two separate 1/16 sequencing reactions as described in section 2.6.

5.2.2. Transformation of *Agrobacterium rhizogenes* K599 with pART27 carrying hairpin cassettes of the *P. thornei* genes *sut-1*, *sut-2* and *sna-2*

Ten microliters of *A. rhizogenes* K599 chemically competent cells (calcium chloride treated) was transformed with approximately 1 µg of plasmid DNA (pART27 carrying the hairpin cassettes) as described in section 2.9.2. *A. rhizogenes* K599 colonies were screened by PCR using 5 pmol of SP6 and 35SART-R primers. Bacterial cultures for colony screening were prepared by suspending cells from a bacterial colony in 15 µL of nuclease free water in PCR tubes. Five microliters of each cell suspension was transferred into each PCR tube, heated at
95°C for 5 minutes before adding to PCR reactions. Template DNA of bacterial colonies transformed with the plasmids produced amplicon of 760 bp, similar to those in Fig. 5.2.1.2.

5.2.3. *Agrobacterium rhizogenes* K599-mediated transformation of carrots with hairpin vectors for *sut-1*, *sut-2* and *sna-2* of *P. thornei*

*A. rhizogenes* K599 carrying pART27 with hairpin cassettes of *sut-1*, *sut-2* and *sna-2* genes were cultured in 10 mL LB broth supplemented with 25 mg/L rifampicin and 100 mg/L spectinomycin for 18 hours at 28°C in the dark on a shaker at 225 rpm: this culture was used to generate hairy roots from carrot discs. *A. rhizogenes* K599 transformed with pART27 without a nematode gene (null vector) and with hairpin cassette carrying 500 bp *gfp* gene (made by Dr. John Fosu-Nyarko) were used as controls for the transformations. Pelleted bacterial suspensions were used for transformations.

Carrot taproots were used as the source tissue to generate hairy roots. Freshly obtained whole carrots (Nantes variety Stefano) were surface sterilised as described in section 2.1.1., peeled, cut transversely into 0.3 mm thick discs and placed on 0.4% water agar in 9 cm diameter tissue culture dishes with the apical side facing upwards. The apical sides of 3-5 discs in one dish were then infected with *A. rhizogenes* K599 by applying cells along the cambial ring on the disc with a pipette tip and spread with an inoculating loop. After inoculation, the plates were sealed with parafilm and kept in the dark, at 25±2°C. Uninfected carrot discs and those infected with unmodified *A. rhizogenes* K599 served as controls.

Hairy roots were excised and transferred to semi solid Murashige and Skoog (MS) medium [4.4g of MS with Gamborg vitamins per liter (PhytoTechnology Laboratories), 30 g sucrose and 8 g Daishin agar Brunschwig Chemie, Amsterdam, pH 5.6-5.8] supplemented with 150 mg/L cefotaxime and with or without 25 mg/L kanamycin in petri dishes. Cefotaxime was added to
the medium to inhibit the growth of *A. rhizogenes* K599 whereas kanamycin was added as a selectable agent for hairy root growth.

Roots induced by *A. rhizogenes* K599 without pART27 were transferred to MS medium supplemented with 150 mg/L cefotaxime and with or without 25 mg/L kanamycin. Roots induced by *A. rhizogenes* K599 transformed with pART27 were transferred to MS medium supplemented with both 150 mg/L cefotaxime and 25 mg/L kanamycin. Four to six roots were placed in one culture dish, sealed with parafilm and kept in the dark at 25±2°C for root growth. Each root was considered a different event. The roots were subcultured every 2 weeks by transferring about a 1 cm piece including the root tip onto similar medium in new culture dishes.

5.2.4. Infection of carrot hairy roots with *P. thornei*

For nematode assays, carrot hairy roots (approximately 1 cm long) induced by *A. rhizogenes* K599 with and without pART27 were aseptically transferred onto MS medium (without any antibiotics) in 9 cm diameter dishes—one root (an event) per dish. There were 5 events for each control and gene and three replicates for each event. Petri dishes were sealed with parafilm and kept in the dark for 3 weeks at 25±2°C for root extension.

Carrot hairy roots were infected with mixed stages of *P. thornei*: the nematodes were harvested from 5-8 weeks old infected carrot discs and surface sterilised as described in sections 2.1.1.1 and 2.1.1.2. Each replicate hairy root was infected with 500 nematodes. On each plate 100 nematodes were placed at five different spots about 1 cm away from root tips. At the point of infection the medium was disturbed using the pipette tip to facilitate nematode movement. After infection, plates were covered with lids, sealed with parafilm and kept in the dark at 25±2°C.
5.2.5. Analysis after infection

The hairy roots were observed using a dissecting microscope 1 day, 2 days, 1 week and thereafter weekly until 45 days after inoculation. After this period, each replicate root was cut into 1-2 cm pieces and placed in 10 mL of sterile water in 9 cm petri dishes to extract *P. thornei*. The plates were gently shaken occasionally and after 2 days the water with nematodes was collected in 10 or 15 mL centrifuge tubes. The nematodes in water were counted under a dissecting microscope. The hairy roots were then removed from the petri dishes, observed under the microscope and the number of nematodes attached to the roots and those on the medium (collected with 5 mL of sterile water) were also counted.

The root segments of each replicate were separately stained with acid-fuchsin (Bybd *et al*., 1983) and observed under a dissecting microscope for the presence of eggs and any nematodes remaining in the root tissue. Briefly, the root segments were immersed in 1.5% sodium hypochlorite for 5 minutes with occasional agitation. The roots were then washed in water for 15 minutes and then immersed in 10-20 mL of acid-fuchsin stain (1 mL of acid-fuchsin stain diluted in 30 mL of water) and boiled for 1 minute. After cooling to room temperature, the roots were washed in water to remove excess stain. Roots were mounted in water on a glass microscope slide covered with a cover slip for observation under the microscope. Since the root densities of replicates in an event were not similar, after observations, the roots were dried in an oven at 55±2°C for 24 hours, the dry weights were measured and the level of nematode infection expressed as the number per dry root weight.

5.3. Results

5.3.1. Carrot hairy root growth

Hairy roots emerged from *A. rhizogenes* K599-infected carrots 13 days after infection (Fig. 5.2). About 24 hrs after emerged roots were transferred to MS medium supplemented with 150
mg/L cefotaxime and with or without 25 mg/L kanamycin, there was up to 1cm extension. None of the roots induced by unmodified *A. rhizogenes* K599 extended further in the presence of kanamycin (Fig. 5.3c). All roots cultured on MS medium without kanamycin extended further and branched well (Fig. 5.3b). From roots induced by *A. rhizogenes* K599 with null vector and *gfp*, 90% extended further on MS medium with kanamycin (Fig. 5.3a). Generally 61%, 57% and 18% of roots induced by *A. rhizogenes* K599 with pAsut-1, pAsut-2 and pAsna-2 respectively grew further and branched well on MS medium with kanamycin (Fig. 5.3d).

**Fig. 5.2.** Carrot discs two weeks after infection with (a) LB broth only (b) *A. rhizogenes* K599 (c) *A. rhizogenes* K599 with pART27-null (d) *A. rhizogenes* K599 with pART27-*gfp* (e) *A. rhizogenes* K599 with pART27-*sut-1* (f) *A. rhizogenes* K599 with pART27-*sut-2* (g) *A. rhizogenes* K599 with pART27-*sna-2*. Root development was present in b-g.
Fig. 5.3. (a) Hairy roots growing on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin, 5 days after transfer from carrot discs inoculated with *A. rhizogenes* K599 with pART27-null. Some roots elongated while others did not. (b) Hairy roots induced by unmodified *A. rhizogenes* K599, growing on MS medium with 150 mg/L cefotaxime, after 3 weeks of transfer from carrot discs. (c) Hairy roots induced by unmodified *A. rhizogenes* K599, not growing on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin, after 3 weeks of transfer from carrot discs. (d) Hairy roots induced by *A. rhizogenes* K599 with pART27-sut-2, after 3 weeks of transfer from carrot discs. Not all roots grew on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin.

There were differences in root growth and vigour in replicates of each event, resulting in variations in root dry weight (2.8-57.3 mg, Fig. 5.4). There were also differences in root colour notably white or brown, even for roots of similar ages (Fig. 5.5).
Fig. 5.4. Average dry weights of carrot hairy roots: (um)-not transformed with pART27, and those transformed with null vector (pANull), gfp (pAgfp), sut-1 (pAsut-1), sut-2 (pAsut-2) and sna-2 (pAsna-2). Each bar represents average of five events ± standard error.

Fig. 5.5. Representative carrot hairy root events and replicates used in this study. Event number is preceded by the gene and vector name.

5.3.2. Nematode behaviour after infection of carrot hairy roots

Within 24 hours after inoculation of the controls and transgenic hairy root events, the nematodes were active and were observed to be moving towards the roots. Three days after inoculation the nematodes could be seen in aggregates parallel to the roots or actively moving
on roots or in the medium. Some individual nematodes lay dormant parallel to and on the outside of hairy roots for long periods of time. Stylet thrusts could be seen on some roots from actively moving nematodes. One week after inoculation most nematodes were inactive in the medium or in close proximity to hairy roots (Fig. 5.6).

**Fig. 5.6.** *P. thornei* that have moved and staying close to carrot hairy roots, 1 week after infection (x40).

### 5.3.3. Nematode survival/reproduction on carrot hairy roots

Although each replicate root was infected with 500 nematodes, the numbers of nematodes extracted from 45 days after inoculation was less than 500 except a replicate root induced by unmodified *A. rhizogenes* K599 (um-9) from which 603 nematodes were extracted. No eggs were observed in any of the roots during the six-week period and no nematode or egg was observed in roots after acid-fuchsin staining.

There was a large variation in the number of nematodes extracted from carrot hairy root events within a control/transgene. The numbers of nematodes extracted from eight control events (um-1, um-3, um-13, pANull-2, pANull-7, pANull-10, pAgfp-9, pAgfp-23) and each event
of hairy roots transformed with hairpins of \textit{sut-1}, \textit{sut-2} and \textit{sna-2}, except \textit{pAsut-1-23} and \textit{pAsna-2-16} were statistically lower than those of roots induced by unmodified \textit{A. rhizogenes} K599 (um-9) (p<0.05, Fig. 5.7a).

The average numbers of nematodes extracted per gram of dry root weight of six control events (um-13, pANull-2, pA-Null-4, pAgfp-9, pAgfp-21, pAgfp-22) and each event transformed with hairpins of \textit{sut-1}, \textit{sut-2} and \textit{sna-2}, except event \textit{pAsut-2-21}, were statistically lower than that of roots induced by unmodified \textit{A. rhizogenes} K599 (um-9) (p<0.05, Fig. 5.7b).

Collectively, the number of \textit{P. thornei} extracted from hairy roots transformed with \textit{sut-1}, \textit{sut-2} and \textit{sna-2} were statistically lower (45-64\% reduction) than those on hairy roots generated by unmodified \textit{A. rhizogenes} (p<0.05, Fig. 5.8a). However, when the average numbers of nematodes per gram root were considered (Fig. 5.8b), the figure was also statistically significantly lower (47-61\% reduction) in the control hairy roots transformed with null vector and \textit{gfp} (p<0.05). The number of \textit{P. thornei} extracted per gram root transformed with SL associated genes were reduced by 70-87\% (p<0.05) compared to those on hairy roots generated by unmodified \textit{A. rhizogenes} K599.
Fig. 5.7. (a) Mean number of nematodes and (b) mean number of nematodes/gram dry root weight extracted from each carrot hairy root events. Each bar represents the mean of three replicates ± standard error. Bars with “Δ” were statistically different (p<0.05) from um-9.
Fig. 5.8. (a) Average number of nematodes extracted from roots and medium and (b) average number of nematodes per gram of dry root weight of hairy roots generated with unmodified *A. rhizogenes* (um), transformed with null vector (pANull), *gfp* (pAgfp), *sut*-1 (pAsut-1), *sut*-2 (pAsut-2) and *sna*-2 (pAsna-2). Each bar represents the average of 5 events ± standard error. Bars with “Δ” were significantly different from the negative control “um”.

In general, the number of nematodes extracted from the medium was higher than the number of nematodes extracted from the roots (Fig. 5.9).
Fig. 5.9. Number of nematodes extracted from medium (blue) and roots (red) per gram of dry root weight. Each bar represents the average of five events ± standard error.

5.4. Discussion

In planta expression of hairpin RNA of nematode genes has been exploited as a control strategy for plant parasitic nematodes (Kyndt et al., 2013; Yang et al., 2013). In this chapter, carrot hairy roots harbouring hairpin RNAs corresponding to partial sequences of the SL-associated genes sut-1, sut-2 and sna-2 of P. thornei were generated using A. rhizogenes K599 and were used to assess the importance of the genes in the reproduction of the nematode.

Variation in hairy root morphology, growth and vigour, as observed in the carrot hairy roots generated in this research, has been reported in carrots (Guivarc’h et al., 1999), cotton (Wubben et al., 2009) and grapes (Yang et al., 2013). Several factors can account for this variation. The levels of auxins produced by the rol genes from the hairy root inducing plasmid of A. rhizogenes could vary between different hairy root lines and this could result in hairy roots with differing vigour and morphology (Nilsson & Olsson, 1997; Yang et al., 2013). Also the site of T-DNA integration into the host genome and T-DNA copy number can affect host gene expression (Nilsson & Olsson, 1997; Alonso et al., 2003).
A wide variation in the number of nematodes extracted from carrot hairy root events within a control/transgene was observed in the nematode infection assays. Similar results, for example root fresh weight (203.6 - 550.0 mg), number of *Meloidogyne incognita* eggs per hairy root (24.0 - 626.8), and number of eggs per gram root (54.8 - 3209.0) have been reported by Yang *et al.*, (2013) when transgenic grape hairy roots were infected with J2s, in a study on the efficacy of 16D10 siRNAs in inhibiting root knot nematode infection. Also for T3 lines of transgenic *Arabidopsis thaliana* expressing hairpin RNA against three *H. schachtii* genes (U2AF, H’ATPase, Ubiquitin) under the control of cauliflower mosaic virus 35S promoter, the level of infection was variable between lines of the same construct and between independently repeated experiments (Kyndt *et al.*, 2013). In the same experiment, they detected a variable expression level of a target gene (U2AF) construct between and within lines, and a decline in gene expression over subsequent generations, an effect that was shown to be correlated with transcriptional gene silencing of the hairpin construct through promoter methylation (Kyndt *et al.*, 2013).

Despite the variation in the number of nematodes on different carrot hairy root events transformed with hairpin RNA constructs of *P. thornei* SL-associated genes, there was a significant reduction (70-87% reduction) compared to untransformed hairy roots. This observation should be studied further with analysis of target mRNA in *P. thornei* fed on hairy roots and expression levels of hairpin RNA constructs in transgenic events.

The results obtained from *in planta* experiments done in this chapter were similar to those obtained when the same SL associated *P. thornei* genes were targeted by soaking *in vitro* (chapter 4), in which there was a significant (55-99%) reduction in nematode numbers compared to the respective controls. In contrast, Vieira *et al.*, (2015) have discussed that *in planta* delivery of dsRNA of unc-87 and pat-10 of *P. penetrans* was less effective than targeting
the same genes by dsRNA soaking (Joseph et al., 2012; Tan et al., 2013) and suggested that the differences could be due to silencing efficiency using dsRNA delivered by the plant versus soaking (Vieira et al., 2015).

Furthermore, Vieira et al., (2015) also argued that as root lesion nematodes are migratory nematodes, the continuous exposure of nematodes to dsRNA delivered by the plant would be reduced during the periods that the nematodes leave the roots, and this factor may allow for some degree of recovery (Vieira et al., 2015). This possible reduced exposure to dsRNA could also explain the variation in numbers of *P. thornei* extracted from carrot hairy root events in this chapter.

The results obtained from the preliminary experiment done in this chapter which is the first evidence of *in planta* RNAi analysis of SL-associated genes of *P. thornei*, provide evidence of genes that can confer some resistance to *P. thornei* by reducing the total number of nematodes associated with carrot hairy roots.
CHAPTER 4

*In vitro* RNA Interference Analysis of Spliceosome Genes of the

Root Lesion Nematode, *Pratylenchus thornei*

4.1. Introduction

Until recently it was not known whether root lesion nematodes were amenable to RNAi. Two publications were seminal in showing that different *Pratylenchus* spp. were indeed amenable to gene silencing using *in vitro* soaking of these nematodes with dsRNA of target genes. The application of RNAi technology for functional analysis of *P. thornei* genes and its subsequent use as a potential strategy for controlling this pest was first demonstrated by Joseph et al., (2012) and Tan et al., (2013). In these experiments, mixed stages of *P. thornei*, *P. zeae* and *P. coffeae* were soaked with dsRNA of *pat-10* and *unc-87* genes, in a soaking medium containing the neurostimulant octopamine. This treatment resulted in paralysis and uncoordinated movements in all three species. There was a greater reduction in transcripts of both genes in *P. thornei* than in *P. zeae*, indicating that the former may be more susceptible to RNAi. For *P. thornei* treated with dsRNA of the two genes there was a significant reduction (77–81%) in nematode reproduction over 5 weeks on carrot mini discs. In addition, dsRNA derived from *P. thornei* and *P. zeae* silenced the corresponding gene in both species implying that cross species control of nematodes *via* RNAi is possible (Tan et al., 2013).

The transcriptome of a mixed stage population of *P. thornei* has been sequenced and annotated (Nicol et al., 2012). Based on assignment of contigs to publicly available datasets, putative transcripts of carbohydrate active enzymes involved in cell wall degradation, neuropeptides and plant nematode parasitism genes were identified (Nicol et al., 2012).
Availability of the transcriptomes of *P. thornei* (Nicol *et al*., 2012), *P. zeae* (Fosu-Nyarko *et al*., 2015) and *P. coffeae* (Haegeman *et al*., 2011) has provided sequence information on various genes that can be exploited for further RNAi studies.

Based on these previous reports, the objective of this chapter was to use RNAi to study the importance of a group of target genes in the development and reproduction of *P. thornei*, and in particular, the potential of these genes as targets for pest control via RNAi. The genes chosen for the study were *C. elegans* orthologues of three spliced leader-associated genes (*sut*-1, *sut*-2, *sna*-2) and nine genes vital for both *cis* and *trans*-splicing (*prp*-4, *prp*-8, *prp*-17, *prpf*-4, *uaf*-1, *uaf*-2, *t08a11.2, *sfa*-1, *swp*-1): as indicated earlier the role of these genes in mRNA splicing and functional (RNAi) analysis data available for *C. elegans* suggest the genes are vital for nematode development. To achieve this objective, gene fragments that were amplified and sequenced (Chapter 3) were cloned and templates used to generate dsRNA *in vitro* with which *in vitro* RNAi was conducted. This was done by soaking mixed stages of *P. thornei* in dsRNA to study the effects of silencing on the behaviour and responses of the nematodes compared to those of controls. This chapter reports the first RNAi study of *P. thornei* genes possibly involved in the *trans*-splicing process.

### 4.2. Materials and methods

#### 4.2.1. *In vitro* transcription of dsRNA

Gene fragments were amplified by PCR from *P. thornei* cDNA and were sequenced to confirm sequence identity as described in section 3.2.2. The DNA fragments of *prp*-4, *prp*-8, *prp*-17, *prpf*-4, *uaf*-1, *uaf*-2, *t08a11.2, *sfa*-1 and *swp*-1 were ligated to pGEM-T easy vector and the plasmid DNA obtained for each was re-amplified in a PCR with gene-specific primers that had T7 promoter sequence appended (Table 4.1.), to generate templates for *in vitro* transcription. Partial cDNA/transcripts of *sut*-1, *sut*-2 and *sna*-2 were amplified from cDNA using primers
carrying *KpnI* (5’GGTACC3’) and *XhoI* (5’CTCGAG3’) restriction enzyme sites at 5’ ends to allow ligation to pDoubler (Table 4.2) from which DNA templates for *in vitro* transcription were generated to synthesise dsRNA.

DNA templates for each of the genes (1-2 μg each) were transcribed using the HiScribe T7 *In Vitro* Transcription kit, DNaseI treated and the quantity and quality assessed for *in vitro* experiments. Between 84 μg and 332 μg of dsRNA was generated from the templates and these were of good quality as shown in Fig. 4.1.

**Table 4.1.** Primers used to obtain template DNA for *in vitro* transcription of *P. thornei* genes.

T7 RNA polymerase promoter sequences are underlined.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| prp-4 | F- TAATACGACTCACTATAGGGCACCGCATCGTTGGACAAAGACCA  
R- TAATACGACTCACTATAGGGCCGGTGAGGATGATGGGCAAC  | 264 |
| prp-8 | F- TAATACGACTCACTATAGGGCTTGTTGTCGACTGTA  
R- TAATACGACTCACTATAGGGCGGCGTCCGATTCCGCAATTTC  | 353 |
| prp-17 | F- TAATACGACTCACTATAGGGCCGCAATTCTGGCTTTCAGCTT  
R- TAATACGACTCACTATAGGGCAATACGTTTGTTGGAG  | 257 |
| prpf-4 | F- TAATACGACTCACTATAGGGCAGCTTGTTGTAATGCGAG  
R- TAATACGACTCACTATAGGGAGAATCTGACACCAATCGAGC  | 423 |
| uaf-1 | F- TAATACGACTCACTATAGGGGCGCTGCTCTGAAATTTGTCCGAGATTTCGATCAGTCCGCAATTTCGAC  
R- TAATACGACTCACTATAGGGCCGGTGAGGATGATGGGCAAC  | 266 |
| uaf-2 | F- TAATACGACTCACTATAGGGCAGCTTGCTCAGTCCGCAATTTCGAC  
R- TAATACGACTCACTATAGGGGCGCTGCTCTGAAATTTGTCCGAGATTTCGATCAGTCCGCAATTTCGAC  | 318 |
| t08a11.2 | F- TAATACGACTCACTATAGGGCAGCTTGCTCAGTCCGCAATTTCGAC  
R- TAATACGACTCACTATAGGGGCGCTGCTCTGAAATTTGTCCGAGATTTCGATCAGTCCGCAATTTCGAC  | 497 |
| sfa-1 | F- TAATACGACTCACTATAGGGGCGCTGCTCAGTCCGCAATTTCGAC  
R- TAATACGACTCACTATAGGGGCGCTGCTCTGAAATTTGTCCGAGATTTCGATCAGTCCGCAATTTCGAC  | 229 |
| swp-1 | F- TAATACGACTCACTATAGGGGCGCTGCTCAGTCCGCAATTTCGAC  
R- TAATACGACTCACTATAGGGGCGCTGCTCTGAAATTTGTCCGAGATTTCGATCAGTCCGCAATTTCGAC  | 219 |
Table 4.2. Primers used to amplify *sut-1*, *sut-2* and *sna-2* genes of *P. thornei* for cloning with pDoubler. *Kpn*I and *Xho*I restriction enzyme sites are underlined.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sut-1</em></td>
<td>F-TCGGGTACCCATAAGGTGTAATCTGTGT  R-TCGCTCGAGCGAAATGGAAAGATGA</td>
<td>560</td>
</tr>
<tr>
<td><em>sut-2</em></td>
<td>F-TCGGGTACCCATTCTTCACCATCGCGA  R-TCACTCGAGTGGTGTGGCCTGTGT</td>
<td>451</td>
</tr>
<tr>
<td><em>sna-2</em></td>
<td>F-TAACGTACCATTGCCATCGACTGCTGCT  R-TCACGTCCCGTGTGTCCGGTTC</td>
<td>345</td>
</tr>
</tbody>
</table>

![Agarose gel photographs](image)

**Fig. 4.1.** 2% agarose gel photographs showing dsRNA in lanes 1-9 and 10-13 as follows: 1:*prp-4*, 2:*prp-8*, 3:*prp-17*, 4:*prp-4*, 5:*uaf-1*, 6:*uaf-2*, 7:*t08a11.2*, 8:*sfa-1*, 9:*swp-1*, 11:*sut-1*, 12:*sut-2*, 13:*sna-2* of *P. thornei* and 10:*gfp*.

### 4.2.2. Conditions for in vitro RNAi of *P. thornei* genes by soaking

For each target gene, 1,000 vermiform *P. thornei* of mixed J2 – adult stages freshly extracted from carrot discs were soaked in M9 buffer (43 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 2 mM NaCl, 4.6 mM NH$_4$Cl) containing 1 mg/mL dsRNA, 50 mM octopamine hydrochloride (Sigma Aldrich), 3 mM spermidine trihydrochloride (Sigma Aldrich) and 0.05% gelatine (Sigma Aldrich) in 1.5 mL centrifuge tubes. There were two control experiments; one set up as described above but without dsRNA and the other with 1 mg/mL dsRNA of the green fluorescent protein (*gfp*) from *Aequorea victoria*. A similar soaking medium was set up which contained 1 mg/mL fluorescein isothiocyanate (FITC) isomer I (Fluka 46952) without any dsRNA: this was used to assess uptake of the soaking solution by *P. thornei*. All the experiments were incubated at 25°C in the dark.
for 16 hours after which the nematodes were washed five times with sterile water and their
behaviour and activity, as well as the presence of FITC in the body of those soaked with the
dye, was observed using an Olympus BX51 photomicroscope.

Two replicate soaking treatments were set up for the genes prp-4, prp-8, prp-17, prpf-4, uaf-1,
uaf-2, t08a11.2, sfa-1 and swp-1 and controls in one independent experiment. For sut-1, sut-2
and sna-2 genes and controls, one replicate was set up in another independent experiment.

4.2.3. Quantification of target mRNA after in vitro RNAi

After washing *P. thornei* with sterile water following soaking, RNA was extracted from 750
nematodes using the ARCTURUS PicoPure RNA Isolation Kit as described in section 2.2.2., and
approximately 100 ng of RNA was reverse transcribed. When *in vitro* RNAi was conducted in
replicates, nematodes were pooled together for RNA extraction. Messenger RNA levels of prp-
4, prp-8, prp-17, prpf-4, uaf-1, uaf-2, t08a11.2, sfa-1 and swp-1 were determined by
quantitative real time PCR from 400 ng of cDNA using expression of 18S rRNA as the reference.

Quantitative PCRs (qPCRs) were performed using the SYBR Green PCR Master Mix (Applied
Biosystems, Life Technologies). Each PCR contained 10 µL of SYBR Green PCR Master Mix, 400
ng of cDNA, 10 pmol of forward and reverse primers (Table 4.3.). There were two control PCR
reactions; one without template DNA and another without primers. The qPCR reactions were
set up in triplicates and were performed in a Corbett Rotor-Gene thermal cycler using the
following temperature profile: 95°C for 10 minutes, 45 cycles at 95°C for 10 seconds and 56°C
for 60 seconds. The results were analysed using the Rotor-Gene Q Series Software 2.1.0 (Build
9) with the threshold Ct value manually set at 0.04. The mRNA levels of sut-1, sut-2 and sna-2
genes were assessed using a semi-quantitative PCR; abundance of transcripts at the 20th, 25th,
30th, 35th and 40th cycles were compared. PCRs were done with approximately 400 ng of cDNA
and 10 pmol of primers (Table 4.2.). 18S rRNA expression was used as a reference.
Table 4.3. Primers used to quantify mRNA of target and reference genes of *P. thornei* after 16 hours of *in vitro* RNAi.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>prp</em>-4</td>
<td>F-CACGTTTGGCCACCGCATCGT</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>R-CGTGGTAGTAATCCACACAGT</td>
<td></td>
</tr>
<tr>
<td><em>prp</em>-8</td>
<td>F-GATCCAGATAAACGGTCAGTGA</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>R-ATGCTCACAATGCGCTCCT</td>
<td></td>
</tr>
<tr>
<td><em>prp</em>-17</td>
<td>F-GCGGCCAATTCTGCTCTTCA</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>R-GTCCGTGGGGCTGTTTCAGAG</td>
<td></td>
</tr>
<tr>
<td><em>prpf</em>-4</td>
<td>F-GTCCGGTTTAGTGTCCGAT</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>R-GACTGCAACCTGAAAGACAGT</td>
<td></td>
</tr>
<tr>
<td>t08a11.2</td>
<td>F-GTGAATGGATGGCAATCTGCT</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>R-CTTTCGCGATATATCCAAATGT</td>
<td></td>
</tr>
<tr>
<td><em>sfa</em>-1</td>
<td>F-CAAGGGTTCCGTTCGACTGAG</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>R-CAGCGGTACAGCATCTCCAGT</td>
<td></td>
</tr>
<tr>
<td><em>swp</em>-1</td>
<td>F-GAAGAACGGTACGTAGCATGA</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>R-CAATGCAGGAATTGACTCATCAT</td>
<td></td>
</tr>
<tr>
<td><em>uaf</em>-1</td>
<td>F-GACAAATGTCGCCAGTGACTA</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>R-TTCATAGCTGAAAGTGCGTCA</td>
<td></td>
</tr>
<tr>
<td><em>uaf</em>-2</td>
<td>F-TCCAGACTTTCACATGC</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>R-ATACGCGCCGAATTGAGAGA</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>F-CAGAACCGACGTATGCG</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>R-GAAAGTGATGAGCAGATC</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F-ATGAGAGGGCAAGTGCTGGT</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>R-GAAAGCCTGTGACACCCAGT</td>
<td></td>
</tr>
</tbody>
</table>

**4.2.4. Effect of *in vitro* RNAi on the reproduction of *P. thornei***

Longer term effects of RNAi on the reproduction of *P. thornei* was studied using mini carrot discs (Fig. 4.2.). After the 16 hr incubation in dsRNA, the nematodes were washed with sterile water and two carrot mini discs were infected each with 100 nematodes from an *in vitro* soaking experiment. The discs were incubated at 21°C for five weeks in the dark. After 5 weeks each carrot mini disc was cut into small pieces and nematodes were extracted using a mist chamber. Nematodes from each treatment were observed and counted using a dissecting microscope.

Carrot mini discs were prepared by surface sterilising whole carrots and cutting 10 mm diameter discs using a sterile cork borer. The discs were lightly flamed using a Bunsen burner.
and placed in wells of a 24 well plate (Greiner Bio-One, Germany; Fig. 4.2). The plates were covered with lids, sealed with parafilm and incubated at 21°C for 2 weeks for callusing and to detect possible contaminations.

4.3. Results

4.3.1. Behaviour of *P. thornei* after 16 hours of soaking in dsRNA

The stylet, the gut and body cavity of *P. thornei* soaked in a medium containing 1 mg/mL FITC for 16 hours fluoresced under a photomicroscope (Olympus BX-51 compound with filter set for fluorescein wavelengths), indicating uptake of the dye by active ingestion through the stylet or otherwise by uptake through the body wall (Fig. 4.3a). Control *P. thornei* soaked with no dsRNA were active and exhibited normal, sinusoidal movement. In contrast, for most genes tested, dsRNA-treated *P. thornei* exhibited various abnormal behaviours: most were inactive or hyperactive depending on the target gene tested. *P. thornei* soaked in *gfp* dsRNA appeared to move faster than the no-dsRNA controls (Fig. 4.3b). Between 85-88% of *P. thornei* soaked in dsRNA of *prpf-4, uaf-1* and *sna-2* were paralysed (Fig. 4.3c and e). *P. thornei* soaked in *uaf-2* dsRNA moved more slowly than the no dsRNA control.
Fig. 4.3. Phenotypes of *P. thornei* 16h after soaking in (a) Presence of fluorescence in stylet, gut and lumen after soaking in 1mg/mL of FITC (b) *P. thornei* soaked in *gfp* dsRNA and (c-f) *P. thornei* showing paralysis (after soaking with dsRNA of *uaf-1, sna-2*) and knots (soaked with dsRNA of *prp-17, sut-1*) (k-knot; p-paralysis).

For *P. thornei* soaked in *sut-1* (Fig. 4.3f), *sut-2* and *sfa-1* dsRNA, 27%, 21% and 5% of the nematodes formed ‘knots’ respectively, while the behaviour of the other nematodes in those experiments did not differ from no-dsRNA controls. Loss of sense of direction of movement of nematodes soaked in dsRNA of *prp-4, prp-8, prp-17* (Fig. 4.3d), *t08a11.2* and *swp-1* was also evident. There were also some nematodes soaked in dsRNA of the following genes that were knotted: 8% for *prp-4*; 10% for *prp-17* and *swp-1*, 18% for *t08a11.2* and 28% for *prp-8*).

4.3.2. Effect of *in vitro* RNAi on target mRNA levels of *P. thornei*

4.3.2.1. Transcript abundance of spliceosome genes of *P. thornei* after *in vitro* RNAi

PCRs to amplify 18S rRNA and actin were done to assess their suitability as references for determining transcript abundance after RNAi. The 18S rRNA gene was amplified successfully from cDNA of nematodes treated with dsRNA of the nine target genes (Fig. 4.4c) while the
actin gene could not be amplified from cDNA of *P. thornei* treated with dsRNA of *prpf*-4, *uaf*-1, *sfa*-1 and *swp*-1 (Fig. 4.4.c). The band intensity of amplicons of *prp*-8, *prp*-17, *prpf*-4, *uaf*-2, *t08a11.2*, *sfa*-1, and *swp*-1 from nematodes soaked with the target dsRNA were very faint compared to those from nematodes of the control set up clearly indicating down-regulation of the genes in the former, after RNAi (Fig. 4.4d).

**Fig. 4.4.** Amplification of actin, 18S rRNA and nine target genes of *P. thornei* (a) PCR bands of genes from cDNA of nematodes not treated with dsRNA, (b) PCR bands of genes from cDNA of nematodes treated with *gfp* dsRNA and (c, d) PCR bands of genes from cDNA of nematodes treated with dsRNA of target genes. (A: Actin, 18S: 18S rRNA, 1:*prp*-4, 2:*prp*-8, 3:*prp*-17, 4:*prpf*-4, 5:*uaf*-1, 6:*uaf*-2, 7:*t08a11.2*, 8:*sfa*-1, 9:*swp*-1).

Since 18S rRNA amplified in all cDNA samples, it was used as the reference in qPCRs to assess transcript abundance after *in vitro* dsRNA soaking. In contrast to the PCR results shown in Fig. 4.4.d, expression of the target genes were detected from the qPCRs (Fig. 4.5.), possibly due to higher sensitivity of qPCRs.

The Ct values of 18S rRNA expression for nematodes of the two controls (7.15±0.1 - 7.56±0.18) were significantly lower than that of *P. thornei* treated with dsRNA of the target genes (ranging from 14.19±0.08 - 21.62±0.08), (T-test, p<0.05). However, the Ct values for expression of all
the target genes, except prp-17, in P. thornei treated with respective dsRNA were significantly higher than those in the two controls (Fig. 4.5.). Consequently, the relative gene expression calculated using ΔΔCt method with expression of the reference gene for controls rather than individual samples indicated an up-regulation of the target genes (except prp-17) after dsRNA soaking. Whereas assessment of fold changes in gene expression using that of the reference gene of nematodes of individual experiment indicated a down-regulation of expression of the genes, except prp-17.

Fig. 4.5. Ct values of expression of target genes in P. thornei after dsRNA soaking. Each bar represents the mean of three replicates ± standard error. Similar letters in data labels indicate no significant difference in the means (p<0.05).

4.3.2.2. Transcript abundance of putative trans-splicing genes sut-1, sut-2 and sna-2 of P. thornei

Transcript abundance of the three putative trans-splicing-specific genes sut-1, sut-2 and sna-2 were determined using semi-quantitative PCRs with transcripts obtained and assessed at cycles, 25, 30, 35 and 40. At cycle 25, there appeared to be a slight down-regulation of 18S rRNA (Fig. 4.6.) amplified from cDNA of nematodes treated with dsRNA of the three genes.
compared with those for nematodes not treated with dsRNA. For nematodes not soaked with
dsRNA, the three trans-splicing genes amplified at cycle 30 with different band intensities.

Based on the intensities of the PCR bands at cycle 30, the sut-1 gene appears to be up-
regulated whereas sut-2 appears to be down-regulated after the in vitro RNAi compared to the
intensity of the band amplified from nematodes soaked with no dsRNA or dsRNA of gfp. There
was no amplification in sna-2 both after soaking with gene specific dsRNA and dsRNA of gfp
(Fig. 4.6.).

There was a slight down-regulation of sut-1 and sut-2 genes in P. thornei soaked in gfp dsRNA,
when the band intensities at cycle 30 was compared with those obtained for P. thornei not
treated with dsRNA. Less intense bands at cycle 25 indicates that expression of 18S rRNA may
have been affected in nematodes soaked with gfp dsRNA compared to sut-1 and sna-1 treated
nematodes.

Fig. 4.6. Expression of 18S rRNA and putative trans-splicing specific genes in P. thornei after
soaking in dsRNA of gfp, sut-1, sut-2 and sna-2 for 16 hours (the number of PCR cycle after
which the sample was collected is indicated above each sample).
4.3.3. Effect of *in vitro* RNAi of spliceosome genes on reproduction of *P. thornei*

After 16 hours of soaking the mixed stage *P. thornei* in dsRNA, 100 nematodes were transferred onto two or four replicates of mini carrot discs per dsRNA treatment and their reproduction assessed over a 5 week period. There was no significant difference in the mean number of nematodes isolated from mini discs infected with nematodes treated with dsgfp and those soaked with no dsRNA (p<0.05); although there was a 22-24% reduction in the average number of *P. thornei* on mini discs infected with nematodes soaked in dsgfp. There was a 55-99% reduction in the average number of *P. thornei* on mini discs infected with nematodes soaked in dsRNA of putative spliceosome genes, compared to no dsRNA controls of the two independent experiments (Fig. 4.7).

Fig. 4.7. Average numbers of *P. thornei* extracted from carrot mini discs 5 weeks after nematodes previously soaked for 16 hours with dsRNA of nine target genes.
The average numbers of nematodes collected after treatment with dsRNA of all the genes except sna-2 were statistically significantly lower than that of *P. thornei* not treated with dsRNA (*p*<0.05). Twitching behaviour was observed in all *P. thornei* treated with t08a11.2 dsRNA after extraction from carrot mini discs at 5 weeks. Behaviour of *P. thornei* soaked in dsRNA of all the target genes except t08a11.2 was not different from no-dsRNA treated control.

### 4.4. Discussion

Undertaking RNAi *in vitro* provides a means of rapid assessment of the potential importance of a target gene in a nematode, based on general and specific effects of silencing, when compared to appropriate controls. Induction of *in vitro* RNAi of spliceosome genes by soaking resulted in changes in *P. thornei* behaviour after 16 hours treatment with 1 mg/mL dsRNA compared with those not treated with dsRNA. There was lower expression for most target genes directly after soaking, and a 55-99% reduction in the number of nematodes collected from carrot mini discs 5 weeks after soaking treatments. These results clearly show that RNAi of the selected spliceosome genes resulted in a marked reduction in the survival and reproduction of *P. thornei*, and this suggests that these genes may well be good targets for control of *P. thornei via* RNAi using host-induced gene silencing, i.e. when a host plant (crop) is used as the dsRNA source.

RNAi of SL associated sna-2 (SL75p) induced by injecting dsRNA into *C. elegans* was 100% lethal, indicating that it is required for viability (MacMorris *et al.*, 2007). Although RNAi of the same gene in *P. thornei* resulted in paralysis and down-regulation of target mRNA, the percentage reduction in the number of nematodes extracted from mini carrot discs after 5 weeks was 55%, which was relatively less than for other spliceosome genes studied in this chapter.
SL associated genes *sut-1* (SL26p) and *sut-2* are essential to suppress the tau neurotoxicity in *C. elegans* (Kraemer & Schellenberg, 2007; Guthrie et al., 2009). For *P. thornei*, RNAi of putative *sut-1* and *sut-2* genes caused 85-99% reduction in the number of nematodes extracted from mini carrot discs after 5 weeks, suggesting that the genes are essential for viability and reproduction of *P. thornei*. In contrast, RNAi of the *sut-1* of *C. elegans*, had no obvious phenotype at 20°C and *sut-1* mutant strain was fully viable (MacMorris et al., 2007).

RNAi of *prp-17*, *t08a11.2* and *sfa-1* of *C. elegans* results in locomotion variants, embryonic lethality and adult lethality (WormBase). RNAi of the same genes of *P. thornei* also resulted in variations in nematode movement (knot formation, loss of direction) and a significant reduction in nematode reproduction on mini carrot discs 5 weeks after treatment compared to controls. These results suggest that these genes in *P. thornei* may also have vital roles in development as in *C. elegans*. RNAi of the subunits of splicing factor U2AF (*uaf-1*, *uaf-2*) in *C. elegans* resulted in changes in mRNA export from the nucleus (WormBase). For *P. thornei*, RNAi of *uaf-1* and *uaf-2* caused paralysis and slow movement respectively, and an 80-94% reduction in nematode populations on mini carrot discs after 5 weeks. More work is required to understand the exact function of each of the *P. thornei* spliceosome genes in mRNA splicing and their interacting proteins, as has been done for *C. elegans* SL associated genes (MacMorris et al., 2007) and *Trypanosoma brucei* splicing factors Prp-31 and Prp-43 (Liang et al., 2006).

Several factors have been reported to influence the levels of gene silencing, particularly in relation to *in vitro* soaking of nematodes with dsRNA, and these include the type of gene targeted, dsRNA concentration, length of dsRNA, position of dsRNA/siRNA within the target gene, base composition of dsRNA, incubation time, *in vitro* delivery method, method of assessment of silencing, susceptibility of the organism to RNAi and the type of target tissue (Lilley et al., 2012; Tan et al., 2013).
Down-regulation of the non-target genes *sut-2*, and *sna-2* when *P. thornei* was soaked in dsRNA of *gfp*, which is a non-nematode gene, could be due to general stress caused by dsRNA processing, overloading the RNAi machinery or off target effects of siRNA (Tschuch *et al.*, 2008), but recovery of affected nematodes clearly occurred in some cases, as evidenced by the number of nematodes extracted after 5 weeks from carrot mini discs, which was not significantly different from nematodes not treated with dsRNA. Tschuch *et al.*, (2008) have reported changes in gene expression of a set of endogenous genes belonging to a diverse set of cellular pathways, by *gfp* siRNA in mammalian cell lines (HEK293, HeLa, U2OS, EVSAT) using expression microarrays and real-time PCRs. These affected genes included those involved in enzymatic activities, DNA binding, RNA binding, dsRNA binding, signal transduction, membrane proteins, RNA polymerase II transcription factor activity, protein folding, actin binding, cytoskeletal protein binding, ATP dependent helicase activity and translation initiation factor activity. Some sequences of the affected mRNA were similar to the *gfp* siRNA used. Since *gfp*, is a frequently used negative control in RNAi experiments, Tschuch *et al.*, (2008) have suggested the identification of off-targets as false positives and excluding them from the results.

Visual comparison of PCR amplicon intensities using agarose gels and qPCRs showed that expressions of the reference genes *actin* and 18S rRNA were affected by RNAi of the some of the spliceosome genes. This may also be due to off target effects of siRNA or overloading of the RNAi pathway machinery. Bakhetia *et al.* (2007) also reported that there was a slight reduction in transcript abundance of the actin gene (used as positive control) in *H. glycines* J2s from 0 to 7 days post infection (dpi) of soybean plants after RNAi of five pharyngeal gland cell genes, but no further decline by the 21 dpi.
In two independent experiments, the numbers of *P. thornei* extracted from mini carrot discs inoculated with mixed populations after soaking with dsRNA of different target genes (Fig. 4.7.) were significantly different. It must be noted that these experiments were conducted at different times (seasons) of the year with different populations of nematodes, cultured on the same cultivar of carrot but obtained at different times of the year. These variations may in part account for the apparent different growth patterns of the nematodes resulting in the different numbers obtained at the end of the 5-week culture. However, on both occasions the nematodes were extracted and surface sterilised using a similar protocol and each batch of experiments had appropriate controls that ensured that valid conclusions could be drawn from the results obtained.
CHAPTER 5

In planta RNA Interference Analysis of Spliced Leader-Associated Genes of Pratylenchus thornei

5.1. Introduction

Plant-derived RNAi of plant parasitic nematode genes that are vital for survival, is a strategy of interest for nematode management aimed at reducing nematode populations and infectivity of important host plants (Sindhu et al., 2009). Agrobacterium rhizogenes-induced hairy roots provide a convenient system for studying gene functions without going through the long process of producing stable transgenic plants (Yang et al., 2013). A. rhizogenes-induced roots grow rapidly, are genetically stable, lack geotropism and are high-branching on phyto-hormone free medium (Hu & Du, 2006). Hairy roots have significant applications in plant genetic engineering: they have been employed for functional analysis of genes, production of secondary metabolites and production of therapeutic proteins (Hu & Du, 2006). In some cases it is possible to regenerate whole plants from hairy roots (Hu & Du, 2006).

Hairy root systems have been used to study the interactions of plants with parasitic nematodes. Examples include Meloidogyne incognita and Rotylenchulus reniformis and resistant cotton (Wubben et al., 2009), Heterodera glycines and soybean expressing tandem inverted repeats of four H. glycines genes (Klink et al., 2009), M. incognita and tomato expressing dsRNA of two M. incognita genes (Charlton et al., 2010), M. incognita and grapevine expressing hairpin RNA of 16D10 effector gene (Yang et al., 2013) and Pratylenchus penetrans and soybean expressing dsRNA of two locomotion related genes of P. penetrans, pat-10 and unc-87 (Vieira et al., 2015).
Whole carrot discs and calli are often used to maintain in vitro cultures of migratory endoparasitic nematodes such as *P. vulnus* (Moody et al., 1973), *P. thornei* (Tan et al., 2013), *P. agilis*, *P. brachyurus*, *P. scribneri*, *Radopholus citrophilus* and *R. similis* (Reise et al., 1987). *Pratylenchus* spp. can complete their life cycle and induce the same root tip hypertrophy symptoms that are characteristic of the infection in the soil when they are co-cultured on carrot hairy roots (Flores et al., 1999). In an attempt to determine the impact of arbuscular mycorrhizal fungus *Glomus intraradices* on the reproduction of *R. similis*, a major root burrowing nematode infecting various economically important crops, in a dixenic culture system, the life cycles of both the fungus and the nematode were completed in the presence of each other with Ri T-DNA transformed carrot roots as host (Elsen et al., 2001).

The objective of this chapter was to use *A. rhizogenes* K599 to generate carrot hairy roots harbouring hairpin RNAs (hpRNAs) corresponding to sequences of the spliced leader-associated genes *sut-1*, *sut-2* and *sna-2* of *P. thornei*. These hairy roots were then used to assess the effect of knocking down these important genes involved in the effective trans-splicing of most *P. thornei* genes on reproduction of the nematode. This chapter provides first evidence of in planta RNAi analysis of spliced leader-associated genes of *P. thornei*.

### 5.2. Materials and methods

#### 5.2.1. Generation of hairpin vectors for *sut-1*, *sut-2* and *sna-2* genes of *P. thornei*

The genes *sut-1*, *sut-2* and *sna-2* were previously ligated to the vector pDoubler. The partial sense and antisense sequences of the genes were digested and ligated sequentially to the vector pCLEaver-NosA (Fig. 2.5.) to obtain the hairpin expression cassettes. The hairpin cassettes were then cloned into the binary vector pART27 (Fig. 2.6.) for plant transformations.
5.2.1.1. Ligation of DNA sequences to vectors

Plasmid DNA - pDoubler carrying DNA fragments of sut-1, sut-2 and sna-2, was extracted from *E. coli* JM109 cultures and each was digested with *KpnI* and *XhoI* and then with *BamHI* and *XbaI* enzymes to obtain the 5’-3’ and 3’-5’ fragments respectively. The fragments were ligated sequentially to pCLEaver-NosA vector using T4 DNA ligase. The ligated fragments were used to transform *E. coli* JM109 and the transformants selected and screened by PCR (sections 2.9.1. and 2.9.3.). Ligation of the sense and antisense DNA fragments to pCLEaver-NosA was confirmed by PCR respectively using the primer pairs SIntron and S35S and ASIntron and ASNosA as described section 2.9.3. To assess successful ligations of the DNA fragments to the vector, the resulting plasmid DNAs were digested with *KpnI/XhoI* and *BamHI/XbaI* in separate reactions to release the respective inserts from the vector.

5.2.1.2. Ligation of hairpin cassettes of sut-1, sut-2 and sna-2 genes to binary vector pART27

Plasmids confirmed by digestion to have both the sense and antisense fragments of target genes ligated to pCLEaver-NosA were digested with *NotI* to release the hairpin cassette (35S promoter, sense sequence, intron, antisense sequence and terminator); this was done so the fragment could be ligated to *NotI*-linearised and dephosphorylated binary vector pART27. About 20 ng of hairpin cassette DNA for each target gene was ligated to 22 ng of pART27 using Quick T4 DNA ligase (NEB). The reactions were incubated at room temperature for 5 minutes and chilled on ice. About 50 µL of competent *E. coli* JM109 were transformed with 5 µL each of the ligation reactions. Transformed colonies were screened by PCR (Fig. 5.1) with primer pair SP6 and 35SART-R as described in section 2.9.3.
Fig. 5.1. Agarose gel photographs showing amplicons obtained after colony PCR with SP6 and 35SART-R primers for (a) pAsut-1 and pAsna-2 (b) pAsut-2.

Successful ligation of hairpin cassettes to pART27 was confirmed by two double digestion reactions as described above using the enzyme pair KpnI/XhoI and the other with BamHI/XbaI, as well as by capillary sequencing of the resulting plasmids to confirm the orientation of inserts. The sense strand of each gene was sequenced and analysed using 3.2 pmol of SIntron and ASIntron primers in two separate 1/16 sequencing reactions as described in section 2.6.

5.2.2. Transformation of Agrobacterium rhizogenes K599 with pART27 carrying hairpin cassettes of the P. thornei genes sut-1, sut-2 and sna-2

Ten microliters of A. rhizogenes K599 chemically competent cells (calcium chloride treated) was transformed with approximately 1 µg of plasmid DNA (pART27 carrying the hairpin cassettes) as described in section 2.9.2. A. rhizogenes K599 colonies were screened by PCR using 5 pmol of SP6 and 35SART-R primers. Bacterial cultures for colony screening were prepared by suspending cells from a bacterial colony in 15 µL of nuclease free water in PCR tubes. Five microliters of each cell suspension was transferred into each PCR tube, heated at
Template DNA of bacterial colonies transformed with the plasmids produced amplicon of 760 bp, similar to those in Fig. 5.2.1.2.

5.2.3. *Agrobacterium rhizogenes* K599-mediated transformation of carrots with hairpin vectors for *sut-1*, *sut-2* and *sna-2* of *P. thornei*

*A. rhizogenes* K599 carrying pART27 with hairpin cassettes of *sut-1*, *sut-2* and *sna-2* genes were cultured in 10 mL LB broth supplemented with 25 mg/L rifampicin and 100 mg/L spectinomycin for 18 hours at 28°C in the dark on a shaker at 225 rpm: this culture was used to generate hairy roots from carrot discs. *A. rhizogenes* K599 transformed with pART27 without a nematode gene (null vector) and with hairpin cassette carrying 500 bp *gfp* gene (made by Dr. John Fosu-Nyarko) were used as controls for the transformations. Pelleted bacterial suspensions were used for transformations.

Carrot taproots were used as the source tissue to generate hairy roots. Freshly obtained whole carrots (Nantes variety Stefano) were surface sterilised as described in section 2.1.1., peeled, cut transversely into 0.3 mm thick discs and placed on 0.4% water agar in 9 cm diameter tissue culture dishes with the apical side facing upwards. The apical sides of 3-5 discs in one dish were then infected with *A. rhizogenes* K599 by applying cells along the cambial ring on the disc with a pipette tip and spread with an inoculating loop. After inoculation, the plates were sealed with parafilm and kept in the dark, at 25±2°C. Uninfected carrot discs and those infected with unmodified *A. rhizogenes* K599 served as controls.

Hairy roots were excised and transferred to semi solid Murashige and Skoog (MS) medium [4.4g of MS with Gamborg vitamins per liter (PhytoTechnology Laboratories), 30 g sucrose and 8 g Daishin agar Brunschwig Chemie, Amsterdam, pH 5.6-5.8] supplemented with 150 mg/L cefotaxime and with or without 25 mg/L kanamycin in petri dishes. Cefotaxime was added to
the medium to inhibit the growth of *A. rhizogenes* K599 whereas kanamycin was added as a selectable agent for hairy root growth.

Roots induced by *A. rhizogenes* K599 without pART27 were transferred to MS medium supplemented with 150 mg/L cefotaxime and with or without 25 mg/L kanamycin. Roots induced by *A. rhizogenes* K599 transformed with pART27 were transferred to MS medium supplemented with both 150 mg/L cefotaxime and 25 mg/L kanamycin. Four to six roots were placed in one culture dish, sealed with parafilm and kept in the dark at 25±2°C for root growth. Each root was considered a different event. The roots were subcultured every 2 weeks by transferring about a 1 cm piece including the root tip onto similar medium in new culture dishes.

5.2.4. Infection of carrot hairy roots with *P. thornei*

For nematode assays, carrot hairy roots (approximately 1 cm long) induced by *A. rhizogenes* K599 with and without pART27 were aseptically transferred onto MS medium (without any antibiotics) in 9 cm diameter dishes-one root (an event) per dish. There were 5 events for each control and gene and three replicates for each event. Petri dishes were sealed with parafilm and kept in the dark for 3 weeks at 25±2°C for root extension.

Carrot hairy roots were infected with mixed stages of *P. thornei*: the nematodes were harvested from 5-8 weeks old infected carrot discs and surface sterilised as described in sections 2.1.1.1 and 2.1.1.2. Each replicate hairy root was infected with 500 nematodes. On each plate 100 nematodes were placed at five different spots about 1 cm away from root tips. At the point of infection the medium was disturbed using the pipette tip to facilitate nematode movement. After infection, plates were covered with lids, sealed with parafilm and kept in the dark at 25±2°C.
5.2.5. Analysis after infection

The hairy roots were observed using a dissecting microscope 1 day, 2 days, 1 week and thereafter weekly until 45 days after inoculation. After this period, each replicate root was cut into 1-2 cm pieces and placed in 10 mL of sterile water in 9 cm petri dishes to extract *P. thornei*. The plates were gently shaken occasionally and after 2 days the water with nematodes was collected in 10 or 15 mL centrifuge tubes. The nematodes in water were counted under a dissecting microscope. The hairy roots were then removed from the petri dishes, observed under the microscope and the number of nematodes attached to the roots and those on the medium (collected with 5 mL of sterile water) were also counted.

The root segments of each replicate were separately stained with acid-fuchsin (Bybd et al., 1983) and observed under a dissecting microscope for the presence of eggs and any nematodes remaining in the root tissue. Briefly, the root segments were immersed in 1.5% sodium hypochlorite for 5 minutes with occasional agitation. The roots were then washed in water for 15 minutes and then immersed in 10-20 mL of acid-fuchsin stain (1 mL of acid-fuchsin stain diluted in 30 mL of water) and boiled for 1 minute. After cooling to room temperature, the roots were washed in water to remove excess stain. Roots were mounted in water on a glass microscope slide covered with a cover slip for observation under the microscope. Since the root densities of replicates in an event were not similar, after observations, the roots were dried in an oven at 55±2°C for 24 hours, the dry weights were measured and the level of nematode infection expressed as the number per dry root weight.

5.3. Results

5.3.1. Carrot hairy root growth

Hairy roots emerged from *A. rhizogenes* K599-infected carrots 13 days after infection (Fig. 5.2). About 24 hrs after emerged roots were transferred to MS medium supplemented with 150
mg/L cefotaxime and with or without 25 mg/L kanamycin, there was up to 1cm extension. None of the roots induced by unmodified \textit{A. rhizogenes} K599 extended further in the presence of kanamycin (Fig. 5.3c). All roots cultured on MS medium without kanamycin extended further and branched well (Fig. 5.3b). From roots induced by \textit{A. rhizogenes} K599 with null vector and \textit{gfp}, 90% extended further on MS medium with kanamycin (Fig. 5.3a). Generally 61%, 57% and 18% of roots induced by \textit{A. rhizogenes} K599 with pAsut-1, pAsut-2 and pAsna-2 respectively grew further and branched well on MS medium with kanamycin (Fig. 5.3d).

\textbf{Fig. 5.2.} Carrot discs two weeks after infection with (a) LB broth only (b) \textit{A. rhizogenes} K599 (c) \textit{A. rhizogenes} K599 with pART27-null (d) \textit{A. rhizogenes} K599 with pART27-\textit{gfp} (e) \textit{A. rhizogenes} K599 with pART27-\textit{sut}-1 (f) \textit{A. rhizogenes} K599 with pART27-\textit{sut}-2 (g) \textit{A. rhizogenes} K599 with pART27-\textit{sna}-2. Root development was present in b-g.
Fig. 5.3. (a) Hairy roots growing on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin, 5 days after transfer from carrot discs inoculated with A. rhizogenes K599 with pART27-null. Some roots elongated while others did not. (b) Hairy roots induced by unmodified A. rhizogenes K599, growing on MS medium with 150 mg/L cefotaxime, after 3 weeks of transfer from carrot discs. (c) Hairy roots induced by unmodified A. rhizogenes K599, not growing on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin, after 3 weeks of transfer from carrot discs. (d) Hairy roots induced by A. rhizogenes K599 with pART27-sut-2, after 3 weeks of transfer from carrot discs. Not all roots grew on MS medium with 150 mg/L cefotaxime and 25 mg/L kanamycin.

There were differences in root growth and vigour in replicates of each event, resulting in variations in root dry weight (2.8-57.3 mg, Fig. 5.4). There were also differences in root colour notably white or brown, even for roots of similar ages (Fig. 5.5).
Fig. 5.4. Average dry weights of carrot hairy roots: (um)-not transformed with pART27, and those transformed with null vector (pANull), *gfp* (pAgfp), *sut-1* (pAsut-1), *sut-2* (pAsut-2) and *sna-2* (pAsna-2). Each bar represents average of five events ± standard error.

Fig. 5.5. Representative carrot hairy root events and replicates used in this study. Event number is preceded by the gene and vector name.

5.3.2. Nematode behaviour after infection of carrot hairy roots

Within 24 hours after inoculation of the controls and transgenic hairy root events, the nematodes were active and were observed to be moving towards the roots. Three days after inoculation the nematodes could be seen in aggregates parallel to the roots or actively moving
on roots or in the medium. Some individual nematodes lay dormant parallel to and on the outside of hairy roots for long periods of time. Stylet thrusts could be seen on some roots from actively moving nematodes. One week after inoculation most nematodes were inactive in the medium or in close proximity to hairy roots (Fig. 5.6).

Fig. 5.6. *P. thornei* that have moved and staying close to carrot hairy roots, 1 week after infection (x40).

**5.3.3. Nematode survival/reproduction on carrot hairy roots**

Although each replicate root was infected with 500 nematodes, the numbers of nematodes extracted from 45 days after inoculation was less than 500 except a replicate root induced by unmodified *A. rhizogenes* K599 (um-9) from which 603 nematodes were extracted. No eggs were observed in any of the roots during the six-week period and no nematode or egg was observed in roots after acid-fuchsin staining.

There was a large variation in the number of nematodes extracted from carrot hairy root events within a control/transgene. The numbers of nematodes extracted from eight control events (um-1, um-3, um-13, pANull-2, pANull-7, pANull-10, pAgfp-9, pAgfp-23) and each event
of hairy roots transformed with hairpins of sut-1, sut-2 and sna-2, except pAsut-1-23 and pAsna-2-16 were statistically lower than those of roots induced by unmodified A. rhizogenes K599 (um-9) (p<0.05, Fig. 5.7a).

The average numbers of nematodes extracted per gram of dry root weight of six control events (um-13, pANull-2, pA-Null-4, pAgfp-9, pAgfp-21, pAgfp-22) and each event transformed with hairpins of sut-1, sut-2 and sna-2, except event pAsut-2-21, were statistically lower than that of roots induced by unmodified A. rhizogenes K599 (um-9) (p<0.05, Fig. 5.7b).

Collectively, the number of P. thornei extracted from hairy roots transformed with sut-1, sut-2 and sna-2 were statistically lower (45-64% reduction) than those on hairy roots generated by unmodified A. rhizogenes (p<0.05, Fig. 5.8a). However, when the average numbers of nematodes per gram root were considered (Fig. 5.8b), the figure was also statistically significantly lower (47-61% reduction) in the control hairy roots transformed with null vector and gfp (p<0.05). The number of P. thornei extracted per gram root transformed with SL associated genes were reduced by 70-87% (p<0.05) compared to those on hairy roots generated by unmodified A. rhizogenes K599.
Fig. 5.7. (a) Mean number of nematodes and (b) mean number of nematodes/gram dry root weight extracted from each carrot hairy root events. Each bar represents the mean of three replicates ± standard error. Bars with “Δ” were statistically different (p<0.05) from um-9.
Fig. 5.8. (a) Average number of nematodes extracted from roots and medium and (b) average number of nematodes per gram of dry root weight of hairy roots generated with unmodified *A. rhizogenes* (um), transformed with null vector (pANull), gfp (pAgfp), sut-1 (pAsut-1), sut-2 (pAsut-2) and sna-2 (pAsna-2). Each bar represents the average of 5 events ± standard error. Bars with “Δ” were significantly different from the negative control “um”.

In general, the number of nematodes extracted from the medium was higher than the number of nematodes extracted from the roots (Fig. 5.9).
Fig. 5.9. Number of nematodes extracted from medium (blue) and roots (red) per gram of dry root weight. Each bar represents the average of five events ± standard error.

5.4. Discussion

In planta expression of hairpin RNA of nematode genes has been exploited as a control strategy for plant parasitic nematodes (Kyndt et al., 2013; Yang et al., 2013). In this chapter, carrot hairy roots harbouring hairpin RNAs corresponding to partial sequences of the SL-associated genes sut-1, sut-2 and sna-2 of *P. thornei* were generated using *A. rhizogenes* K599 and were used to assess the importance of the genes in the reproduction of the nematode.

Variation in hairy root morphology, growth and vigour, as observed in the carrot hairy roots generated in this research, has been reported in carrots (Guivarc’h et al., 1999), cotton (Wubben et al., 2009) and grapes (Yang et al., 2013). Several factors can account for this variation. The levels of auxins produced by the rol genes from the hairy root inducing plasmid of *A. rhizogenes* could vary between different hairy root lines and this could result in hairy roots with differing vigour and morphology (Nilsson & Olsson, 1997; Yang et al., 2013). Also the site of T-DNA integration into the host genome and T-DNA copy number can affect host gene expression (Nilsson & Olsson, 1997; Alonso et al., 2003).
A wide variation in the number of nematodes extracted from carrot hairy root events within a control/transgene was observed in the nematode infection assays. Similar results, for example root fresh weight (203.6 - 550.0 mg), number of *Meloidogyne incognita* eggs per hairy root (24.0 - 626.8), and number of eggs per gram root (54.8 - 3209.0) have been reported by Yang *et al.*, (2013) when transgenic grape hairy roots were infected with J2s, in a study on the efficacy of 16D10 siRNAs in inhibiting root knot nematode infection. Also for T3 lines of transgenic *Arabidopsis thaliana* expressing hairpin RNA against three *H. schachtii* genes (U2AF, H’ATPase, Ubiquitin) under the control of cauliflower mosaic virus 35S promoter, the level of infection was variable between lines of the same construct and between independently repeated experiments (Kyndt *et al.*, 2013). In the same experiment, they detected a variable expression level of a target gene (U2AF) construct between and within lines, and a decline in gene expression over subsequent generations, an effect that was shown to be correlated with transcriptional gene silencing of the hairpin construct through promoter methylation (Kyndt *et al.*, 2013).

Despite the variation in the number of nematodes on different carrot hairy root events transformed with hairpin RNA constructs of *P. thornei* SL-associated genes, there was a significant reduction (70-87% reduction) compared to untransformed hairy roots. This observation should be studied further with analysis of target mRNA in *P. thornei* fed on hairy roots and expression levels of hairpin RNA constructs in transgenic events.

The results obtained from *in planta* experiments done in this chapter were similar to those obtained when the same SL associated *P. thornei* genes were targeted by soaking *in vitro* (chapter 4), in which there was a significant (55-99%) reduction in nematode numbers compared to the respective controls. In contrast, Vieira *et al.*, (2015) have discussed that *in planta* delivery of dsRNA of *unc-87* and *pat-10* of *P. penetrans* was less effective than targeting
the same genes by dsRNA soaking (Joseph et al., 2012; Tan et al., 2013) and suggested that the
differences could be due to silencing efficiency using dsRNA delivered by the plant versus
soaking (Vieira et al., 2015).

Furthermore, Vieira et al., (2015) also argued that as root lesion nematodes are migratory
nematodes, the continuous exposure of nematodes to dsRNA delivered by the plant would be
reduced during the periods that the nematodes leave the roots, and this factor may allow for
some degree of recovery (Vieira et al., 2015). This possible reduced exposure to dsRNA could
also explain the variation in numbers of P. thornei extracted from carrot hairy root events in
this chapter.

A possibility that was not followed in this work is the possibility of tracking nematodes
movements in or out of roots using fluorescence. In this respect, Goto et al., (2010) have
shown that 0.001% fluorescein diacetate (FDA) can be applied to label individual Meloidogyne
hapla, Meloidogyne javanica and Pratylenchus penetrans nematodes fluorescently for direct
non-destructive observation of their movement inside plant roots.

The results obtained from the preliminary experiment done in this chapter, which is the first
evidence of in planta RNAi analysis of SL-associated genes of P. thornei, provide evidence of
genes that can confer some resistance to P. thornei by reducing the total number of
nematodes associated with carrot hairy roots.
CHAPTER 6

*In vitro* and *In planta* RNA Interference Analysis of Spliceosome Genes of the Beet Cyst Nematode, *Heterodera schachtii*

6.1. Introduction

The beet cyst nematode (*Heterodera schachtii* Schm.) is a major parasite of sugar beet in many sugar beet growing regions. Most susceptible hosts for this nematode are in the Chenopodiaceae (e.g. beets, spinach) and Cruciferae (e.g. cabbage, broccoli, cauliflower, brussel sprouts, turnip, radish, mustard) families (Amiri *et al*., 2002). *H. schachtii* severely damages roots, especially during summer. The life cycle of cyst nematodes is described in section 1.1.3.2. White cysts about the size of a pin head attached to the roots mature to produce lemon-shaped, reddish-brown cysts (Beet cyst nematode in vegetables, 2016).

The transcriptome of the pre-parasitic J2 stage of *H. schachtii* has been sequenced and annotated using sequences from both free-living and parasitic nematodes (Fosu-Nyarko *et al*., 2016). The transcriptome included genes involved in general metabolism, root entry, migration through host tissues, evasion or modification of host defenses, effectors thought to be required for induction of syncytial cells, spliceosome genes and RNAi pathway genes.
Annotated transcripts were more similar to those of *H. glycines* sequences than sequences of *Globodera pallida* and root knot nematodes (*Meloidogyne* spp.) (Fosu-Nyarko et al., 2016).

Amenability of the soybean cyst nematode *H. glycines* and the potato cyst nematode *Globodera pallida* to RNAi *in vitro* in the presence of octopamine has previously been demonstrated by Urwin et al., (2002) and Alkharouf et al., (2007). Reduced infectivity has been reported on *Arabidopsis thaliana* and soybean by host delivered RNAi for essential genes both for *H. glycines* (Steeves et al., 2006; Klink et al., 2009; Li et al., 2010) and *H. schachtii* (Sindhu et al., 2009; Hamamouch et al., 2012; Kyndt et al., 2013). As an example, *in planta* RNAi of four parasitism genes of *H. schachtii* has been achieved using *A. thaliana*, in which the nematode target mRNA abundance was reduced, and the number of mature nematode females feeding on transgenic plant roots was also reduced between 23% to 64% compared to controls, although complete resistance was not observed (Sindhu et al., 2009).

In this chapter two SL-associated genes (*sut*-*1, sna*-*1*) and seven genes involved in both trans and cis-splicing (*prp*-4, *prp*-8, *prp*-17a, *prp*-17b, *prp*-21, *uaf*-1, *uaf*-2) of *H. schachtii* were first studied by inducing RNAi *in vitro* for 16 hours using 1 mg/mL dsRNA in M9 soaking buffer with octopamine, spermidine and gelatine. Gene silencing effects on the behaviour, target mRNA levels and reproduction of the nematode were then assessed. After this, *A. thaliana* T2 plants transformed with hairpin expression cassettes of the genes *sut*-1, *sna*-1, *prp*-4, *prp*-8, *prp*-17a, *prp*-17b, *uaf*-2 and *cpf*-1 were infected with *H. schachtii* J2s and reproduction in the first and second generations of the nematode were assessed.
6.2. Materials and methods

6.2.1. DNA template and *in vitro* transcription

DNA of each of the target genes was ligated to pDoubler using *KpnI* and *XhoI*. From plasmids obtained, a linear fragment of the cloned gene flanked by T7 promoter regions were digested with *EcoRI* or *NotI* and the cleaned template used for *in vitro* transcription. DsRNA was synthesised using the T7 high yield RNA synthesis kit (New England Biolabs Inc.), according to the manufacturer’s instructions. After transcription and DNaseI treatment, dsRNA was cleaned, precipitated as described in section 2.2.1.1 and the quality checked on 2% agarose gel (Fig. 6.1.).

![Agarose gel photograph showing dsRNA of gfp, P. thornei and H. schachtii genes (P. thornei genes- sut-1, sut-2, sna-2; H. schachtii genes – sut-1, sna-1, prp-4, prp-8, prp-17a, prp-17b, prp-21, uaf-1, uaf-2).](image)

**Fig. 6.1.** Agarose gel photograph showing dsRNA of *gfp*, *P. thornei* and *H. schachtii* genes (*P. thornei* genes- *sut*-1, *sut*-2, *sna*-2; *H. schachtii* genes – *sut*-1, *sna*-1, *prp*-4, *prp*-8, *prp*-17a, *prp*-17b, *prp*-21, *uaf*-1, *uaf*-2).

6.2.2. *In vitro* RNAi analysis of *H. schachtii* genes by soaking

Sterile second stage juveniles (J2s) of *H. schachtii* were obtained from cysts maintained *in vitro* on white mustard roots. Cysts were collected with sterile forceps with fine tips, crushed and hatched in 3.14 mM zinc chloride in a 50 mL sterile falcon tube. Hatched J2s were then
collected with a 38 µm sieve into a sterile 50 mL falcon tube and the number estimated. For *in vitro* RNAi, 2000 J2s were soaked with 1 mg/mL dsRNA of each of the *H. schachtii* genes in M9 buffer containing 50 mM octopamine hydrochloride, 3 mM spermidine trihydrochloride and 0.05% gelatine in 1.5 mL Eppendorf tubes, and incubated at 25±2°C in darkness for 16 hours. There were two controls for the experiments: one set up had no dsRNA, and for the other 1 mg/mL dsRNA of *gfp* was used. Uptake of the soaking medium was confirmed by microscopic observation of 1 mg/mL of FITC ingested by *H. schachtii* J2s soaked in a similar medium with no dsRNA.

**6.2.2.1. Analysis of gene silencing**

After 16 hours soaking, the J2 nematodes were washed five times with sterile water. Two hundred nematodes were used to observe RNAi phenotypes using an Olympus BX51 photomicroscope. Uptake of the soaking medium was confirmed by observing *H. schachtii* soaked with FITC using the FITC filter of the same microscope. Possible gene knockdown was assessed in the nematodes by PCR. To do this, 1000 of the soaked J2s were washed and frozen in liquid nitrogen after which total RNA was extracted using ARCTURUS PicoPure RNA Isolation Kit (Life Technologies) as described in section 2.2.2. Total RNA (approximately 98 ng) was reverse-transcribed using MultiScribe Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions and semi quantitative PCRs performed using My Taq Red DNA polymerase (Bioline) at 55°C with approximately 400 ng of cDNA and 10 pmol of primers (Table 6.1.). Transcript abundance of target genes and actin was assessed at 25, 30, 35 and 40 cycles.

**Table 6.1.** Primers used to quantify mRNA of actin and spliceosome genes in *H. schachtii* J2s after 16 hours of *in vitro* RNAi.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>F-CGTGACCTCCTGACCTACCT&lt;br&gt;R-GCACAACCTTCTCCCTTGATGT</td>
</tr>
<tr>
<td>sut-1</td>
<td>F-ATCAATTTCGGTTCGAGCTA&lt;br&gt;R-TAGCTCGCAACCGAAATTGT</td>
</tr>
<tr>
<td>sna-1</td>
<td>F-TGTGATGGGACAATTGGAAGGAACTG&lt;br&gt;R-CGTGTAAGAAACGGGTGGAAGTTG</td>
</tr>
<tr>
<td>prp-17a</td>
<td>F-TCG AGA TCT ATCCGACATACACTGGACA&lt;br&gt;R-TAC AGG CCT TTGTACATCGACGATTGGGA</td>
</tr>
<tr>
<td>prp-17b</td>
<td>F-TCG AGA TCT CAAATCGAATTGTCCTTTTCCA&lt;br&gt;R-TAC AGG CCT CATTGGATTACATTGTCCTCCATC</td>
</tr>
<tr>
<td>uaf-1</td>
<td>F-TCG AGA TCT GGAACATTCCATTGCATTCAATG&lt;br&gt;R-TAC AGG CCT CATTGGAAATGTCATCCCCGGAT</td>
</tr>
<tr>
<td>uaf-2</td>
<td>F-TCG AGA TCT TATGATGCGACCGATGTCA&lt;br&gt;R-TAC AGG CCT TAGCGGCACCACCGATTGGTGTA</td>
</tr>
</tbody>
</table>

6.2.2.2. Longer term effect of *in vitro* RNAi on *H. schachtii*

The effect of RNAi of each of the genes on survival and reproduction of *H. schachtii* was assessed for up to 5 weeks after infection. Eight replicates of 3 week old *A. thaliana* wild type plants growing on modified Knop medium in square tissue culture plates (10 cm x 10 cm) were each infected with 100 J2s 16 hr after soaking in dsRNA and washing. Four plants per plate were infected, the roots partially covered with a paper strip to reduce light and facilitate infection, and kept at 24±2°C at 16/8 light/dark cycle. The plants were observed weekly for root and cyst development. Between the third and fifth weeks after infection, the cysts were observed weekly under a dissecting microscope and the number of developing cysts counted.

6.2.3. *In planta* RNAi analysis of *H. schachtii* genes

6.2.3.1. Selection of *A. thaliana* T2 seeds

*In planta* RNAi of *H. schachtii* orthologues of seven splicing genes (*sut-1, sna-1, prp-4, prp-8, prp-17, uaf-2, cpf-1*) were assessed using transgenic *A. thaliana* plants. Second generation (T2) seeds of *A. thaliana* expressing hairpins of the target genes were generated as described by Fosu-Nyarko and Jones (2015b). The seeds were selected on MS medium supplemented with either 50 mg/L kanamycin, when the plants were transformed with the binary vector pART27,
or 5 mg/L ammonium glufosinate, when floral dip transformations were done with the binary vector pBART containing the bar gene (Fosu-Nyarko, J., unpublished). Wild type *A. thaliana* and *A. thaliana* transformed with binary vectors without a nematode gene or hairpin were used as negative controls during plant infection. About 100 µL packed volume of seeds were sterilised as described in section 2.1.2. Transgenic seeds were spread on selective medium with sterile 0.4% water agar mixed with appropriate antibiotics and the plates sealed with parafilm and kept at 24±2°C at 16/8 light/dark.

### 6.2.3.2. Infection of *A. thaliana* T2 seedlings with *H. schachtii* J2s

Nine to 10 days after antibiotic selection, healthy transformed T2 seedlings were transferred to modified Knop medium in square tissue culture plates. It took two weeks to properly select transgenic seeds transformed with the binary vector pBART which contained a hairpin of the second gene fragment of *prp-17* (*prp-17b*); the T2 seeds were hence selected with 5 mg/L ammonium glufosinate. The plants were kept on Knop medium for another two weeks after which 8-12 replicates for each transgenic event and controls, four plants per plate, were infected with 100 freshly-hatched *H. schachtii* J2s. The plates were sealed with parafilm, the roots partially covered with a paper strip to facilitate infection and kept at 24±2°C at 16/8 light/dark. Cyst development on *A. thaliana* roots was observed weekly. The number of cysts developed on each root was counted from the third through to the fifth week after infection under a dissecting microscope.

### 6.2.3.3. Analysis of RNAi in second generation J2s obtained from females cultured on *A. thaliana* T2 seedlings

About 10-15 weeks after infection in culture, when the cysts had turned brown, J2s that hatched from the eggs were used to infect wild type *A. thaliana* to study the effect of *in planta* RNAi on the progeny that hatched from nematodes that had fed on transgenic *A. thaliana*. The cysts that developed on roots of all replicate plants of each control and the T2 transgenic
events were collected with sterile forceps, crushed and the eggs hatched in 3.14 mM zinc chloride in 1.5 mL sterile eppendorf tubes kept in dark at 24±2°C. After 3 days eggs and J2s were counted and the number of eggs per cyst and percentage of eggs that had hatched per cyst were calculated.

Infectivity of the second generation J2s was assessed by inoculating 1-5 replicates of wild type A. thaliana plants, each with 100 J2s in vitro, the roots covered and the plates kept at 24±2°C at 16/8 light/dark. Cyst development on each root was observed weekly and the number counted from the third through to the fifth week after infection under a dissecting microscope.

6.3. Results

6.3.1. Behaviour of H. schachtii J2s after 16 hours of RNAi in vitro

Uptake of the soaking medium by J2s was evident since FITC was clearly visible in the stylet of most nematodes soaked with the dye (Fig.6.2.a). Soaking in dsRNA of five of the nematode genes, sut-1, sna-1, prp-21, uaf-1 and uaf-2, did not seem to affect the behaviour of the nematodes as there was no visible difference in movement from the J2s soaked without dsRNA (Fig. 6.2.b) or with dsRNA of gfp; almost all the nematodes were alive and appeared to exhibit normal sinusoidal movement. On the other hand, all the J2s soaked in dsRNA of prp-4 and prp-17b appeared paralysed (Fig. 6.2.c). DsRNA of prp-8 and prp-17a affected a small percentage of the nematodes; 90% appeared actively moving while about 10% were paralysed (Fig. 6.2.d).
6.3.2. Effect of *in vitro* RNAi on target mRNA levels of *H. schachtii* J2s

According to the PCR results, less intense bands at cycle 25 suggested that the reference gene actin was down-regulated in all the dsRNA treated J2s compared with J2s not treated with dsRNA (Fig. 6.3.). In contrast, the putative SL-associated gene fragments *sut*-1 and *sna*-1 appeared to be up-regulated when the band intensities at cycle 30 were compared with those obtained for J2s not treated with dsRNA. It appeared to be the same for *prp-17a*, *prp-17b* and *uaf*-1 genes when the band intensities at cycle 25 were compared. No difference could be observed visually in band intensities obtained for *uaf*-2 at cycle 30 with reference to that obtained for J2s not treated with dsRNA (Fig. 6.3.).

In J2s soaked in *gfp* dsRNA, there was a slight down-regulation of *sut*-1 when the band intensities at cycle 35 were compared with those obtained for J2s not treated with dsRNA. Apparently *prp-17b* and *uaf*-2 were also down-regulated in *gfp* dsRNA treated J2s according to band intensities at cycle 30. Seemingly *sna*-1, *prp-17a* and *uaf*-1 were not amplified in J2s soaked in *gfp* dsRNA (Fig. 6.3.).
Fig. 6.3. Expression of actin, sut-1, sna-1, prp-17a, prp-17b, uaf-1 and uaf-2 genes in *H. schachtii J2s* after 16 hours of RNAi treatment *in vitro*. The number of PCR cycle after which the sample was collected is indicated above each sample.
6.3.3. Longer term effects of *in vitro* RNAi on reproduction of *H. schachtii*

The *H. schachtii* J2s previously soaked in dsRNA for 16 hours were used to inoculate wild type *A. thaliana* to assess if possible knockdown of the target genes persisted and affected reproduction of the nematodes. About 48 hours after inoculation the nematodes were observed under a dissecting microscope: nearly all treated nematodes except those soaked in dsRNA of *prp*-4 and *prp*-17b had moved from the infection site towards the roots.

Five weeks after inoculation, all the cysts that had formed were white and appeared full of eggs under a dissecting microscope (Fig.6.4.a-e) with the exception of the cysts developed from nematodes previously soaked in dsRNA of *uaf*-1, which appeared empty (Fig.6.4.f). Male nematodes could also be observed close to some cysts, except for J2s previously soaked with dsRNA of *prp*-8 and *uaf*-1. All the cysts, except for the ones developed from *uaf*-1-treated nematodes, turned brown within 5-8 weeks.

![Fig. 6.4. *H. schachtii* cysts (x40) developing on wild type *A. thaliana* roots after 5 weeks, from J2s previously soaked in dsRNA for 16 hours. Male nematodes are indicated by the arrows.](image)
There was a wide variation in the number of cysts developed on replicate *A. thaliana* roots (0-17). For plants infected with J2s soaked in a medium without dsRNA or treated with *sna-1* dsRNA, at least one cyst developed on each root. Whereas for plants infected with nematodes treated with dsRNA of *gfp, sut-1, prp-4, prp-8, prp-17a, prp-17b, prp-21, uaf-1* or *uaf-2*, there were some roots on which no cyst developed.

There was a 9-81% reduction in the numbers of cysts developed on *A. thaliana* roots from J2s treated with dsRNA of seven of the nine putative spliceosome genes (except *uaf-2* and *sna-1*) compared to J2s not treated with dsRNA (Fig.6.5.). However, the average numbers of cysts developed (mean of eight replicates) after treatment with dsRNA of *sut-1, prp-21, prp-17a* and also *gfp, uaf-2, sna-1* were not statistically different (p<0.05) from that of the control nematodes soaked without dsRNA. The averages were statistically lower for nematodes treated with dsRNA of *prp-4, prp-8, prp-17b* and *uaf-1* (Fig.6.6.).

![Fig. 6.5. Total number of cysts (blue) and males (red) that developed on eight wild type *A. thaliana* roots in 5 weeks from J2s previously soaked in dsRNA for 16 hours.](image-url)
Fig. 6.6. Average number of cysts (blue) and males (red) that developed per wild type *A. thaliana* root, in 5 weeks, from J2s previously soaked in dsRNA for 16 hours.

6.3.4. *In planta* RNAi analysis of *H. schachtii* feeding on T2 transgenic *A. thaliana*

6.3.4.1. Number of *H. schachtii* cysts developed on transgenic *A. thaliana* T2 roots

No phenotypic difference was observed during *A. thaliana* T2 seed selection and growth of the selected replicates of any event. There was a wide variation in the number of cysts developed on replicate plants of any event (0-19). The maximum number of cysts developed on a replicate plant was 19, which was on a replicate of *sut*-1 transgenic event 4.

The numbers of cysts developed on *A. thaliana* wild type and on vector only transformed plants (Kakan) were not statistically different (p<0.05) in the four experiments. There was a significant reduction (73-99%) (p<0.05) in the number of cysts developed on *A. thaliana* transformed with the SL specific gene *sut*-1 events 1, 5 and 6 compared to cysts on wild type and Kakan *A. thaliana* plants. However, the number of cysts developed on events, 2, 3 and 4 were not statistically different from the controls (Fig. 6.7.a). There was 3-99% reduction in the number of cysts developed on eight events of *sna*-1 (SL specific gene) *A. thaliana* (Fig. 6.7.a), except for event 2. From those eight events, the average of cysts on events 3, 5, 7, 8 and 9 were statistically lower than those on control plants (p<0.05).
On *A. thaliana* T2 events of *prp-4* (all nine events), *prp-8* (all 13 events), *prp-17a* (all 10 events), *prp-17b* (all 13 events), *uaf-2* (all 13 events) and *cpf-1* (all except event 7) there were 70-100%, 41-98%, 90-100%, 87-100%, 97-100% and 65-100% significant reductions in the number of cysts respectively (Figs. 6.8.a, 6.9.a, 6.10.a) compared with controls. Observed cysts on these plants appear to have developed normally, turning brown after 5-8 weeks.

**6.3.4.2. Number of eggs per cyst of adult females on transgenic *A. thaliana* T2 roots**

Generally, the number of eggs per cyst on wild type *A. thaliana* ranged between 76 and 307 in the four experiments. The numbers on the Kakan controls were between 95 and 264 eggs per cyst.

The number of eggs per cyst of mature females on transgenic *A. thaliana* events was also highly variable (0-325). The number of eggs was reduced in all six *sut-1* transgenic events by 47-100% per cyst compared to the number of eggs in females on wild type *A. thaliana* plants (Fig. 6.7.b). For *sna-1* it was 15-100% reduction on eight out of nine events on which cysts were developed (Fig. 6.7.b). Similarly, there were 23-100% (all seven events), 23-98% (11 out of 13 events), 20% (only one event), 15-100% (seven out of eight events), 29-93% (three out of five events) and 37-87% (four out of six events) reductions in eggs per cyst in females those were feeding on *prp-4, prp-8, prp-17a, prp-17b, uaf-2* and *cpf-1* transgenic events respectively, compared to the control (Figs. 6.8.b, 6.9.b, 6.10.b). The highest reduction in the number of eggs per cyst was from females fed on *sut-1* transgenic events.

The variability of the number of eggs per cyst was independent of the number of cysts on transgenic events. For example there was only one cyst on each of the *uaf-2* events 7, 8, 9, 11 and 15 (total of 8 replicates) but the numbers of eggs per cysts respectively were 10, 40, 140,
98 and 174. There was one cyst each on *sut-1* event 1 (total of replicates), *prp-4* event 3, *prp-17b* events 5, 7 and six cysts on *sna-1* event 3 but the cysts did not contain any eggs.

6.3.4.3. Percentage of J2s hatched from eggs of adult females fed on T2 *A. thaliana* roots

Percentage of J2s hatched varied between 32 and 90 per cyst from females developed on wild type *A. thaliana* in the four experiments. For Kakan it was 31-88%, which was not different from the wild type *A. thaliana* control.

The percentage of J2s hatched per cyst was highly variable (0-95%) among the transgenic events of all eight genes (Figs. 6.7.c, 6.8.c, 6.9.c, 6.10.c). Compared to the wild type *A. thaliana* control, on five *sut-1* events on which there were females with eggs, there was 16-49% reduction in J2s hatched per cyst. Similarly, compared to the controls, there was 13-100% (eight events), 1-77% (five out of six events), 0-100% (ten out of thirteen events), 31% (only one event), 8-17% (two out of five events), 10-100% (five events) and 27-67% (five out of six events) reduction in J2s hatched per cyst developed on *sna-1, prp-4, prp-8, prp-17a, prp-17b, uaf-2* and *cpf-1* transgenic events respectively. From eggs of females developed on eight out of thirteen *prp-8* events of *A. thaliana*, 84-95% hatched similar to the control (90%), regardless of the number of eggs per cyst. For the events 1, 5 and 6, in contrast, only 0-3% hatched.

All the J2s hatched from eggs of females fed on wild type and transgenic *A. thaliana* roots were alive and active. No J2s hatched from eggs of adult females that developed on *sna-1* event 9, *prp-8* events 1, 6 and *uaf-2* events 7, 8 and the number of eggs in those females was 4-43 per cyst.
6.3.4.4. Assessment of infectivity of second generation of nematodes feeding on transgenic

*Arabidopsis T2* roots

The infectivity of J2s hatched from adult females that survived after feeding on transgenic plants of the eight genes were assessed by infecting five replicates of wild type *Arabidopsis*, each with approximately 100 J2s and their development and reproduction examined for 5 weeks. Second generation J2s from adult females feeding on wild type *Arabidopsis* produced 5-8 cysts per wild type *Arabidopsis* plant. The second generation J2s from *sut-1* events 2, 3, 4; *sna-1* events 2, 6; *prp-4* event 9; *prp-8* events 2, 4, 7, 8, 9; *prp-17b* events 1, 6, 8; *uaf-2* event 15 and *cpf-1* events 1, 5, 7, 12 all developed into cysts on wild type *A. thaliana* roots (Figs. 6.7.d, 6.8.d, 6.9.d, 6.10.d). The number of cysts varied between one to 13 per *Arabidopsis* plant.

Cysts developed by J2s from adult females feeding on *sut-1* and *sna-1* transgenic *A. thaliana* appear to have developed faster as they were visible from the second week after infection of wild type *A. thaliana* compared to second generation J2s from transgenic plants of the other genes and wild type J2s which were only visible three weeks after infection. The number of cysts on wild type *A. thaliana* infected by second generation J2s hatched from the no gene vector control transgenic plants (Kakan) were significantly lower in two experiments than the number of cysts produced by J2s from adult females feeding on wild type *A. thaliana* (Figs. 6.7.d, 6.9.d).
Fig. 6.7. (a) The total number of cysts on 12 replicates of *sut-1* and *sna-1* transgenic *A. thaliana* events (b) The number of eggs per cyst developed on transgenic *A. thaliana* events (c) The percentage of J2s hatched per cyst obtained from infected transgenic *A. thaliana* events (d) The total number of cysts developed from second generation J2s obtained from transgenic plants on five replicates of wild type *A. thaliana*. 
Fig. 6.8. (a) The total number of cysts on 12 replicates of prp-4 and prp-8 transgenic A. thaliana events (b) The number of eggs per cyst developed on transgenic A. thaliana events (c) Percentage of J2s hatched per cyst obtained from infected transgenic A. thaliana events (d) The average number of cysts developed from second generation J2s obtained from transgenic plants per wild type A. thaliana.
**Fig. 6.9.**

(a) The total number of cysts on 12 replicates of *prp-17a* and *prp-17b* transgenic *A. thaliana* events

(b) The number of eggs per cyst developed on transgenic *A. thaliana* events

(c) Percentage of J2s hatched per cyst obtained from infected transgenic *A. thaliana* events

(d) The average number of cysts developed from second generation J2s obtained from transgenic plants per wild type *A. thaliana.*
Fig. 6.10. (a) The total number of cysts on eight replicates of $uaf$-$2$, $cpf$-$1$ and $prp$-$17b$ transgenic $A. thaliana$ events (b) The number of eggs per cyst developed on transgenic $A. thaliana$ events (c) Percentage of J2s hatched per cyst obtained from infected transgenic $A. thaliana$ events (d) The average number of cysts developed from second generation J2s obtained from transgenic plants per wild type $A. thaliana$. 
6.4. Discussion

The objective of this chapter was to use RNAi to study the importance of selected spliceosome genes in the development and reproduction of *H. schachtii*, and the potential of these genes as targets for the control of *H. schachtii*. Studying the development of females (cysts) of these amphimictic nematodes is important because they feed longer (from J2 to adult stage, about 21 days) than males (from J2 to J3, about 9 days) and cause more damage to host plants (Colgrove & Niblack, 2005). Generally, there was a 9-81% reduction in the numbers of cysts developed on wild type *A. thaliana* roots over 5 weeks after *in vitro* RNAi of seven of the nine putative spliceosome genes (except *uaf-2* and *sna-1*) compared to J2s not treated with dsRNA.

The greatest reduction in the cyst number (81%), was observed when *H. schachtii* was treated with *uaf-1* dsRNA, although the mRNA level did not appear to have reduced. *Uaf-1*, encodes the large subunit of the splicing factor U2 Auxiliary Factor (U2AF). The U2AF protein facilitates the interaction of U2 snRNP with the branch point in introns and also recognises the 3’ splice site “AG” (Zorio & Blumenthal, 1999). In contrast, the number of cysts developed over 5 weeks was not different from those of J2s not treated with dsRNA when *H. schachtii* was treated with dsRNA of *uaf-2*, which encodes the small subunit of U2AF. The next highest reduction in the number of cysts developed over 5 weeks were observed from *H. schachtii* J2s soaked in *prp-17b* (78%) and *prp-4* (75%): these were all paralysed immediately after RNAi induction. *Prp-17* is a splicing factor involved in pre-mRNA processing. Although *prp-17a* and *prp-17b* were amplified from two different segments of the same gene and both appeared to be up-regulated, the percentage reductions in cyst development differed, and were 47% and 78% respectively. When *H. glycines* was soaked in dsRNA corresponding to two different non-overlapping regions within the coding region of the pectate lyase gene (*pel-1*), which is expressed in the subventral oesophageal gland cells, the 267 bp construct showed a higher fold reduction in *pel-1* transcript level than did the 285 bp construct (Sukno et al., 2007). The
Inhibition of *G. pallida* motility induced by 227 bp was more profound than that of the 316 bp (with overlapping regions) construct after soaking in FMRFamide-like peptide 6 (flp-6) dsRNA (Kimber *et al.*, 2007). In contrast, both 271 bp (full length transcript) and 42 bp dsRNAs (coding region) of the oesophageal gland peptide 16D10, led to 93-97% transcript reduction in *M. incognita* J2s and suppression of nematode reproduction by 74-81% on *Arabidopsis* (Huang *et al.*, 2006). Effects of RNAi on phenotype, target mRNA levels and cyst development (reproduction) depend on the type of gene targeted, length of dsRNA and more importantly the position within the targeted gene (Kimber *et al.*, 2007; Lilley *et al.*, 2012).

In *H. schachtii* J2s soaked in *gfp* dsRNA, all the genes studied were in fact down-regulated, yet they subsequently developed into cysts, the numbers of which did not differ from those developed from J2s soaked without dsRNA. Since the *gfp* gene is not present in *H. schachtii*, the occurrence of down-regulation of other non-related genes may be due to a general stress during the soaking process, but the nematodes may then have recovered after they were removed from the dsRNA containing solution. The *gfp* induced down-regulation was in contrast to results of Dalzell *et al.*, (2009) in which they did not find a reduction in transcript abundance of endogenous genes like β-actin, β-tubulin and *flp* (FMRFamide-like peptide) in *M. incognita* or *G. pallida* following soaking in 0.1 mg/mL dsRNA of nine non-nematode derived genes, including *gfp* for 24 hours. Endogenous gene regulation of *gfp* dsRNA-treated nematodes and down-regulation of actin in all the dsRNA-soaked nematodes may be attributed to rate limiting components of the RNAi pathway which can become saturated, hence inhibiting processing of endogenous small RNAs leading to changes in normal gene regulation (Lilley *et al.*, 2012).

The time taken to recover from RNAi effects after the RNAi trigger has been removed may also have an effect on subsequent development and reproduction of J2s in an appropriate host.
Recovery time may depend on the target gene silenced and the effect of RNAi on important processes of the nematode. In this research, the levels of mRNA of target genes were quantified after soaking in corresponding dsRNA for 16 hours. This may not be the time when transcript depletion actually happens (Lilley et al., 2012). Two *M. incognita* genes expressed in the subventral oesophageal glands; calreticulin (*Mi-crt*) and polygalacturonase (*Mi-pg-1*) showed maximum silencing 20 and 44 hours after removal from 4-hour exposure to dsRNA. The transcript levels of both genes returned to normal after 68 hours (Rosso et al., 2005). Up-regulation of target genes in this experiment can be a cellular response that may have occurred in an attempt to maintain normal mRNA levels after gene silencing was induced. The changes in mRNA levels of *H. schachtii* J2s soaked in dsRNA of *gfp* and spliceosome genes observed in this research may be further confirmed by quantifying the mRNA levels at different times during and after soaking in dsRNA, and studying the reproduction of the J2s that hatch from eggs of females soaked in dsRNA.

The variation in the number of cysts on replicate plants (0-17) infected with J2s soaked in dsRNA for 16 hours may be due to the varying number of the nematodes that may have recovered from RNAi, the number that were able to reproduce after RNAi and/or the number of J2s that were directed by environmental and genetic factors to be females in each inoculum of 100 J2s. The target gene and its function, the effect of RNAi of a gene on growth and development of J2s, recovery time along with the genotypic diversity in the nematode population may also contribute to the variation in the number of cysts developed on host plants from replicate inocula with J2s.

Since plants serve as a continuous source of dsRNA for feeding nematodes, using plants as a source of dsRNA may be a more efficient way to deliver RNAi triggers and to assess the effect of RNAi on nematode survival, reproduction and inheritance of the RNAi effect. When the J2s
were cultured on transgenic plants, some may have survived any RNAi effect in that they retained the ability to reproduce leading to development into females and production of mature cysts with viable eggs. Once again the J2s may be genetically different in each 100 J2s inocula that was used to infect replicate plants, or possibly the transgenic plants may have been genetically different, and/or may have processed the nematode dsRNA into different small RNA triggers with different efficiencies for RNAi in infecting nematodes (Yang *et al.*, 2013). These could be some of the reasons for the variation in the number of cysts on different transgenic events and their replicates.

In this research, among the transgenic events harbouring hairpins of each of the genes studied there were events on which the number of developing *H. schachtii* cysts was reduced by 99-100%. Amongst these, nine out of ten events of *prp-17a* had the most significant reduction (100%) compared to the wild type *A. thaliana*. Efficacy of targeting the *prp-17* gene in the closely related *H. glycines* Ichinohe feeding on transgenic soybean plants in reducing cyst and egg numbers has also been reported (Li *et al.*, 2010).

On *A. thaliana uaf-2* transgenic events there was a 97-100% reduction in cyst development, an observation that was contrasted by the *in vitro* RNAi results of *uaf-2*, in which the treated J2s appeared unaffected and relatively normal numbers of cyst developed. It is possible that in the latter case, the *uaf-2* gene was not down-regulated after 16 hours of soaking in dsRNA or the nematodes may have recovered rapidly from possible effects of RNAi after they were removed from the RNAi trigger. The same could apply for *sna-1*, one of the two SL specific genes studied, as *in planta* RNAi of *sna-1* was more effective in reducing the cyst formation of *H. schachtii* in both first and second generations than *sut-1* although the cyst development by *sna-1* dsRNA treated J2s did not differ from the control and *sut-1* dsRNA treated J2s after RNAi *in vitro*. 

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The reduction in cyst development by the second generation J2s from females that were cultured on transgenic plants producing dsRNA of most of the genes, regardless of the number of eggs per cyst and the percentage of eggs hatched per cyst, may be an indication that the effect of RNAi inheritance may occur through more than one generation. Studying female development through generations is more important because reduction in the population of these plant parasites with time would be useful in reducing their populations in agricultural fields.
Spliceosomes, the multi-megadalton RNA-protein complexes that carry out pre-mRNA splicing, to generate mature mRNA for protein biosynthesis by the ribosomes are dynamic molecular machines, assembling de novo for each splicing event by the stepwise recruitment of subunits on a substrate (Wahl & Lührmann, 2015).

The overall aim of this research was to study the effects of silencing selected spliceosome genes of the plant parasitic nematodes *P. thornei* (a root lesion nematode) and *H. schachtii* (beet cyst nematode), in vitro and in planta using RNAi. Individual components of the research involved: (i) identifying and characterising, in silico, transcripts of spliceosome genes of *P. thornei* and *H. schachtii* based on sequence homology to *C. elegans* genes and RNAi data, (ii) functional characterisation of spliceosome genes of *P. thornei* and *H. schachtii* using in vitro supplied dsRNA to induce RNAi, followed by (iii) a study of the effects of silencing selected genes of both nematode species using transgenic plants as a source of the dsRNA.

In this research, each of the components of the overall aims was achieved, and valuable new information has been provided that will contribute to future control of these serious plant pests.

Comparative analysis of sequences of plant nematode species with those of *C. elegans* identified putative transcripts of 55 spliceosome genes of the root lesion nematodes *P. thornei*, *P. zeae* and *P. coffeae*, and putative ESTs of 44 spliceosome genes of the cyst nematodes *H.*
glycines and H. schachtii. The difference in number of spliceosome genes identified is probably a result of differences in the amounts of sequence data available for each species. Amongst the transcripts/ESTs identified, there were sequences of SL-associated genes, indicating that SL trans-splicing of mRNA is indeed present in these plant parasitic nematodes. It was evident from sequence analysis that the P. thornei spliceosome transcript sequences were more similar to those of parasitic nematodes than to free-living nematodes. Orthologues for SL specific sequences were found only amongst sequences of nematodes, an observation of significance since it increases the possibility of targeting these genes specifically by RNAi, that is, it minimises the possibility of affecting non-target species. The latter reasoning or possibility is important when considering potential future translation of results into commercial practice.

Soaking of motile stages of plant parasitic nematodes in dsRNA is regarded as an effective method for initial screening of gene function and discovery of candidate target genes that are suitable for plant-delivered RNAi for nematode control (Fosu-Nyarko & Jones, 2015a). When the root lesion nematode P. thornei and the cyst nematode H. schachtii were soaked in 1 mg/mL dsRNA targeting selected spliceosome genes for 16 hours, changes in nematode behaviour and regulation of target mRNA levels were observed. Persistence of the effects of transient gene silencing by soaking in dsRNA was also evident for most of the genes studied. It was manifested by a reduction in reproduction of P. thornei and cyst development of H. schachtii compared to nematodes not soaked in dsRNA, over a 5 week period of culture after the initial treatment of nematodes with dsRNA.

Although the mode of dsRNA delivery (soaking), dsRNA concentration (1 mg/mL) and time duration for which RNAi was induced (16 hours) were the same for P. thornei and H. schachtii, the effects of RNAi induction were different for the two species, even when the same gene was targeted. P. thornei showed abnormal movements when soaked in dsRNA of all genes,
including gfp dsRNA used as a non-specific control dsRNA, whereas H. schachtii J2s were only paralysed after they had been soaked in prp-4, prp-8 and prp-17 dsRNA.

For both species, soaking in gfp dsRNA appeared to result in initial down-regulation of most spliceosome genes and the non-target reference genes (actin and 18S rRNA). However, the number of P. thornei collected from carrot mini discs and the number of H. schachtii cysts which developed on wild type A. thaliana roots were not significantly different from control nematodes (not treated with dsRNA) over 5 weeks after the dsRNA treatment, and this indicates that both species were able to recover from the effects of treatment with a non-target dsRNA.

Most spliceosome genes of P. thornei were down-regulated after dsRNA treatment, except for prp-17, while for H. schachtii, spliceosome genes for which the PCR analysis was done, were in fact up-regulated immediately after soaking in dsRNA for 16 hours, compared to the nematodes not treated with dsRNA. The sut-1 and prp-17 genes were up regulated in both species. Expression of the reference genes actin and 18S rRNA was down regulated in both species treated with dsRNA of most of the target genes.

Although there were differences in the immediate responses to dsRNA treatments between these two species, for example, after 16 hours of soaking in dsRNA, P. thornei uaf-1 was down-regulated whilst in H. schachtii it was up-regulated, nevertheless for both species there was an 80-81% reduction in the number of nematodes/cysts counted after 5 weeks culture on host plants. Uaf-1 and uaf-2 encode subunits of the splicing factor U2 Auxiliary Factor (U2AF). P. thornei, uaf-2 was down-regulated whilst there was no change in expression in H. schachtii. It is interesting to note that in this case the longer term effects on reproduction of the two species mirrored the initial effects on target gene expression. After RNAi of uaf-2 had been induced by soaking, there was a 94% reduction in the number of P. thornei extracted from mini
carrot discs after 5 weeks, whereas for similarly treated *H. schachtii* there were actually more cysts on wild type *A. thaliana* than those not soaked with dsRNA.

Even though the SL specific *sut-1* appear to be up-regulated in both species after dsRNA treatment, there was about 85% reduction in the number of *P. thornei* extracted from mini carrot discs, whilst there was only a 9% reduction in the number of *H. schachtii* cysts developed on wild type *A. thaliana* after 5 weeks. Generally, following dsRNA treatment of SL specific genes *sut-1*, *sut-2* and *sna-2*, there was a 55-99% reduction in the number of *P. thornei* extracted from mini carrot discs after 5 weeks. In contrast, following dsRNA treatment for the SL specific genes *sut-1* and *sna-1* of *H. schachtii*, there was no significant reduction in the number of cysts developed on wild type *A. thaliana*. This could be the result of rapid recovery of SL specific gene expression in *H. schachtii* after removing the RNAi trigger. This explanation could be confirmed by studying mRNA levels in the nematodes at different time intervals during and after the RNAi induction.

It became clear from the results of the *in vitro* RNAi experiments, that effects of RNAi induction were different between the root lesion nematode and the cyst nematode studied, even when the same gene was targeted. It has been reported that susceptibility to RNAi of closely related species of nematodes can differ, for example between *P. thornei* and *P. zeae* (Tan *et al.*, 2013). When both nematodes were fed with the same amounts and dsRNA of *pat-10* and *unc-87* under the same conditions, the behaviour of a greater percentage of *P. thornei* was affected than for *P. zeae*, and there was also a greater reduction in transcripts for both genes in *P. thornei* (Tan *et al.*, 2013). Fosu-Nyarko and Jones, (2015a) suggest that, from limited gene silencing data available for *Pratylenchus* species, it would appear that in general *Pratylenchus* species are more amenable to control by RNAi, possibly because they do not form a feeding tube. These authors also comment that it appears that *Meloidogyne* spp. are more amenable to RNAi than cyst nematode *Heterodera/Globodera* species. A possible
explanation for the latter is that there are differences in the structures of feeding tubes formed by these two sedentary endoparasitic genera which may, in some way, differentially affect uptake of siRNAs or longer dsRNAs from host cell cytoplasm when feeding.

As indicated earlier, factors such as the target gene, targeted region of a gene, the length of the dsRNA used, the concentration of dsRNA, time of incubation, mode of delivery, possible recovery from RNAi and whether there are compensating pathways for loss of a particular function may influence the effectiveness of RNAi in different nematodes (Lilley et al., 2012; Tan et al., 2013; Fosu-Nyarko and Jones, 2015a).

The final area of study was to deliver dsRNA to nematodes via transgenic plants, since this is the most likely mode of delivery for a commercial crop. There are some differences in the delivery of dsRNA via such plants: in contrast to transient introduction of long dsRNA by soaking of nematodes, plant delivery of dsRNA to silence the expression of genes vital for nematode reproduction or survival provides dsRNA continuously if expressed in host cells from a constitutive promoter. However, the plant cell is likely to process long dsRNA to siRNAs, so that the form of dsRNA ingested by the nematode differs from that supplied by soaking. There may be subtle differences in the way that plants and nematodes process long dsRNA, and plant delivery of plant-processed siRNAs could reduce the effectiveness of control by transgenic plants. Nevertheless plant control of nematodes using RNAi is clearly a potential approach to confer nematode resistance, in a sustainable and environment friendly manner (Urwin et al., 2002; Kamath et al., 2003; Tan et al., 2013, Fosu-Nyarko & Jones, 2015a).

RNAi technology has the potential to improve public acceptance to GM crops. This is because dsRNA does not encode a message for a functional protein. Therefore, in RNAi-mediated resistance, non-target effects should be lower than those of highly specific transgenic proteins
such as β-endotoxins of *Bacillus thuringiensis* (Bt), which are already deployed widely to control insects in some crops (Bakhetia *et al*., 2005). In addition, dsRNA is not new to the human diet. Virus-infected crops, that produce molecules similar to those used in RNAi technology, are commonly consumed (Bakhetia *et al*., 2005). Non-target species that feed on the plant can be protected by using dsRNA that has no effect when ingested by other invertebrates such as insects by avoiding targeting a gene family that is highly conserved across the plant and animal kingdoms (Bakhetia *et al*., 2005). Delivering dsRNA by using promoters that are active only within roots or feeding cells may offer biosafety (Lilley *et al*., 2004). Grafting transgenic rootstocks with a non-transgenic scion may also reduce any risk factors (Fosu-Nyarko & Jones, 2015).

Presence of many small RNAs in rice grains with sequence identity to regions in human and several animal genomes have been found by Heisel *et al*., (2008), which supports the idea that there has been safe consumption of RNA by humans and animals over a long period of time. Petrick *et al*., (2013) have concluded that nucleic acids are natural components of all animal and plant-related foods and also have a long history of safe consumption. There are biological barriers (such as salivary RNases in the mouth, acidic pH and digestive enzymes in the stomach, endosomal and lysosomal degradation) for cellular uptake of nucleic acids from foods and rapid catabolism and/or excretion of nucleic acids. Therefore it has been concluded that crops using RNA-mediated gene regulation are safe for human and animal consumption (Petrick *et al*., 2013).

In this research, there was a significant reduction in *P. thornei* populations cultured on carrot hairy roots transformed with SL specific *sut-1, sut-2* and *sna-2* genes over 5 weeks, and these results were similar to the results obtained when *P. thornei* was soaked in dsRNA of those genes for 16 hours. These results suggest that soaking *P. thornei* in dsRNA of target genes is a
useful approach for screening for the potential effectiveness of different gene targets for nematode control. However, for *H. schachtii*, even though cyst development after J2s were soaked with *sut-1* and *sna-1* dsRNA was not significantly lower than controls, and expression of the genes were up-regulated immediately after soaking, there were nevertheless significantly fewer cysts that developed on some *A. thaliana* events transformed with *sut-1* and *sna-1*. Depending on the site of transgene insertion, copy number, promoter strength and construct design, a set of different transgenic plant events transformed with the same construct usually demonstrate a range of the desired property, with some events more effective than others (Fosu-Nyarko & Jones, 2015a).

*H. schachtii* soaked in *uaf-2* dsRNA produced the most cysts after infection of wild type *A. thaliana* roots, in contrast, the greatest reduction of cyst development was achieved with *H. schachtii* feeding on *uaf-2* transformed *A. thaliana* T2 seedlings. It appears likely that *H. schachtii* soaked in *uaf-2, sut-1* and *sna-1* dsRNA exhibited a recovered response soon after the 16 hours soaking period.

The viability of nematodes in the generation following dsRNA treatment was also investigated to find out if effects of dsRNA treatment were persistent. Although the number of eggs per cyst and the number of J2s hatched per cyst were variable among events of a control/gene, reduction in the *H. schachtii* population in the second generation was apparent. This is significant because in a field situation this would also help reduce nematode populations over time. Overall results from host mediated RNAi may also depend on model or crop plant species, their genotype, number of events generated and studied and the nematode genotype (Fosu-Nyarko & Jones, 2015a).
In terms of potential acceptance of RNAi as a method of nematode control in crop plants, there have been some promising developments and precedents for the acceptance of RNAi for commercial use. These include evidence of long term stability of RNAi traits and the commercial production and release in Canada and the USA of non-browning ‘Arctic’ apples in which RNAi has been used to down-regulate the expression of polyphenol oxidase (http://www.okspecialtyfruits.com/). Similarly there has been down-regulation of the omega-6 fatty acid desaturase gene in soybean, resulting in increased levels of monounsaturated (oleic) fatty acids in soybean seeds. The high–oleic oil soybeans are beneficial for human health and industrial oil production. Other nutritionally enhanced products being developed include tomatoes with increased carotenoids, high-amylose and reduced-gluten wheat, and oranges with higher levels of beta-carotene (http://www.thescientist.com/?articles.view/articleNo/43020/title/Improving-Crops-with-RNAi/).

This research both complements previous research in this field (Klink et al., 2009; Joseph et al., 2012; Tan et al., 2013; Yang et al., 2013; Kyndt et al., 2013; Vieira et al., 2015) and provides new data. From the results presented in this thesis it can be concluded that the spliceosome genes studied here are potential target genes that can be used to achieve effective transgenic resistance in host plants to control both root lesion and cyst nematodes via RNAi technology.

In addition, the potential exists to use RNAi technology to achieve cross species control of nematodes by targeting regions conserved among species or including more than one target sequence in the dsRNA, to develop broad spectrum transgenic resistance in one construct, and this is one of the next steps for this research. Tan et al., 2013 have reported that dsRNA derived from either nematode species (P. thornei and P. zeae) silenced the corresponding gene in both species in vitro. Targeting the parasitism gene 16D10, which is highly conserved (95-98% nucleotide identity) in root-knot nematode species, for silencing by expressing dsRNA in
transgenic *Arabidopsis* resulted in transgenic plants that were resistant to multiple root-knot nematode species, *M. incognita, M. javanica, M. arenaria* and *M. hapla* (Huang et al., 2006).

As indicated in section 3.3.5 in this thesis, transcripts putatively encoding most *P. thornei* spliceosome proteins such as *prp-17* had high similarities to such sequences of other plant parasitic nematodes, suggesting the possibility of achieving broad spectrum resistance.

Overall, the research presented in this thesis successfully achieved the major aims at the start of the project, and provides a firm basis for future application of the genes studied to control nematode pests in crop plants.
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APPENDIX 1

Results of in silico analysis of putative sequences (of amplicons) encoding spliceosome units of P. thornei

(a) Hypothetical protein WUBG_02756, partial [Wuchereria bancrofti]
Sequence ID: gb|EFW6331.1| Length: 288 Number of Matches: 1
Range 1: 151 to 280

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Subjct 151 LESRRFSIPPEPFLSYKHDKLLLIALLERKASEYVKRNLSDREELALDLQXNSPH

Query 230 ALSRIKHMLTMRSFKVEIEFDLYLPSVQIEPMELKVDIALDYAVLVY ГDКRRLF
Subjct 211 ALSRIKHMLTMRSFKVEIEFDLYLPSVQIEPMELKVDIALDYAVLVY ГDКRRLF

(b) PRP-8 [Caenorhabditis elegans]
Sequence ID: ref|MP_498785.1| Length: 2329 Number of Matches: 1

See 2 more title(s)
Range 1: 659 to 946

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Features:

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Subjct 659 GVAKTVKORVESHDLLRAVYMDHIDMPPEGIKQMKARVQLHASENRCWAKANIPW

Query 61 KVPGLPTPEVNLTRVVKAKADWTSAYNHREVRARGVDKTVCCKNLGRRTLYLKA
Subjct 719 KVPGLPTPEVNLTRVVKAKADWTSAYNHREVRARGVDKTVCCKNLGRRTLYLKA

Query 121 EDEQROHYLKDGEYTSFSAEAVYVTIVVVHLESRRFSIPPEPFLSYKHDKLLLIALLERKASEYVKRNLSDREELALDLQXNSPH
Subjct 779 EDEQROHYLKDGEYTSFSAEAVYVTIVVVHLESRRFSIPPEPFLSYKHDKLLLIALLERKASEYVKRNLSDREELALDLQXNSPH

Figure A1. Alignments of a 351 bp fragment putatively encoding P. thornei Prp-8 (query) with (a) W. bancrofti hypothetical protein (WUBG_02756) (subject) and (b) C. elegans Prp-8 protein (subject).
Figure A2. Alignments of a 410 bp fragment putatively encoding *P. thornei* T08A11.2 (query) with (a) *W. bancrofti* hypothetical protein (WUBG_09184) (subject) and (b) *C. elegans* T08A11.2 protein (subject).
Figure A3. Alignment of a 276 bp fragment putatively encoding *P. thornei* Uaf-2 (query) with *W. bancrofti* U2 (RNU2) small nuclear RNA auxiliary factor 1 (subject).

Figure A4. Alignments of a 409 bp fragment putatively encoding *P. thornei* Swp-1 (query) with (a) *Necator americanus* Surp module (subject) and (b) *C. elegans* Swp-1 isoform d (subject).
Figure A5. Alignments of a 192 bp fragment putatively encoding *P. thornei* Sfa-1 (query) with (a) a KH domain containing protein of *Haemonchus contortus* (subject) and (b) *C. elegans* Sfa-1 (subject).

Figure A6. Alignment of a 389 bp fragment putatively encoding *P. thornei* Prpf-4 (query) with *B. malayi* Prpf-4 isofrom a (subject).
**Figure A7.** Alignment of a 267 bp fragment putatively encoding *P. thornei* Prp-4 (query) with *C. elegans* prp-4 mRNA (subject).
Figure A8. Alignment of a 371 bp fragment putatively encoding *P. thornei* *Uaf*-1 (query) with *C. elegans* *uaf*-1 mRNA (subject).