Host-regulated gene expression in plant parasitic nematodes

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The candidate confirms that the work submitted is their own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Plant parasitic nematodes are major economic pests, causing significant yield losses in crop plants worldwide. These nematodes modify or degrade cell wall components to facilitate host entry, migration, and in the case of sedentary endoparasites, feeding site formation. Previous research has demonstrated that *Pratylenchus coffeae* exhibits host-specific gene expression of cell wall-degrading enzymes when exposed to various host root exudates. This research aimed to investigate whether or not other plant parasitic nematodes exhibit similar capabilities. Similar genes encoding cell wall-degrading enzymes featuring signal peptides, cellulase catalytic domains, and sometimes cellulose binding domains were identified in *Globodera pallida* and *Meloidogyne incognita*. Initial exposure to root exudates induced transcriptional responses of these genes in both species, accompanied by behavioural modifications. Subsequent RNA-seq analysis revealed host-specific gene expression patterns in *G. pallida* and *M. incognita* when exposed to root exudates from different host and non-host plants. Notably, responses in *M. incognita* were unique and there was limited overlap in the genes that were up- or down-regulated in response to each root exudate. Conversely, *G. pallida* displayed a strong response to its preferred host, potato, but not to other plants. Specifically, 1,412 were differentially expressed upon exposure to potato root exudate, compared to only 12 and 4 genes in response to maize and carrot root exudates, respectively. In contrast, *M. incognita* exhibited responses to a variety of root exudates. Spatial expression of a subset of up-regulated genes was revealed to be in the pharyngeal glands and amphids of both *G. pallida* and *M. incognita*, suggesting potential roles as effectors. Successful gene silencing using RNAi resulted in reduced parasitic success in both nematode species. The results from this research advances our understanding of the molecular strategies employed by *G. pallida* and *M. incognita* during plant parasitism, offering insights into their adaptability.

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5-HT	5-hydroxytryptamine
ANOVA	Analysis of variance
BLAST	Basic logarithmic alignment search tool
bp	Base pair
cDNA	Complimentary DNA
CDS	Coding sequence
CEP	C-Terminally encoded peptide (CEP) plant peptide mimic
CPM	Counts per million
CS	Cleavage site
Ct	Cycle threshold
CTAB	Hexadecyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOG Box	Dorsal gland motif
Dpi	Days post infection
dsRNA	Double-stranded RNA
DsiRNA	Dicer-substrate small interfering RNA
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EDTA	Eethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FC	Fold change
FDR	False discovery rate
GH	Glycosyl hydrolase
GO	Gene ontology
GPCR	G-protein coupled receptor
IBM	International Business Machines
IPTG	Isopropylthio-β-galactoside
Kb	Kilo-base pair

kDa	Kilo-daltons
LB	Luria-Bertani
N/A	Not applicable
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
nm	Nanometer
ns	Not significant
NTA	Nitrilotriacetic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RCF	Relative centrifugal force
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
RPM	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl-sulphate
Sec/SP	Secretory signal peptide
SEM	Standard error of the mean
siRNA	Small interfering RNA
SPRYSEC	Sp1a and Ryanodine receptors (SPRY) domain containing proteins
SPSS	Statistical package for the social sciences
SSC	Sodium citrate
TAE	Tris/Acetate/EDTA
Tm	Melting temperature

Chapter 1
Introduction

1.1 Nematoda

Nematodes, classified under the phylum Nematoda, have recently been classified as being the most ancient animals on Earth (Shatilovich *et al*., 2023). They are a diverse group of roundworms that have been recorded in almost every habitat worldwide (Hodda, 2022). With more than 30 thousand species identified (Kiontke and Fitch, 2013), nematodes comprise one of the most abundant animal groups in existence. Among them, free-living nematodes, such as the model organism *Caenorhabditis elegans,* play important roles in various ecological processes such as decomposition and nutrient cycling (Gebremikael *et al*., 2016, Neher, 2001, Neher, 2010). However, while the majority of nematodes are free-living and provide beneficial ecological services, some are parasitic and they inflict disease on the animal or plant hosts they depend on for survival (Jones *et al*., 2013a, Cole and Viney, 2018).

1.2 Plant parasitic nematodes

The plant parasitic lifestyle is thought to have independently emerged within four of the twelve distinct nematode clades (Blaxter *et al*., 1998). Among these four clades, more than 4,100 species of plant parasitic nematode have been described (Decraemer and Hunt, 2006). Over the course of evolution, nematodes have adopted two main modes of parasitism: endoparasitism and ectoparasitism. Sedentary endoparasites, which encompass the cyst and root-knot nematodes, penetrate and migrate through the root and establish a single feeding site, from which they feed throughout their parasitic life stages (Jones *et al*., 2013a). Similarly, migratory endoparasitic nematodes, such as *Pratylenchus* and *Radopholus* species, also enter the root system. However, unlike the sedentary parasites, they do not form permanent feeding sites. Instead, they feed on tissue as they travel through the roots (Jones *et al*., 2013a). In contrast, ectoparasitic nematodes such as *Trichodorous* species, spend their entire lives in the soil, puncturing the roots only when necessary to extract nutrients (Jones *et al*., 2013a).

Despite the differences in parasitic mechanism, the majority of plant parasitic nematodes have a similar odontostyle known as a stylet (Figure 1.1). Whilst its evolutionary origins may differ among nematode clades, its purpose remains consistent across species. The stylet facilitates the withdrawal of nutrients during feeding (Smant *et al*., 2018) and is also used to inject specialised secretions known as effectors, produced in the pharyngeal glands (Figure 1.1), directly into the host in order to facilitate host parasitism (Rehman *et al*., 2016, Vieira and Gleason, 2019).

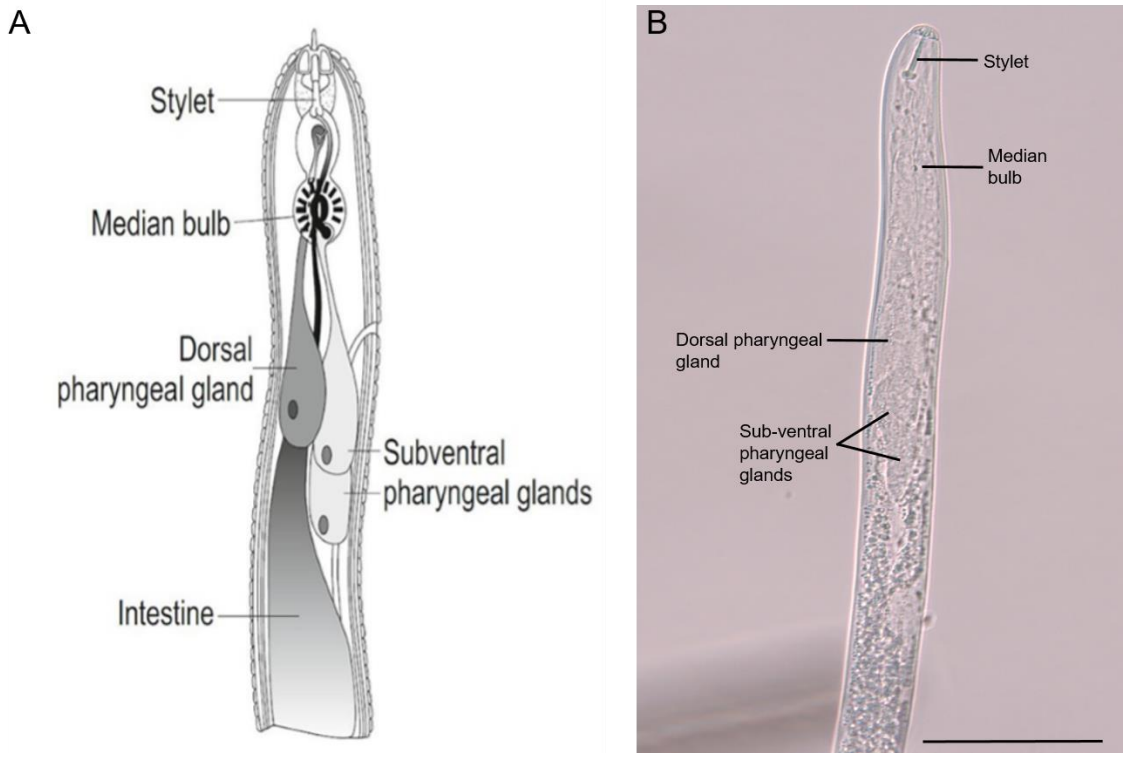


Figure 1.1- Anatomy of a plant parasitic nematode.

A) Schematic of a plant parasitic nematode. The styl*et al*lows the nematode to feed on plant material which is passed into the intestine. The stylet is also used to inject effectors into the host which are produced in the dorsal and sub-ventral pharyngeal glands.

B) Photograph of plant parasitic nematode. Photograph taken using Zeiss AXIO Scope A1 microscope. Major structures labelled. Size marker is 50 µm.

Plant parasitic nematodes present a major threat to global crop production and food security, ranking among the most economically important plant pests worldwide (Jones *et al*., 2013a). Resulting yield losses due to crop damage are estimated to range between 12.3 and 14 percent (Singh *et al*., 2015, Zwart *et al*., 2019), with economic losses thought to be between $80 and $157 billion annually (Chitwood, 2003, Abad *et al*., 2008, Nicol *et al*., 2011, Bonfil *et al*., 2004). The true extent of the damage is likely surpass these values as they are substantially under-estimated due to the challenges associated with identifying parasitic infection. Lack of awareness and the manifestation of non-specific symptoms, which often resemble abiotic stress, hinders timely diagnosis (Jones *et al*., 2013a).

As the global population continues to expand, so does the demand for food, placing significant strain on agricultural systems to enhance yields of staple crops including potatoes, maize, rice, and wheat. However, this challenge is compounded by the threat if crop losses due to plant parasitic nematode infection, which are likely to exceed current estimates. Therefore, it is becoming increasingly more important that effective strategies of control for these pests are developed.

1.2.1 Cyst nematodes

Cyst nematodes, belonging to the *Globodera* and *Heterodera* genera, in the family *Heteroderidae* in the order Tylenchida and class Secernentea, infect the root systems of their host plants. Their name is derived from the generation of a cyst, which is formed from the tanned body of the female nematode, containing her eggs within its structure. Although cyst nematodes infect a wide range of crop plants between them (Jones *et al*., 2013a), each cyst nematode species exhibits a limited capacity for infecting only a select range of hosts (Moens *et al*., 2018). For some cyst nematode species, their narrow host ranges are confined to a specific family of plants. *Heterodera avenae,* for example, exclusively infects cereal plants within the *Poaceae* family (Smiley *et al*., 2011, Al-Hazmi *et al*., 2001). Similarly, the potato cyst nematodes, *Globodera* species, have a distinct preference for plants within the *Solanaceae* family (Price *et al*., 2021, Whitehead, 1985). For others, host-specificity is more pronounced. The pea cyst nematode, *Heterodera goettingiana*, for example, has a preference for only one host and this species of nematode solely parasitises peas (*Pisum sativum)* (Di Vito and Greco, 1986).

The life-cycle of a cyst nematode begins when the first-stage juvenile (J1) moults to become a second-stage juvenile (J2) within the egg (Figure 1.2). The eggshell, which is composed of an outer vitelline layer, middle chitinous layer and an inner glycolipid layer

(Perry and Trett, 1986), provides a protective barrier for the nematode during embryogenesis and throughout the first moult.

Hatching of the pre-penetrative J2 from the egg occurs within the cyst in response to environmental cues ranging from temperature fluctuations (Den Nijs and Lock, 1992), to soil moisture content, and oxygen availability (Masler and Perry, 2018). The hatching of cyst nematodes with narrow host ranges requires further stimulation from more host-specific cues, such as root exudates (Masler and Perry, 2018). Root exudates are released from the roots of living plants into their surrounding soil environments. They contain primary metabolites including sugars, carbohydrates, and amino acids, which are secreted passively by diffusion into the soil, mainly from the root tip (Badri and Vivanco, 2009, Canarini *et al*., 2019, Koo *et al*., 2005, Nguyen, 2009). These metabolites mix with water in the soil and create a concentration gradient away from the root surface (Cooper *et al*., 2018). The distance that these extend from the root varies with soil water content, size of metabolite, and soil type (Watt *et al*., 2006). The hatching of potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis,* is almost entirely dependent on stimulation from hatching factors within the root exudates of their *Solanaceae* hosts (Den Nijs and Lock, 1992, Devine and Jones, 2003). In this context, hatching factors refer to host-specific compounds that play a role in inducing the hatching process. Individual compounds within root exudates have been identified through fractionation (Byrne *et al*., 1998, Devine and Jones, 2003) and some of the hatching factors that have been isolated from potato roots include glycoalkaloids (Byrne *et al*., 1998) and Solanoeclepin A (Tanino *et al*., 2011). Research suggests that sugars found in potato root exudates, including glucose, fructose and arabinose, independently induced hatching in *G. pallida* (Bell *et al*., 2021). However, it is noteworthy that no single sugar induced hatching to the same extent as potato root exudate did (Bell *et al*., 2021). This suggests that a combination of root exudate components contribute to the stimulation of nematode hatching.

The degree to which cyst nematodes rely on root exudates to hatch varies greatly and could be reliant on the host ranges of individual species (Masler and Perry, 2018). Those with broader host ranges such as *Heterodera schachtii, Heterodera glycines,* and *H. avenae,* have been shown to hatch in the absence of root exudate (Masler and Perry, 2018). *H. avenae* responds to changes in temperature instead (Greco, 1981), whilst *H. glycines* shows ‘incomplete’ host-mediated hatching, with some individuals hatching in water, but others requiring stimulation from soybean root exudate (Thapa *et al*., 2017).

The hatching process involves three key steps: modifications to eggshell permeability, activation of the J2, and eclosion (Perry and Moens, 2011). In reaction to external stimuli,

permeability of the eggshell increases in a calcium-dependent manner (Duceppe *et al*., 2017). This leads to a subsequent release of trehalose, which partially rehydrates the J2, making it more active and prompting the initiation of stylet thrusting which punctures the eggshell, allowing hatching to take place (Palomares-Rius *et al*., 2016). *In vitro* experiments have observed the presence of potato cyst J2s in the soil within 14 days of planting suitable hosts (Mimee *et al*., 2015).

In *G. rostochiensis,* recent discoveries have highlighted the significance of an annexin-like protein in governing eggshell permeability (Price *et al*., 2023). This protein was found to be localised to the eggshell and there is evidence that it engages with lipids within the inner glycolipid layer to help modulate permeability (Price *et al*., 2023). The lipid binding capabilities of the annexin have been observed to be influenced by host root exudates (Price *et al*., 2023) which indicates a potential link between this protein and the requirement of root exudate stimulation for *G. rostochiensis* hatching.

Pre-penetrative J2s must sense and respond to root exudate gradients within the soil in order to locate a suitable host. The amphids, located at the anterior end of the nematode, and phasmids (posterior end) are involved in environmental detection and are able to perceive root exudates from a distance (Ochola *et al*., 2021). Amphids are the primary sensory organs in nematodes and contain 12 sensory neurones which are involved in the sensing of a range of environmental cues including olfactory and thermal stimuli. Phasmids have a similar structure to the amphids but are smaller in size; they have roles in mechanoreception and chemorepulsion behaviour in nematodes (Altun and Hall, 2010).

The detection of root exudates in this manner triggers the J2s to move through the soil by undulatory propulsion (Burr and Robinson, 2004) towards the host plant (Masler and Perry, 2018). As endoparasites, the nematodes then penetrate the host tissue near to the root tip using their stylet and pharyngeal gland secretions (Moens *et al*., 2018). Inside the host root, the J2, now in its parasitic state, employs its stylet to migrate intracellularly through plant cells. As it does so, it inflicts substantial damage to the tissues spanning from the entry site to the vascular cylinder, where it initiates the formation of a permanent feeding site (Figure 1.2) (Abad and Williamson, 2010, Moens *et al*., 2018). A single pericycle cell is selected and effectors produced in the pharyngeal glands are injected to initiate the formation of a syncytium (Golinowski *et al*., 1997). The syncytium results from the merging of hundreds of plant cells facilitated by partial cell wall breakdown and nuclear division in the absence of mitosis within the cell (Jones and Northcote, 1972).

Cyst nematodes reproduce by amphimixis, resulting in the production of both males and females (Figure 1.2). Sex determination of adults is finalised at the J2 stage, just before the second moult to third-stage juvenile (J3) (Grundler *et al*., 1991b). At this stage, the male J3 already has a single testis and the female J3 already has ovaries and has become saccate (Grundler *et al*., 1991b). A further moult to fourth-stage juvenile (J4) occurs before the nematodes reach adulthood (Figure 1.2). Once mature, the males are vermiform and migrate out of the root back into the soil, where they can survive for up to 10 days (Evans, 1970). In contrast, the females remain sedentary at the feeding site for several weeks (Moens *et al*., 2018), growing until their bodies ultimately protrude through the root surface (Figure 1.2).

Once outside of the host, males move through the soil in pursuit of females which they are attracted to because of the sex pheromones they produce (Green, 1980, Green and Plumb, 1970). The exact composition of cyst nematode pheromones is largely unknown, however, *H. glycines* females secrete vanillic acid which has been found to serve functions as a sex pheromone (Jaffe *et al*., 1989).

The male fertilises the female and she produces hundreds of eggs which are retained within her body. When the female dies, her cuticle undergoes polyphenol oxidase tanning, forming a protective layer around the embryonated eggs, termed a cyst (Figure 1.2). *In vitro* studies have observed mature *G. rostochiensis* cysts in the soil between 42 and 63 days after planting of suitable hosts (Moens *et al*., 2018).

Cyst nematodes can remain dormant within their eggs in states of diapause or quiescence for long periods of time to evade unfavourable environmental conditions. These are stages of arrested development and they are similar to the dauer stage in *C. elegans* where development is halted at the second moult (Hu, 2007). Diapause is a strategy used to overcome long term problems such as the absence of a suitable host or longer term seasonal changes (Masler and Perry, 2018). There are two principal forms of diapause: obligate and facultative. Obligate diapause is mandatory and it occurs within the initial developmental stages (Perry and Moens, 2011). It is believed to have evolved as an adaptation to ensure survival in environments where suitable hosts might be scarce (Moens *et al*., 2018). Certain cyst nematodes, including *G. pallida, G. rostochiensis* and *H. avenae,* undergo a compulsory diapause during their first development season (Moens *et al*., 2018). In contrast, facultative diapause is not obligatory, instead it is influenced by environmental cues (Perry and Moens, 2011). For instance, in *G. rostochiensis*, declining temperatures and reduced day length have been identified as triggers for facultative diapause (Hominick *et al*., 1985). Similarly, in *H. avenae,* the induction of diapause exhibits regional variability but it is also primarily influenced by temperature fluctuations (Abidou *et al*., 2005).

In contrast, quiescence is a state of temporary dormancy driven by short-lived, deteriorating environmental conditions including temperature fluctuations and reduced water availability (Masler and Perry, 2018). Within this state, the metabolic and developmental activities of the J2 temporarily cease, until the environment becomes more favourable. Unlike diapause, quiescence is a direct response to the immediate environment, rather than a deliberate strategic choice (Perry *et al*., 2013). Cyst nematodes are capable of surviving upwards of 20 years in states of arrested development (Lane and Trudgill, 1999).

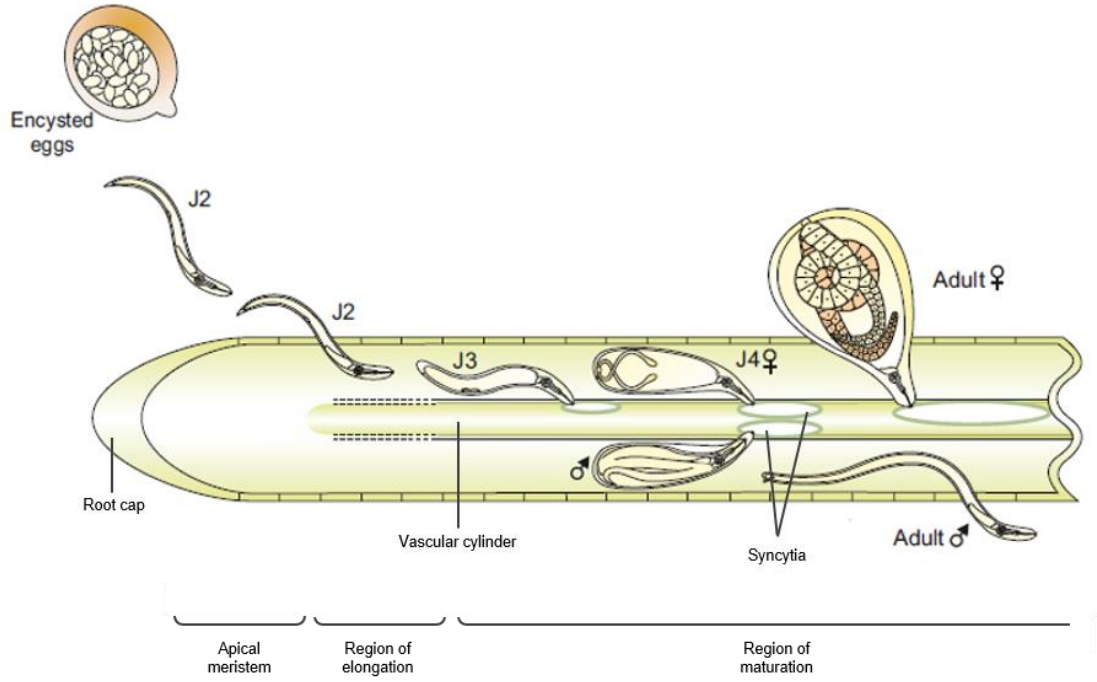


Figure 1.2- Life-cycle of cyst nematodes.

Cyst nematodes (*Globodera* and *Heterodera* species) moult from J1 to J2 within the encysted egg. Once they have hatched in the soil, they locate and penetrate host roots close to the root tip. They then migrate intracellularly through the plant cells to the vascular cylinder, causing significant cell damage. Here, they induce the formation of a syncytium from a single pericycle cell which acts as the permanent feeding site. The nematode then undergoes three moults to the adult stage (male or female). Males are vermiform and migrate out of the root, whereas females remain sedentary at the feeding site and grow until their bodies are protrude through the root surface. Males fertilise the females which they are attracted to by the emission of sex pheromones. Hundreds of embryonated eggs are produced within the female body. When she dies, her cuticle tans, forming a protective layer around the eggs, termed a cyst.

1.2.2 Root-knot nematodes

Root-knot nematodes are classified in the *Meloidogyne* genus within the *Heteroderidae* family of the order Tylenchida and class Secernentea, setting them apart from cyst nematodes. While taxonomically distinct from cyst nematodes, root-knot nematodes exhibit the same mode of parasitism as sedentary endoparasites. These nematodes are so called because of the galls which resemble knots that they induce on the roots. They comprise almost one hundred *Meloidogyne* species (Subbotin *et al*., 2021) which, together with cyst nematodes, are the most economically damaging classes of plant parasitic nematode (Jones *et al*., 2013a, Sasser and Freckman, 1987).

In contrast to cyst nematodes, root-knot nematodes generally have very broad host ranges and between them, they can infect almost all species of vascular plant (Moens *et al*., 2010). For instance, *Meloidogyne incognita* alone has been found to infect over three thousand species of plants (Greco and Di Vito, 2009).

The life-cycle of a root-knot nematode begins when eggs are laid by the mature female into a gelatinous matrix in the soil (Figure 1.3). Similar to cyst nematodes, *Meloidogyne* species undergo their first moult from J1 to J2 whilst still inside the egg, where the eggshell, consisting of the same vitelline, chitinous and glycolipid layers as the cyst nematode's, provides protection (Moens *et al*., 2010). The hatching biology of root-knot nematodes is less well understood than that of cyst nematodes. However, it is known that the process comprises the same three steps described previously for cyst nematodes (Perry, 2002). In contrast, in *Meloidogyne* species, the initiation of the hatching process is triggered by the activation of the J2, rather than altered permeability of the eggshell (Perry, 2002). The permeability changes of the eggshell is suggested to be caused by protein secretions from the pharyngeal glands of the J2s themselves (Bird, 1968, Premachandran *et al*., 1988). This is highlighted in the cases of *M. incognita* and *Meloidogyne artiellia,* where enzymes likely to erode the lipid and chitin layers of the eggshell, such as chitinases and lipases, have been recognised as having a role in facilitating egg shell permeability (Perry *et al*., 1992, Mkandawire *et al*., 2022, Fanelli *et al*., 2005).

Root-knot nematodes hatch from their eggs into the soil as pre-penetrative J2s when environmental conditions such as temperature, to soil moisture content, and oxygen availability are optimal (Curtis *et al*., 2010). The spectrum of optimal conditions varies hugely between species and among different populations of the same species. This is particularly evident in the context of optimal temperatures, which can diverge significantly between populations inhabiting different global regions or in response to annual

fluctuations in temperature (Stephan, 1983, Khan *et al*., 2014, Vela *et al*., 2014). Unlike cyst nematodes, the impact of root exudates on the hatching process of root-knot nematodes is comparatively minor (Curtis *et al*., 2010). *In vitro* studies suggest that the majority of J2s in this genus hatch in water, without stimulation from root exudates (Curtis *et al*., 2010). Nevertheless, certain environmental conditions suggest a potential role for them. Notably, the hatching of *M. chitwoodi* eggs, laid by females living on young plants exhibited no reliance on root exudate stimulation, whereas eggs produced by females living on older plants, towards the end of the plant's growing season did require root exudates to hatch (Wesemael *et al*., 2006). This could represent a survival strategy employed by this species to ensure that J2s hatch exclusively when the proximity of a suitable host is guaranteed. In contrast, the presence of root exudate exhibited no discernible influence on the hatching of *Meloidogyne chitwoodi* (Wesemael *et al*., 2006), while in *M. incognita,* the presence of root exudate has been observed to actively suppress the hatching process in a number of studies (Yang *et al*., 2016, Kalaiselvam and Devaraj, 2011, Danahap and Wonang, 2015). In general, the necessity of root exudates for the hatching of *Meloidogyne* species is not a universal requirement.

Once hatched, pre-penetrative J2s detect root exudate gradients within the soil using their amphids and phasmids (Curtis *et al*., 2010), alerting them to the nearby presence of a suitable host (Trudgill, 2003). Like cyst nematodes, root-knot nematodes also move through the soil by undulatory propulsion (Burr and Robinson, 2004). They are able to migrate up to 30 cm within three days in this way in sand-loam to reach the roots of a suitable host (Flaherty *et al*., 1992). Here, they use their stylet to penetrate the root cortex close to the differentiation zone at the root tip (Wyss and Grundler, 1992, Abad *et al*., 2003). Once inside, the now parasitic J2 moves intercellularly between cortical cells, descending to the meristem and then turns 180° to move up into the vasculature (Wyss and Grundler, 1992). As they move intercellularly, unlike cyst nematodes, root-knot nematodes do not inflict damage as they migrate through the root. When they reach the vascular cylinder, root-knot nematodes briefly feed on protoxylem and protophloem cells (Khan *et al*., 2023). As sedentary parasites, they then prompt the re-differentiation of these cells, triggering the formation of a specialised feeding site supported by dorsal and sub-ventral gland secretions (Khan *et al*., 2023). This site consists of several "giant cells" which are functionally similar to the syncytia formed by cyst nematodes (Moens *et al*., 2010). These giant cells exhibit a heightened metabolic activity to accommodate the demands of the nematode's nutrient uptake and development (Abad *et al*., 2010). They are also considerably enlarged and can reach almost 400 times the size of normal vascular root cells (Abad *et al*., 2010), which causes

the root to swell, causing the characteristic swellings of infected roots. The nematode feeds from each of these cells sequentially for between three and eight weeks (Abad *et al*., 2010). During this time, the J2 absorbs nutrients from the plant into its intestine (Figure 1.1), which causes the nematode to swell. After this period, the J2 moults rapidly to become J3 and then J4 larval stages (which do not feed), before their final moult to become adults (Figure 1.3) (Moens *et al*., 2010).

Within the *Meloidogyne* genus, three distinct modes of reproduction exist. Similar to cyst nematodes, some root-knot nematodes reproduce by amphimixis, in which male and female gametes fuse in the embryonated egg (Chitwood and Perry, 2010). This mode of reproduction is only seen in *Meloidogyne carolinensis*, *Meloidogyne microtyla*, *Meloidogyne megatyla* and *Meloidogyne pini* (Chitwood and Perry, 2010), which are considered minor root-knot nematodes due to their restricted distribution and limited host range and opportunism (Jepson, 1987). For the majority of other species within this genus, reproduction takes place through one of two types of parthenogenesis. Facultative meiotic parthenogenesis or automixis, occurs when reproduction involves the fusion of male and female gametes when males are present (van der Beek *et al*., 1998). In the absence of males, the female gamete still undergoes a process of reduction through meiosis, but instead of fusing with the male gamete, it fuses with the second polar body produced in the reduction, thereby re-establishing the somatic chromosome number (van der Beek *et al*., 1998, Triantaphyllou, 1966). *Meloidogyne hapla, Meloidogyne exigua* and *M. chitwoodi* are examples of root-knot nematodes that reproduce in this way. In contrast, mitotic parthenogenesis, or apomixis is observed in the most prevalent and economically significant species, including *M. incognita, Meloidogyne javanica* and *Meloidogyne arenaria* (Triantaphyllou, 1962, Triantaphyllou, 1963, Triantaphyllou, 1981). In this process, the female gamete develops without undergoing meiosis, and the unfertilised egg originates from an unreduced oocyte (Castagnone-Sereno, 2006). In circumstances of environmental stress that lead to the production of males, they are not involved in fertilisation for these apomictic species; instead they influence population density and dynamics (Triantaphyllou, 1973). The only known nematodes to reproduce in this way are root-knot nematodes and threadworms (*Strongyloides ratti*) (Viney, 1994).

Consequently, the majority of *Meloidogyne* J4 juveniles ultimately develop into adult females. After several weeks of feeding from the giant cells, the mature female becomes saccate in shape (pyriform) (Moens *et al*., 2010). Anatomically, her ovaries are in contact with the intestine, which allows for the direct transfer of nutrients to developing eggs (Eisenback and Hunt, 2010), of which she can lay 30-40 per day (Karssen and Moens, 2006). Whilst her head remains at the feeding site, her posterior

body protrudes out of the root (Figure 1.3). Unlike cyst nematodes, the deposition of her eggs occurs directly onto the root surface within a protective gelatinous matrix consisting of a network of glycoproteins (Figure 1.3) (Sharon and Spiegel, 1993).

Much like cyst nematodes, root-knot nematodes have the ability to enter phases of arrested development or dormancy. These phases occurs during either the embryonic or larval stage, while the nematodes remain inside the egg before they hatch (De Guiran and Ritter, 1979). These nematodes exhibit comparable quiescence and diapause stages to those observed in cyst nematodes. These stages are entered into as a response to unfavourable environmental conditions (Evans and Perry, 2010).

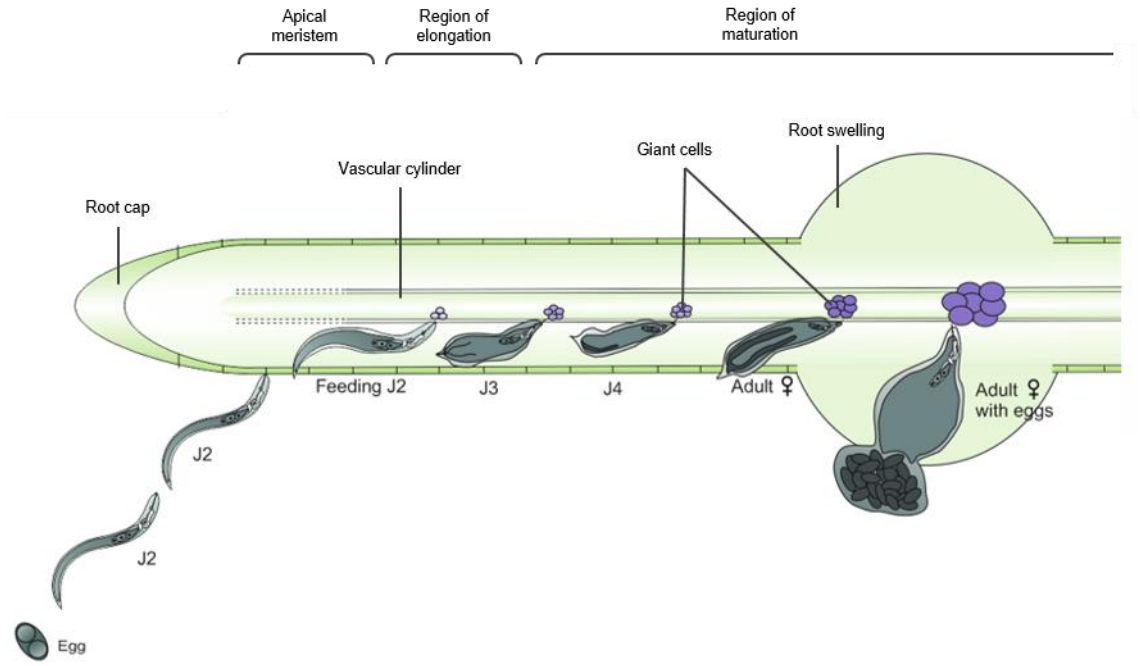


Figure 1.3- Life-cycle of root-knot nematodes.

Meloidogyne species undergo the first moult from J1 to J2 within the egg and hatch in the soil as pre-penetrative J2s. They migrate through the soil to the host plant root where they penetrate close to the root tip and migrate intracellularly to the vascular cylinder. Here, they induce the formation of “giant cells” which act as a permanent feeding site. Root-knot nematodes then undergo a further two moults to J3 and J4 larvae before their final moult to adults. The majority of adults in the economically important species are females, which are saccate in shape. They are sedentary endoparasites, remaining inside the host to feed from giant cells which causes galling of the root. Reproduction is primarily by parthenogenesis, and although the majority of these nematodes are female, a small number of males (not shown) are vermiform and migrate out of the root.

1.3 Nematode effectors

Effectors are specialised proteins secreted by pathogens that exert influence over the function and structure of host cells (Hogenhout *et al*., 2009). In plant parasitic nematodes, effectors are typically secreted, directly into cells of the plant, through the stylet (Hussey *et al*., 2002). They are produced mainly in the dorsal and sub-ventral pharyngeal glands (Rehman *et al*., 2016). However, certain effectors have been found to be synthesised in the amphids (Eves-van den Akker *et al*., 2014b, Vieira *et al*., 2011) or even secreted from the hypodermis directly onto the nematode cuticle (Jones *et al*., 2000, Spiegel *et al*., 1997). As reviewed by Vieira and Gleason (2019), effectors facilitate the successful parasitism of plant parasitic nematodes by enabling host entry and modulation of the host immune response. In sedentary endoparasites, such as cyst and root-knot nematodes, they are also involved in the development and maintenance of the feeding site (Lilley *et al*., 2018, Verma *et al*., 2018, Truong *et al*., 2015).

Effectors involved in host invasion and migration are most likely to be synthesised in the sub-ventral pharyngeal glands (Figure 1.1), as effectors produced here are known to have roles in early parasitism (Thorpe *et al*., 2014, Jones and Mitchum, 2018). Conversely, the formation and maintenance of the feeding site in sedentary endoparasites is likely to be orchestrated by effectors produced in the dorsal pharyngeal glands (Figure 1.1), as research highlights that effectors produced here contribute to later stages of the parasitic process (Mitchum *et al*., 2013).

Extensive research has been dedicated to identifying nematode effectors and there has been a particular focus on those produced by cyst and root-knot nematodes (Haegeman *et al*., 2012). In 2010, over 60 different types of effector had been described in these plant parasitic nematodes (Abad *et al*., 2010), but since then, a collection of over 100 have been identified *M. incognita* alone (Da Rocha *et al*., 2021). There are many different types of nematode effector and nematodes are thought to have evolved a distinct repertoire, tailored to their specific hosts. For example, effectors specific to cyst nematodes known as SPRYSECs (Sp1a and Ryanodine receptors (SPRY) domain containing proteins), have been found to play important roles during early parasitism by suppressing of the host's immune response (Diaz-Granados *et al*., 2016). Conversely, C-Terminally encoded peptide (CEP) plant peptide mimics (CEPs) play a role throughout the life-cycle of root-knot nematodes in imitating endogenous host peptides to help maintain the feeding site (Hu and Hewezi, 2018). Moreover, an assortment of cell wall-degrading enzymes are involved in manipulating host cell walls during various nematode life stages, from the initial root penetration process to the sedentary life stages in both cyst and root-knot nematodes (Bohlmann and Sobczak, 2014).

1.3.1 Cell wall-degrading enzymes

Plant cell walls are made up primarily of cellulose (Albersheim *et al*., 2010) and the success of plant parasitic nematodes depends entirely on their ability to overcome this physical barrier. The majority of pre-penetrative J2s, regardless of species, rely heavily on cell wall-degrading enzymes, a class of effector, to facilitate this. Expression profiles of genes encoding cell wall-degrading enzymes show that highest expression is in the early to late infective stages of many plant parasitic nematodes (Rai *et al*., 2015b). This suggests that they are utilised during early parasitism to degrade cellulose, and other polymers, of the plant cell wall in order to gain entry into the host and for cyst and root-knot nematodes to subsequently migrate to the vascular cylinder to form feeding sites.

The first cell wall-degrading enzyme to be identified in animals was an endo-1, 4-β-glucanase which is a cellulose-degrading enzyme (Smant *et al*., 1998). It was detected in the cyst nematodes: *G. rostochiensis* and *H. glycines* (Smant *et al*., 1998). Since then, cellulases that hydrolyse the 1,4-β linkages in cellulose, have also been found in sedentary plant parasitic nematodes, including: *Meloidogyne, Globodera*, and *Heterodera* species, as well as in migratory plant parasitic nematodes (*Pratylenchus, Radopholus,* and *Ditylenchus* species) (Kyndt *et al*., 2008, van Megen *et al*., 2009). Three distinct enzyme classes are encompassed by the term "cellulase", they are endoglucanases, cellobiohydrolases, and β-glucosidases (Patel *et al*., 2019). Interestingly, all nematode cellulases are members of the same glycosyl hydrolase family: GH5 (Kyndt *et al*., 2008). Glycosyl hydrolases represent a class of enzymes involved in the hydrolysis of glycosidic bonds in various carbohydrates, including cell wall polysaccharides (Bourne and Henrissat, 2001). Spanning across a spectrum of organisms including bacteria, fungi, plants and animals, these enzymes derive their nomenclature from their individual substrates (Bourne and Henrissat, 2001). For example, cellulases catalyse the breakdown of cellulose and xylanases target xylan. The CAZy database currently documents 128 distinct glycosyl hydrolase families (Drula *et al*., 2022), exemplifying the extensive diversity and importance of this enzyme category. As all of the cellulases found in nematodes belong to the same glycosyl hydrolase family it suggests that the genes encoding these enzymes were transferred to a common ancestor and passed down through evolutionary history to a broad range of plant parasitic nematode species. However, cellulases in *Bursaphelenchus xylophilus* belong to GH45 (Kikuchi *et al*., 2004), which are unrelated to GH5 cellulases, suggesting a different origin of these genes for this species.

Other cell wall-degrading enzymes that have been characterised in plant parasitic nematodes include xylanases, pectate lyases, and polygalacturonases which degrade

different components of the cell wall to facilitate host entry (Rai *et al*., 2015b, Macharia *et al*., 2023). The exact complement of effectors in plant parasitic nematodes is heavily dependent on host range and is tailored to the specific characteristics of the host cell walls. For instance, the pine wood nematode, *B. xylophilus,* infects *Pinus* species which possess highly lignified cells. This particular plant parasitic nematode has therefore evolved lignin-degrading enzymes (Rai *et al*., 2015b) to overcome this barrier. These enzymes are notably absent in other species like cyst nematodes that do not infect such hosts.

In the absence of cell wall-degrading enzymes, plant parasitic nematodes have a reduced ability to penetrate and therefore parasitise their host plants. For example, the reduction in transcript by RNA interference (RNAi) of genes encoding the cell wall-degrading enzymes *hg-pel* (pectate lyase) and *hg-eng-1* (β-1, 4-endoglucanase) in *H. glycines* reduces the ability of J2s to penetrate the root successfully (Bakhetia *et al*., 2007). Similarly, silencing of cellulase genes in J2s of *G. rostochiensis* also decreased their ability to infect the roots of their hosts (Chen *et al*., 2005, Rehman *et al*., 2009). Furthermore, the knock-down of cellulose binding proteins in *H. schachtii* by *in planta* RNAi led to significant reduction in infection of *A. thaliana* (Joshi *et al*., 2022). These studies evidence an essential role of cell wall-degrading enzymes in early parasitism (Thorpe *et al*., 2014). If plant parasitic nematodes are unable to penetrate the host, they are unable to become established and therefore fail as parasites.

It is generally accepted that many of the plant parasitic nematode genes encoding cell wall-degrading enzymes have been acquired through horizontal gene transfer (Rai *et al*., 2015b, Yan *et al*., 1998). This is the process by which genes or genetic material is transferred from one organism to another and is different from vertical inheritance where genetic material is passed from a parent to its offspring. Soil bacteria and fungi are the most likely sources of transfer due to the homology observed between cell wall-degrading enzyme encoding genes in bacteria, fungi, and plant parasitic nematodes (Rai *et al*., 2015b, Danchin *et al*., 2010, Jones *et al*., 2005). This assumption is supported by the fact that the free-living nematode, *C. elegans,* and the animal parasitic nematode, *Brugia malayi,* lack these enzymes (Kikuchi *et al*., 2004).

1.4 Responses of plant parasitic nematodes to detection of host-derived signals

Root exudates are known to encourage the growth of beneficial soil microorganisms (Meier *et al*., 2017, Mommer *et al*., 2016), which implies that plant parasitic nematodes could similarly respond to such stimulation. As previously stated, root exudates contain compounds that not only trigger the hatching of some plant parasitic nematodes, but also

direct them towards the roots of their host plants. It is therefore plausible that detection of these host-derived signals induces changes in nematode behaviour and/or gene expression, potentially enhancing their parasitic success.

1.4.1 Behavioural modifications

There is evidence that plant parasitic nematodes alter their feeding behaviour in response to the detection of root exudate as they migrate towards and penetrate the roots of their host plants to feed. Feeding behaviour involving movement of the stylet is known to be regulated by serotonin in cyst and root-knot nematodes, and more recently, a role for this neurotransmitter has been observed in the feeding behaviour of *Pratylenchus penetrans* (Han *et al*., 2017). Root exudates from the model plant organism *Arabidopsis thaliana* contain active compounds, known as kairomones, (Huettel, 1986) which initiate stylet thrusting in pre-penetrative *M. incognita* J2s *in vitro*, similar to that observed at the root surface (Teillet *et al*., 2013). It is suggested that these kairomones could activate serotonin signalling pathways leading to the changes in behaviour observed in *M. incognita.*

Likewise, *H. schachtii* showed a similar response to mustard root exudate *in vitro* (Grundler *et al*., 1991a). Increased stylet thrusting and aggregation of pre-penetrative J2s were recorded for this species when exposed to root exudates from these plants on agarose gel discs (Grundler *et al*., 1991a). Stylet thrusting *in vitro* was more pronounced when the stylet was in contact with another nematode or the bottom of the petri dish, perhaps mimicking enhanced stylet activity at the root surface (Grundler *et al*., 1991a). Similar results were also seen when exposing *Pratylenchus scribneri* to corn root exudate (Tsai and Van Gundy, 1990), suggesting this phenomenon is common in many plant parasitic nematode species.

Information regarding altered chemotaxis behaviour in plant parasitic nematodes in response to root exudates is contradicting in many cases and much of the data has been obtained through *in vitro* behavioural experiments. Although studies suggest that many species of plant parasitic nematodes are attracted to root exudates (Liu *et al*., 2019, Grundler *et al*., 1991a, Wuyts *et al*., 2006, Farnier *et al*., 2012), *in vitro* studies such as these are unable to artificially replicate stimulus gradients in the soil generated by expulsion of compounds by the plant and so, findings therefore cannot be generalised to the field.

1.4.2 Transcriptional regulation

Differential gene expression is likely to drive the changes in physiology and behaviour discussed. Many animal parasitic nematodes have been shown to alter their gene

expression to upregulate the transcription of proteins that allow them to successfully parasitise their hosts. This is seen in *Ancylostoma caninum,* for example, where genes encoding lysozymes, proteases, and lectins are upregulated when the nematodes are inside the gut of their canine hosts (Mulvenna *et al*., 2009). Similarly, *Heligmosomoides polygyrus,* a parasitic nematode of rodents, has been shown to differentially regulate the expression of protease and lysozyme coding genes upon host detection (Zarowiecki and Berriman, 2015). The upregulation of these genes is thought to increase gut permeability in the host, which increases the availability of nutrients to the parasites.

This occurrence is also seen in plant parasitic nematodes and the detection of root exudates by pre-penetrative J2s is known to cause changes in gene expression. Differential expression of *M. incognita* genes was observed when exposing J2s to roots and root exudates from *A. thaliana* plants compared to when not exposed to a host (Teillet *et al*., 2013). Stimulation of protein secretion was also reported in this species in response to legume root exudates (Zhao *et al*., 2000) and similar responses have been seen in *G. rostochiensis* in response to potato root exudate (Smant *et al*., 1998). Similarly, when populations of *G. pallida* J2s are exposed to a susceptible host, distinct gene expression patterns are seen compared to populations exposed to resistant hosts (Kooliyottil *et al*., 2019, Kud *et al*., 2022). Recent research by Bell *et al*. (2019) indicates that gene expression changes could vary depending on the host. Results revealed the host-specific expression of two genes encoding cell wall-degrading enzymes in *Pratylenchus coffeae.* Upon detection of root exudate, each of the genes exhibited distinct expression patterns, with higher concentrations of their specific substrates leading to more significant up-regulation (Bell *et al*., 2019). It is possible that other plant parasitic nematodes may also have this ability.

It is not yet understood exactly how sensing of root exudate components leads to the altered gene expression in the pharyngeal gland cells that has been observed in plant parasitic nematodes. As previously mentioned, serotonin signalling is involved in nematode behaviour (Han *et al*., 2017) and it hypothesised that differential gene expression might also be attributed to neurological pathways. Stimuli are probably perceived by sensory neurones in the amphids and phasmids, and signalling pathways are activated to induce the changes observed. G-protein coupled receptors might be involved in subsequent signal transduction as, compared to ionotropic receptors, the binding of neurotransmitters to G-protein coupled receptors, mediates slower and longer lasting effects, such as changes to transcription (Koelle, 2018). As this type of receptor responds to external stimuli to generate intracellular responses, it is likely that they are involved in the responses to the detection of root exudates observed in previous studies.

Host-derived cues trigger a series of complex behavioural and molecular changes in plant parasitic nematodes. While the exact mechanisms governing these alterations remain partially elusive, understanding the reasons behind these changes is relatively straightforward. These adaptations are believed to play a role in priming nematodes for host penetration and equipping them for a successful parasitic lifestyle within the host plant. Understanding the mechanisms underpinning the nematode's response to potential host detection will provide valuable insights into their pathogenicity. Ultimately, the characterisation of specific genes or pathways involved in their host could lead to the development of targeted control strategies capable of disrupting or hindering the parasitic life-cycle. As many of the current management strategies for plant parasitic nematodes are ineffective, the development of novel control methods would help to mitigate yield losses and contribute to food security by enhancing agricultural productivity.

This project therefore endeavours to uncover the previously unexplored molecular mechanisms governing the host-parasite interactions of the most economically damaging plant parasitic nematodes. The cyst nematode, *G. pallida,* and the root-knot nematode, *M. incognita,* were selected for investigation as they represent species with narrow and broad host ranges, respectively. The hypothesis suggests that these nematodes will respond to host-derived cues in a host-specific manner, and that the extent of the responses may correspond to host range. By investigating and characterising the specific behavioural and transcriptional modifications in these species in response to host-specific cues, novel targets for control could be revealed. If this objective is achieved, this project aims to further characterise potential gene targets to reveal their potential roles within the context of parasitism. This exploration aims to inform precise and targeted control strategies, aimed at mitigating the economic impact of these plant parasitic nematodes.

1.5 Project aims

- To elucidate previously unknown aspects of the molecular mechanisms that underpin host-parasite interactions in two distinct nematode species: *G. pallida* and *M. incognita.*

- To determine whether or not *G. pallida* and *M. incognita* respond to host-specific cues in the form of host root exudates.

- To verify whether or not *G. pallida* and *M. incognita* have the ability to alter gene expression in a host-specific manner and to determine the extent of this using RNA-Seq.

- To further characterise genes that could be involved in parasitism and investigate their potential roles using RNAi.

Chapter 2
General Materials and Methods

2.1 Biological material

2.1.1 Plant maintenance

Potato (*Solanum tuberosum* "Désirée") plants were grown from tubers in a 1:1 sand/loam (Norfolk Topsoil; Bailey's of Norfolk) mixture in glasshouses at approximately 20 °C with 50-60 percent relative humidity and 16 hours of light per day. Cabbage (*Brassica oleracea* "Golden Acre Primo II") and carrot (*Daucus carota* "Nantes 2") plants were grown from seed in the same glasshouses in compost.

Tomato (*Solanum lycopersicum* "Ailsa craig") and maize (*Zea mays* "Earlibird F-1") plants were grown in potting compost (No.2 Potting Supreme; Petersfield Growing Mediums) from seed in glasshouses at 24 °C with 50-60 percent relative humidity with 16 hours of light per day.

2.1.2 Root exudate preparation

Plants approximately four weeks old were removed from soil/compost and the roots of the intact plant were washed in tap water. Roots were then immersed in sterile tap water for 16 hours at room temperature. After this duration, the roots were removed from the plant and subsequently weighed. Adjustments were made to the volume to ensure a final concentration of 80 g/L. Immediately after they were prepared, root exudates were filtered through filter paper (Whatman W1) before they were filter sterilised using a syringe filter (0.22 µm) and stored at 4 °C until required. This method was employed to prepare root exudate from cabbage, carrot, maize, potato and tomato plants.

2.1.3 Nematode maintenance and extraction

2.1.3.1 *Globodera pallida*

Globodera pallida from the "Lindley" population were cultured on the susceptible potato cultivar, Désirée, at an inoculum level of 20-30 eggs/g growing in 50:50 sand:loam soil in a glasshouse at 20 °C with 16 h day length. Cysts that had undergone diapause at 4 °C were separated from soil using the Fenwick can method (Fenwick, 1940, Camacho *et al*., 2018), where soil containing cysts was washed into a Fenwick can through a wide-meshed sieve. Cysts floated to the top and were therefore washed over the edge of the Fenwick can and collected on a fine-meshed sieve (150 µm). Cysts were then transferred in water to a sealed funnel containing a fast-flow filter paper and encouraged to the edges. The clamp sealing the funnel outlet was released and water flowed out of

the funnel, leaving *G. pallida* cysts (and other floating debris) on the filter paper at the previous water line.

A stereo-binocular microscope was then used to collect the cysts from the filter paper before they were surface sterilised using hexadecyltrimethylammonium bromide (CTAB) (0.5 mg/ml) and chlorhexidine digluconate (0.1 %) for 30 minutes. Cysts were washed in sterile tap water and placed on top of a nylon mesh (30 µm) in potato root exudate (80 g/L). They were then incubated in darkness to hatch at 20 °C. After approximately five days, J2s were collected and the root exudate was replaced. Daily collection of J2s continued for up to a week after the first hatching of eggs was observed and J2s were washed with sterile tap water and maintained at 10 °C until required.

2.1.3.2 *Meloidogyne incognita*

Meloidogyne incognita VW6 population were cultured on tomato roots ("Ailsa Craig") that were grown in potting compost (No.2 Potting Supreme; Petersfield Growing Mediums) in glasshouses at 24 °C with 50-60 percent humidity. In order to infect new plants, previously infected roots with obvious egg masses were chopped into short pieces and mixed with fresh potting compost, which was used to re-pot four week old tomato plants.

J2s of *M. incognita* were collected from the roots of tomato plants that had been infected for eight weeks. The roots were removed from above ground tissue, cleaned, and transferred to a nylon mesh covered with a layer of tissue paper, above a funnel. This was placed inside a misting chamber where the spray of water (five minutes each hour) encouraged nematodes to hatch and J2s to migrate out of the root and down through the funnel into 50 ml collection tubes. Due to the slow rate of water flow, J2s settled to the bottom of the tube. Nematodes were collected at daily intervals and were washed with sterile tap water before being maintained at 10 °C until required.

An alternative method of *M. incognita* J2 extraction was employed when large numbers of J2s were required. Roots were collected from tomato plants that had been infected for eight weeks and they were cut into 2-3 cm pieces. These root pieces were shaken in 1 % sodium hypochlorite solution for two minutes to dissolve the gelatinous matrix of the egg masses. The hypochlorite solution containing the released *M. incognita* eggs was then poured over nested 150, 63 and 25 µm pore sieves and washed with water. Eggs and fine root debris were washed from the 25 µm pore sieve into 50 ml collection tubes and centrifuged at 1,200 rcf for 10 minutes to generate a pellet. This pellet was re-suspended in 20 ml 40 % sucrose before 10 ml water was layered on top. The solution was centrifuged at 1,200 rcf for 10 minutes and the eggs were collected from the sucrose-water interface using a glass Pasteur pipette. The sucrose was diluted by transferring the eggs into a large volume of water. They were recovered by passing them

through a 25 µm sieve. The eggs were then surface sterilised using a solution of CTAB (0.5 mg/ml), chlorhexidine digluconate (0.1 %) and Tween-20 (0.01 %) for 30 minutes. Eggs were washed in sterile tap water and placed on top of a nylon mesh (20 µm) in sterile tap water. They were incubated in darkness to hatch at approximately 25 °C. J2s were collected daily and they were maintained at 10 °C until required.

2.2 General molecular techniques

2.2.1 Total RNA extraction

For each nematode species, total RNA was extracted from approximately 20,000 J2s using E.Z.N.A® Plant RNA Kit (Omega Bio-tek) following the manufacturer's protocol. Briefly, snap-frozen nematode tissue was ground by means of a TissueLyser LT (Qiagen) using 2 mm and 5 mm beads which disrupted the nematode and released cellular components. RB buffer (mixed with 2-mercaptoethanol) was added to stabilise the samples and to denature RNases and other cellular proteins. The mixture was transferred through a homogeniser column to remove debris. Ethanol (70 %) was combined with the supernatant and this was passed through a HiBind® RNA Mini Column which bound RNA to the membrane surface. The optional DNase I digestion step was performed to eliminate genomic DNA and the samples were then washed with a series of RNA wash buffers to remove impurities. Total RNA was eluted in diethyl pyrocarbonate (DEPC)-treated water. Whenever RNA was used for quantitative real-time polymerase chain reaction (qRT-PCR), an additional DNase digestion was carried out using a TURBO™ DNA-free kit (Invitrogen™). Briefly, RNA was incubated with 0.1 volume 10 X TURBO™ Buffer and TURBO™ DNase (1 µl) at 37 ºC for 20 minutes. 0.1 volume DNase Inactivation Reagent was then added and samples were incubated at room temperature (~20 ºC) for five minutes. Spectrophotometry (Thermo Scientific Nanodrop™ 1000) was then used to determine the quality and the concentration (ng/µl) of the RNA. It was then stored at -80 °C until required.

2.2.2 cDNA synthesis

Unless otherwise stated, first strand cDNA for amplification of full-length coding regions was synthesised using SuperScript™ II reverse transcriptase (Invitrogen™) from total RNA (~250 ng) previously extracted from J2 nematodes. 1 µl Oligo-dT primers (5'-AAGCAGTGGTATCAACGCAGAGTACT(30)) and 1µl dNTPs (10 mM) was added to the RNA which was incubated for five minutes at 65 °C. Samples were then chilled on ice whilst 4 µl 5 X first strand buffer and 2 µl dithiothreitol (DTT) (0.1 M) were added. A short incubation (2 minutes) at 42 °C preceded the addition of 1 µl reverse transcriptase (200 U/µl) and the final incubation at the same temperature for 50 minutes.

The reaction, with a total volume of 20 µl, was inactivated at the end with a 15 minute incubation at 75 °C.

cDNA for the amplification of genes by qPCR was synthesised from ~250 ng RNA using iScript™ cDNA synthesis kit (BioRad). iScript™ Reverse Transcriptase (1 µl) and 5 X iScript™ reaction mix (4 µl) containing dNTPs and Oligo-dT primers was added to the RNA in a final volume of 20 µl. The mixture was incubated at 25 °C for 5 minutes to allow for priming, 42 °C for 20 minutes to allow for reverse transcription, and then 95 °C for 1 minute to inactivate the reverse transcriptase enzyme.

cDNA was stored at -20 °C.

2.2.3 Polymerase chain reaction (PCR)

cDNA was used as a template for the amplification of genes of interest throughout this thesis using a range of DNA polymerases, depending on the experiment and the fidelity required. These include Phusion™ high-fidelity DNA polymerase (New England Biolabs), MyTaq™ polymerase (Bioline) and OneTaq® (New England Biolabs). General cycling parameters were as follows: 30-60 seconds denaturing phase at 94-98 °C, followed by 30 cycles of 5-10 seconds at 94-98 °C, 20-30 seconds at the specific annealing temperature for each primer pair and 20-30 seconds at 72 °C. The final extension step was between five and 10 minutes at 72 °C. Specific DNA polymerase details and incubation times and temperatures are provided in each chapter.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to determine the size of DNA fragments throughout this thesis. 1 % w/v agarose was dissolved in TAE (Tris/Acetate/EDTA) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) by microwaving. GelRed (1:40,000 dilution) (Biotium) was added and the mixture was allowed to set in an appropriate gel tray with comb. An aliquot of amplified DNA or plasmid digest was combined with a loading buffer (6 X) (New England Biolabs) and loaded into separate wells within the gel before it was electrophoresed in TAE buffer at 80 Volts for approximately 40 minutes, or until relevant bands could be visualised under UV light. Fragment size (kb) was determined with the aid of a molecular ladder (1 kb) (New England Biolabs).

2.2.5 Purification of PCR products

DNA fragments matching the expected size of the specific genes of interest were purified from the remaining portion of successful amplification reactions using an E.Z.N.A® Cycle Pure Kit (Omega Bio-tek). CP buffer (four times the volume of the PCR

product) was added to the PCR product and this was passed through a HiBind® Mini Column to bind DNA to the membrane. The column was washed with a wash buffer to remove salt residues and purified DNA was eluted in 30 µl elution buffer.

When the fragment of interest was accompanied by other fragments, representing non-specific PCR products, a gel extraction was performed to purify the DNA using an E.Z.N.A® Gel Extraction Kit (Omega Bio-tek). Briefly, the fragment of interest was cut from the gel using a scalpel. This was combined with binding buffer (XP2) and incubated at 60 °C until the gel had melted. The mixture was vortexed and passed through a MicroElute® LE DNA column to bind the DNA to the membrane. The membrane was washed with wash buffer and DNA was eluted with 20 µl of elution buffer.

All purified DNA was stored at -20 °C.

2.2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

In this thesis, the relative expression of genes of interest was determined using qRT-PCR performed using a CFX Connect™ Real-Time System (Bio-Rad). Before evaluating the experimental samples, the efficiency of a primer pair for each gene of interest was assessed by generating a standard curve using a 3-fold dilution series of cDNA. All primer pairs subsequently used for qRT-PCR had an efficiency between 95 and 105 percent.

Each qRT-PCR reaction was set up in individual wells of a 96-well polyethylene plate, consisting of cDNA template (5 µl) (generated using iScript™ cDNA synthesis kit (Section 2.2.2), Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad) (10 µl), combined primer pair (7.5 µM each) (0.8 µl) and distilled water (4.2 µl). Cycling parameters included a 10 minute denaturing phase at 95 ºC, followed by 40 cycles of 30 seconds at 95 ºC and 10 seconds at 60 ºC. Fluorescence was recorded at each 60 ºC stage. After the 40 cycles, a dissociation curve was plotted for each reaction by measuring the fluorescence of each well during a temperature increase from 60 ºC to 95 ºC. Each sample was run in technical triplicate, and negative controls containing purified water instead of cDNA were included for each gene of interest.

The CFX Manager™ software (Bio-Rad) was utilised to determine the Ct value for each sample which represents the cycle number at which the threshold fluorescence is reached. The Ct value is indicative of the amplification level and allows the estimation of the relative abundance of the target transcripts. For example, a change of one Ct value corresponds to a two-fold difference in transcript abundance. Ct values from each sample were used to calculate the fold change in gene expression relative to a control using the Livak ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Briefly, the difference in

the Ct value (ΔCt) of each gene compared to the reference genes was calculated for each sample using the following equation:

$$\Delta Ct = Ct(gene\ of\ interest) - Ct(geometric\ mean\ of\ reference\ genes)$$

The ΔCt was then used to calculate the difference in gene expression between the treated and control samples (ΔΔCt) using the following equation:

$$\Delta\Delta Ct = \Delta Ct(treated\ sample) - \Delta Ct(control\ sample)$$

In all qRT-PCR experiments described in this thesis, the geometric mean of two reference genes was used in the calculation of the ΔCt. For *G. pallida,* Elongation factor 1A (*Gp-EF1-A*) and Initiation factor 1 (*Gp-EIF1*) were selected as reference genes, while for *M. incognita,* Actin 2 (*Mi-Act-2*) and PTP (*Mi-PTP*) were selected. The reference genes were chosen because their expression levels were confirmed to be stable in J2 nematodes (Hu and DiGennaro, 2019, Sabeh *et al*., 2018).

2.2.7 *In situ* hybridisation

2.2.7.1 Preparation of single-stranded digoxigenin-labelled DNA probes for *in situ* hybridisation

The first step in *in situ* hybridisation is the generation of small DNA templates (150-200 bp) for the single-stranded digoxigenin-labelled DNA probes. Briefly, cDNA was synthesised using an iScript™ cDNA synthesis kit (Bio-Rad) from 200 ng total RNA extracted from J2 nematodes. Each gene fragment of interest from *G. pallida* and *M. incognita* was then amplified from 2 µl cDNA by PCR using 10 µl MyTaq™ Red Mix (Bioline) and 1 µl each sense and antisense primers (10 µM) (detailed in specific chapters). The total reaction volume was 20 µl. Cycling parameters were three minutes denaturing phase at 95 °C followed by 30 cycles of: 15 seconds at 95 °C, 15 seconds at 55 °C and ten seconds at 72 °C. This was followed by a final extension step of ten minutes at 72 °C. The resultant products were visualised using agarose gel electrophoresis. They were then purified using the E.Z.N.A® Cycle Pure kit (Section 2.2.5) before being used as templates for subsequent probes.

Single-stranded digoxigenin-labelled antisense DNA probes of between 150 and 200 bp in length were then generated for these genes from the gene-specific DNA templates. In a total volume of 20 µl, template DNA was combined with 1.5 µl 10 X digoxigenin DNA labelling mix (Roche, Germany), 4 µl antisense primer (10 µM), 0.2 µl OneTaq® DNA polymerase, and 4 µl 5 X OneTaq® reaction buffer (New England Biolabs). Cycling parameters were as follows: two minutes at 94 °C followed by 30 cycles of 15 seconds at 94 °C, 15 seconds at the appropriate annealing temperature and 90 seconds at 72 °C.

The final extension was at 72 °C for four minutes. Control, sense probes were generated in the same way, using sense primers (10 µM) (detailed in specific chapters), in separate reactions. Agarose gel electrophoresis was used to visualise the probes.

2.2.7.2 Fixation, permeabilisation, hybridisation and staining of nematodes

The fixation, permeabilisation, hybridisation and staining of J2s was performed according to the protocol published by Kud *et al*. (2019). Briefly, approximately 50,000 J2s were fixed in formaldehyde in M9 buffer (2 %) for 18 hours at 4 ºC and for a further four hours at room temperature (~20 ºC). They were then cut into two or three pieces using a razor blade and digested with proteinase K (0.5 mg/ml) in M9 buffer for 30 minutes. The nematodes were frozen for 15 minutes on dry ice and then re-suspended in ice cold methanol for 30 seconds, followed by ice cold acetone for one minute. Nematodes were slowly rehydrated with RNase free water and then hybridised with heat-denatured single-stranded digoxigenin (DIG)-labelled DNA probes overnight at 50 ºC.

Hybridised nematodes were washed three times with 4 X saline sodium citrate buffer (SSC) and three times with 0.1 X SSC/0.1 % SDS at 50 ºC. Washed nematodes were incubated in blocking reagent (1 %) in maleic acid buffer (Roche, Germany) for 30 minutes. The nematodes were then labelled for two hours with anti-digoxigenin-AP fab fragments diluted 1:1000 in blocking reagent.

Nematodes were stained with nitro blue tetrazolium (337 µg/ml; Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (175 µg/ml) in alkaline phosphatase detection buffer (Roche) overnight at 4 ºC. Stained nematodes were washed twice in Tween-20 (0.01 %) and viewed under a microscope (Zeiss AXIO Scope A1). Images were captured on an Axicam 305 Color using Zen (2.6 blue edition) imaging software. To verify gland cell expression, the staining location was compared to that in published images.

2.3 General solutions

2.3.1 Tris/Acetate/EDTA (TAE) buffer

0.5 M EDTA (pH 8) was generated by dissolving EDTA disodium salt in distilled water (0.186 g/ml) and autoclaving. A 50 X stock solution of TAE buffer was then prepared by adding 242 g Tris base to 600 ml distilled water before adding 100 ml 0.5 M EDTA and 57.1 ml glacial acetic acid. The volume was then made up to 1 L with distilled water. The 50 X stock solution was diluted 1:50 in distilled water to generate a 1 X working solution for use in agarose gel electrophoresis.

2.3.2 M9 buffer

A 10 X stock solution of M9 buffer was prepared by dissolving 30 g KH_2PO_4, 60 g Na_2HPO_4 and 5 g NaCl in 1 L distilled water. This solution was autoclaved at 121 °C and once cooled, 1 ml $MgSO_4$ (1 M) was added. This was then diluted 1:10 in distilled water to generate a 1 X working solution for use in *in situ* hybridisation.

2.4 General media

2.4.1 Luria-Bertani (LB) growth medium

LB growth medium, or broth, was prepared by dissolving tryptone (10 g/L), NaCl (5 g/L) and yeast extract (5 g/L) in distilled water and autoclaving at 121 °C. To generate solid media, 1 % bacteriological agar (w/v) was added before autoclaving.

2.5 Antibiotics

Antibiotic name	Working concentration
Ampicillin	100 µg/ml
Kanamycin	25 µg/ml

Table 2.1- List of antibiotics

Chapter 3
Identification and characterisation of cell wall-degrading enzymes in *G. pallida* and *M. incognita*

3.1 Introduction

Mature plant cell walls are extremely strong and complex structures consisting of a diverse array of polymers including cellulose, hemicelluloses, pectins, lignin and proteins (Figure 3.1) (Alberts *et al*., 2008, Pettolino *et al*., 2012). In the root tip, where growth is actively occurring, the primary cell walls of newly synthesised plant cells, termed primary cell walls, are thin and elastic to accommodate cellular expansion (Cosgrove and Jarvis, 2012). As cells move into the maturation zone of the root, they undergo a transformation. Here, the secondary cell wall is formed through the successive deposition of additional layers of matrix within the pre-existing primary cell wall once cell growth has stopped (Cosgrove and Jarvis, 2012). The secondary cell wall differs significantly in composition from the primary cell wall and its distinct composition and augmented thickness enhances its mechanical strength and rigidity, allowing it to withstand mechanical stresses and provide structural support to the plant cells (Alberts *et al*., 2008).

Each of the components of the plant cell wall plays a unique role. Cellulose constitutes approximately 95 percent of plant cell walls (Figure 3.1) (Albersheim *et al*., 2010, Alberts *et al*., 2008) and this polysaccharide confers the tensile strength to the cell wall. Hemicelluloses, such as xylan, xyloglucan and mannan play an important role in reinforcing the cell wall by cross-linking with the cellulose (Figure 3.1) (reviewed by Scheller and Ulvskov (2010)). Pectin contributes to cell-cell adhesion and water retention which facilitates intercellular communication and maintenance of cell turgor (Shewry, 2003). Lignin, found in secondary cell walls, confers rigidity and structural support to the plant tissues (Liu *et al*., 2018).

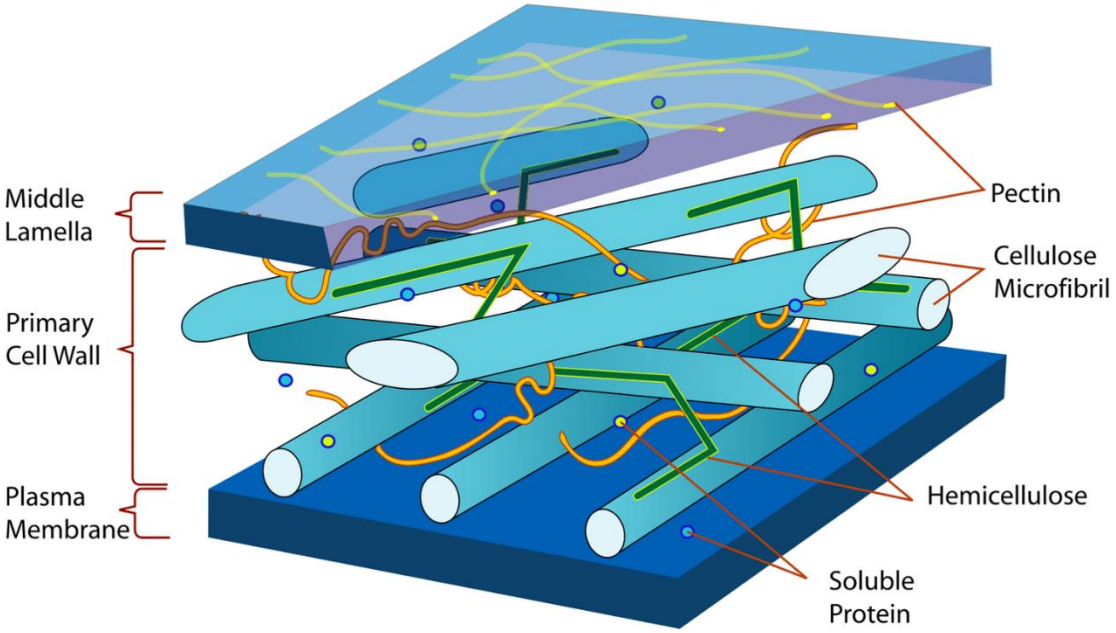


Figure 3.1- Structure of the plant cell wall.

Schematic of the structure of the plant cell wall (Sticklen, 2008).

The success of plant parasitic nematodes depends entirely on their ability to overcome this physical barrier and the majority of pre-penetrative J2s, regardless of species, rely heavily on cell wall-degrading enzymes, a class of effector, to facilitate this. Cell wall-degrading enzymes, such as cellulases, have roles in modifying and degrading the plant cell wall. The first cell wall-degrading enzyme to be identified in nematodes was an endo-1, 4-β-glucanase. It functions by catalysing the breakdown of cellulose through targeting of the β-glycosidic bonds within its structure. This enzyme was initially observed in *G. rostochiensis* and *H. glycines* (Smant *et al*., 1998). Subsequently, other cell wall-degrading enzymes have been characterised in plant parasitic nematodes including cellulases, endoxylanases, pectate lyases, and polygalacturonases, each specialising in the degrading distinct components of the cell wall to facilitate host entry (Rai *et al*., 2015b). In the absence of these enzymes, plant parasitic nematodes, regardless of species, have a reduced ability to parasitise their host plants (Bakhetia *et al*., 2007, Chen *et al*., 2005, Joshi *et al*., 2022, Rehman *et al*., 2009, Thorpe *et al*., 2014). If plant parasitic nematodes are unable to penetrate the plant cell wall, they are unable to establish themselves and consequently cannot thrive as parasites.

Expression profiles of genes encoding cell wall-degrading enzymes show that highest expression is in the early to late infective stages of many plant parasitic nematodes (Rai *et al*., 2015b). This suggests that they are utilised during early parasitism to degrade polymers of the plant cell wall to gain entry into the host, and for cyst and root-knot nematodes, to subsequently migrate to the vascular cylinder where they form their feeding sites.

Variations in types and quantities of cell wall-degrading enzymes between nematode species suggests a potential relationship with host range specificity. Differences in the cell wall-degrading enzyme repertoires could enable different nematode species to target the specific cell wall components of their chosen hosts. The diversity of cell wall-degrading enzymes present in some nematodes may contribute to their ability to infect a wide range of hosts, or to exhibit host-specificity.

Cellulases are made up of three classes of enzyme including endoglucanases, cellobiohydrolases and β-glucosidases (Patel *et al*., 2019). These enzymes work together to modify and degrade cellulose with in the plant cell wall. Endoglucanases facilitate the cleavage of internal β-glycosidic bonds within the cellulose chain, while cellobiohydrolases further disassemble the cleaved cellulose into cellobiose. Finally, β-glucosidases complete the process by breaking down cellobiose into its constituent components (Patel *et al*., 2019). Cellulase enzymes have been found in sedentary plant parasitic nematodes, including: *Meloidogyne, Globodera*, and *Heterodera* species, as

well as in migratory plant parasitic nematodes (*Pratylenchus, Radopholus,* and *Ditylenchus* species) and interestingly, they are all members of the same glycosyl hydrolase family: glycosyl hydrolase family 5 (GH5) (Kyndt *et al*., 2008). Glycosyl hydrolases act to break down carbohydrates by hydrolysing the glyosidic bonds holding glucose monomers together (Henrissat and Davies, 1997). They specifically target β-1,4-linked polysaccharides, including cellulose (Henrissat and Davies, 1997).

In contrast, endoxylanases belong to a separate family of glycosyl hydrolases, GH30, which is a diverse family of enzymes found in a variety of organisms, including bacteria, fungi, and nematodes (Mitreva-Dautova *et al*., 2006). GH30 enzymes are involved in the break-down of various carbohydrates, and like GH5 enzymes, they are involved in the hydrolysis of glycosidic bonds. Specifically, endoxylanases have endo-β-1,4-xylanase activity against xylan, the most abundant hemicellulose found in plant cell walls (Albersheim *et al*., 2010, Alberts *et al*., 2008). Genes belonging to this family are not as well characterised as those in the GH5 family and have only been described in the literature for *M. incognita* (Mitreva-Dautova *et al*., 2006), *M. javanica* (Macharia *et al*., 2023), *P. penetrans* (Vieira *et al*., 2015), *R. similis* (Vieira *et al*., 2021) and *B. xylophilus* (Shinya *et al*., 2021).

Recent findings suggest that plant parasitic nematodes alter the transcription of specific genes that encode cell wall-degrading enzymes in response to host-specific cues. Bell *et al*. (2019) found that when exposed to root exudates, *P. coffeae* displays differential expression of a cellulase-coding gene (*Pc-eng-1*) and an endoxylanase-coding gene (*Pc-xyl*). Notably, this differential expression was seen to be host-specific, with the variations in expression correlating with the concentrations of each of the enzyme substrates within the root exudates (Bell *et al*., 2019).

It was hypothesised that this capability might extend to other plant parasitic nematodes, potentially encompassing those with different lifestyles. *G. pallida* and *M. incognita* represent two sedentary endoparasitic species with a narrow and broad host range, respectively. Building on the current knowledge, the initial aims were to identify and further characterise genes encoding cell wall-degrading enzymes in these species that are similar to those that displayed host-specific expression in *P. coffeae.* This would provide insights into the repertoires of cell wall-degrading enzymes within these species and could offer a more comprehensive understanding of their functions and potential roles in host infection. Ultimately, this will allow for an increased understanding of the molecular basis of parasitism in these species which could provide insights into potential avenues of targeted management strategies.

3.2 Aims

- To identify genes encoding cell wall-degrading enzymes in *G. pallida* and *M. incognita* similar to those observed to be differentially regulated in *P. coffeae.*

- To clone putative cellulase and endoxylanase genes from *G. pallida* and *M. incognita.*

- To determine spatial expression of putative cellulase and endoxylanase genes in *G. pallida* and *M. incognita* using *in situ* hybridisation.

- To confirm enzyme activity of putative cellulase and endoxylanase genes in *G. pallida* and *M. incognita* using a bacterial protein expression system.

3.3 Materials and Methods

3.3.1 Identification of genes encoding cell wall-degrading enzymes in *G. pallida* and *M. incognita*

It was previously shown that an endoglucanase gene (*Pc-eng-1*) and an endoxylanase (*Pc-xyl*) gene are differentially expressed in *P. coffeae,* in a host-specific manner (Bell *et al*., 2019). Orthologues of these genes were identified in *G. pallida* and *M. incognita* through a combination of literature and Basic Local Alignment Search Tool (BLAST) searches.

A number of sequences encoding cell wall-degrading enzymes, including cellulases and endoxylanases have previously been reported in *M. incognita* and *G. pallida* (Haegeman *et al*., 2010, Huang *et al*., 2004, Ledger *et al*., 2006, Rosso *et al*., 1999, Rybarczyk-Mydłowska *et al*., 2012, Thorpe *et al*., 2014). In addition, orthologues of the previously published endoglucanase and endoxylanase genes in *P. coffeae* were searched for amongst the annotated genes of *G. pallida* (Cotton *et al*., 2014) and *M. incognita* (Blanc-Mathieu *et al*., 2017) genome assemblies using the BLAST on WormBase ParaSite v14 (WS271) (Howe *et al*., 2017). BLASTp searches explore protein databases for amino acid sequences that show similarity to the query sequence. The predicted amino acid sequences of *Pc-eng-1* and *Pc-xyl* were used as the query sequences in these searches.

Amino acid sequences for the genes of interest selected from the results of the literature and BLAST searches were aligned in a multiple sequence alignment using the Clustal Omega alignment tool (EMBL-EBI) (Sievers *et al*., 2011). Protein and catalytic domains were then identified in the amino acid sequences of the genes of interest using InterPro scan (Quevillon *et al*., 2005), which were mapped onto the sequences. Following this, Signal P (v5.0) (Nielsen, 2017) was used to confirm whether or not the genes of interest encoded a signal peptide.

RNA-Seq data concerning life stage expression for the putative cellulase and endoxylanase genes in *G. pallida* (Cotton *et al*., 2014) and *M. incognita* (Choi *et al*., 2017) were then examined to determine relative expression levels across the life-cycle and to evaluate the accuracy of the gene models.

3.3.2 Molecular Cloning

cDNA was generated using SuperScript™ II reverse transcriptase (Invitrogen™) from total RNA extracted from J2s of *G. pallida* and *M. incognita* (Section 2.2), and molecular

cloning was carried out for the full-length cDNA sequences of putative cellulase and endoxylanase genes of *G. pallida* and *M. incognita.*

3.3.2.1 Primer design

Primers (Table 3.1) for the amplification of genes of interest in *G. pallida* and *M. incognita* were designed from the coding sequences (CDS) available on WormBase ParaSite (manually corrected if necessary based on homology and RNA-Seq coverage). The parameters of each primer pair were analysed using Sigma-Aldrich OligoEvaluator™ (http://www.oligoevaluator.com/LoginServlet) to ensure they were suitable for amplification and to determine appropriate annealing temperatures.

3.3.2.2 Amplification of genes of interest

Genes of interest were amplified from previously synthesised cDNA using PCR (Section 2.2.3). Template cDNA and relevant primers (0.5 mM) (Table 3.1) were combined with Phusion™ high-fidelity DNA polymerase (New England Biolabs), dNTPs (0.2 mM), and 5 X Phusion™ HF buffer (New England Biolabs). Cycling parameters were as follows: 30 seconds denaturing phase at 98 °C, 30 cycles of 5 seconds at 98 °C, 20 seconds at the relevant annealing temperature (Table 3.1), and 20 seconds at 72 °C. The final extension incubation was for 10 minutes at 72 °C.

DNA products were analysed using agarose gel electrophoresis (Section 2.2.4) and purified using the E.Z.N.A® Cycle Pure or Gel Extraction Kits (Omega Bio-Tek) (Section 2.2.5).

3.3.2.3 A-tailing of purified DNA fragments

Phusion™ high-fidelity DNA polymerase (New England Biolabs) produces blunt ends during DNA amplification and so it was necessary to A-tail the PCR products for use in cloning into a vector with T-overhangs. A-overhangs were generated by incubating purified PCR products with 1 µl Taq polymerase, dATP (0.62 mM) and Taq reaction buffer (10 X) (New England Biolabs) at 72 °C for 20 minutes.

3.3.2.4 DNA Ligation

Resultant A-tailed PCR product (3 µl) was directly ligated into a pGEM® T Easy vector (50 ng) (Promega) using T4 DNA ligase (Promega) (1 µl) and rapid ligation buffer (2 X) (Promega). The 10 µl ligation reaction was incubated at room temperature (~20 °C) for approximately one hour.

Nematode Species	Gene Name	Forward Primer Sequence	Reverse Primer Sequence	Annealing temperature used (°C)
G. pallida	GPLIN_000313600	ATGGCCTTCGCTTTACCATTTC	TCATCCGGAGCATCCGCTC	64
G. pallida	GPLIN_000536400	ATGAATGCCGGCATCAAATT	TTAGCCCGAACATGCCGTT	62
G. pallida	GPLIN_000536400 (1)	AGTAATCGACGGGTCGGCCTAA	TTAGCCCGAACATGCCGTT	66
G. pallida	GPLIN_000536400 (2)	ATGTGTCGTCTCCATTCTCT	TTAGCCCGAACATGCCGTT	59
G. pallida	GPLIN_000755100	ATGTCCATTGTACTGCTTGC	TTATATTTTTCTAATTTTGGCAAGCT	60
G. pallida	GPLIN_000755200	ATGTCCATTGTACTGCTTGCCGT	TTAACCCGAGCAGCTGACGC	68
G. pallida	GPLIN_001111200	ATGTGCGCCTTGATTTGTGC	TCAACCGCGGCAACTTACTC	65
M. incognita	*mi-eng-1*	ATGAATTCTCTCTTATTAATAGCATTT	TCAATGAACGCATTTTCCAT	59
M. incognita	*mi-eng-2*	ATGTTAATAATTAAAAATATTTTTAT	CAACCAGTTATACCAT	50
M. incognita	*mi-eng-2* (1)	ACCACCATGTGGTTAAAATA	TTAACAACCAGTTATACCATTATT	48

Table 3.1- Primer sequences used in molecular cloning.

M. incognita	*mi-eng-4*	ATGTTTTCCCTCTCATTAGT	CTAAGTGTTGTTTTTGGGACC	57
M. incognita	*mi-xyl*	ATGAAATTATTTAATTTTTTCTTTTTA	TTAATTAACGGAAACAAAAGTAAC	56
M. incognita	*mi-xyl* (1)	GATAATATAGCAAAAATAAATTCTGA	TTAATTAACGGAAACAAAAGTAAC	54

Table 3.1 continued- Primer sequences used in molecular cloning.

3.3.2.5 Generation and transformation of ultra-competent E. coli cells

The method for the generation of ultra-competent *E. coli* cells for transformation was modified from Inoue *et al*. (1990). Briefly, 10-12 *E. coli* (DH5α) colonies were cultured in 250 ml super optimal broth at 19 °C with vigorous shaking until the OD_{600} was 0.5. Once this was reached, the cells were cooled on ice for 10 minutes before being pelleted by centrifugation at 4,000 rpm for 10 minutes at 4 °C. Pelleted cells were re-suspended in 80 ml ice cold TB buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl) and placed on ice for a further 10 minutes. Centrifugation was repeated with the same conditions as before to re-pellet the cells. The pellet was then re-suspended in 20 ml ice cold TB buffer combined with 1.4 ml dimethyl sulfoxide (DMSO). Cells (100-200 µl) were aliquoted into 1.5 ml micro-centrifuge tubes, snap-frozen in liquid nitrogen and stored at -80 °C until required.

To transform ultra-competent *E. coli,* 5 µl ligation (generated previously in Section 3.3.2.4) was added to the thawed cells and chilled on ice for 5 minutes. The cell mix was then pipetted directly onto pre-warmed Luria-Bertani (LB)-agar (1% w/v) plates supplemented with ampicillin (100 µg/ml) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase (X-gal) (40 µg/ml). The cells were spread on the plate and left to dry before being incubated overnight at 37 °C.

3.3.2.6 Colony screening

Blue-white screening was utilised in the first instance to identify successful ligations. Positive white colonies (ie. those likely containing an insert) were verified by means of colony PCR. Eight (seven positive white and one blue control) reactions were prepared for each transformation.

Template DNA was created by mixing a single colony with purified water (100 µl) and 1 µl of the resulting cell suspension was combined in a total volume of 20 µl with 2 X MyTaq™ Red Mix (BioLine) and M13 forward and reverse primers (500 nM) (Table 3.2). Cycling parameters were three minutes denaturing phase at 95 °C (this step also lysed the bacterial cells) followed by 30 cycles of 15 seconds at 95 °C, 15 seconds at 55 °C and 10 seconds at 72 °C. A final extension step of 10 minutes at 72 °C completed the reaction.

Agarose gel electrophoresis (Section 2.2.4) was used to analyse the PCR products.

3.3.2.7 Plasmid extraction

Positive colonies confirmed by colony PCR were cultured in LB broth (5 ml) supplemented with ampicillin (100 µg/ml) overnight at 37 °C with vigorous shaking at 200 rpm.

Plasmids were extracted from cultured *E. coli* using an E.Z.N.A® Plasmid DNA Mini Kit (Omega Bio-tek). Briefly, bacterial cells were pelleted by centrifugation (10,000 rcf) and a series of solutions were added to lyse cells. Cellular debris was pelleted by centrifugation (13,000 rcf). The supernatant (containing plasmid DNA) was passed through a HiBind® DNA Mini Column and HBC buffer was added to the column to reduce the solubility of the plasmid DNA. This was then washed with DNA wash buffer and plasmid DNA was eluted and stored at -20 °C.

3.3.2.8 Plasmid Digestion

To confirm the success of the cloning, extracted plasmids were digested. EcoRI restriction sites within the pGEM® T Easy vector (Promega) that flank the cloning site were targeted with Eco-RI restriction enzyme and CutSmart® buffer (10 X) (New England Biolabs). Incubation at 37 °C for between one and two hours separated the insert from the remaining plasmid. Digestion products were electrophoresed (Section 2.2.4) in order to visualise the results.

3.3.2.9 Sequencing

Plasmids (50 ng/µl) were prepared and sent to GeneWiz for sequencing of inserts by Sanger sequencing using either M13 or T7 primers (Table 3.2).

Vector (source)	Primer Name	Primer Sequence	Annealing temperature used (°C)
pGEM® T Easy (Promega)	M13 Forward	TGTAAAACGACGGCCAGT	55
pGEM® T Easy (Promega)	M13 Reverse	GGAAACAGCTATGACCATG	55
pGEM® T Easy (Promega)	T7 Forward	TAATACGACTCACTATAGGG	51
pGEM® T Easy (Promega)	T7 Reverse	GCTAGTTATTGCTCAGCGG	51

Table 3.2- Vector primer sequences used in molecular cloning.

3.3.3 *In situ* hybridisation

Single-stranded digoxigenin-labelled antisense DNA probes were generated for genes of interest from plasmid DNA as described in Section 2.2.7.1. Control sense probes were generated in the same way in separate reactions. Primers used in these reactions are detailed in Table 3.3.

Nematodes were fixed, permeabilised, hybridised and stained as previously described in Section 2.2.7.2.

3.3.4 Confirming enzymatic activity of genes of interest

To determine the enzymatic activity of the putative cellulase and endoxylanase genes identified in *G. pallida* and *M. incognita,* protein expression and purification was attempted.

3.3.4.1 Ligation of genes of interest into pQE-30 expression vector

Primers incorporating either BamHI, HindIII or SalI restriction sites were designed to amplify the coding sequence, without the signal peptide, of previously cloned genes of interest from *G. pallida* and *M. incognita* (Table 3.4). The incorporation of a restriction site into the primer sequence allowed for direct cloning of the coding region into an expression vector that would allow future protein expression from the genes of interest in *E. coli*. The primers were designed such that once cloned, the coding sequence would be in-frame with the plasmid encoded 6xHis purification tag. Amplification of the gene of interest was achieved by PCR from previously generated plasmid DNA (Section 2.2.3) using Phusion DNA polymerase. Cycling parameters were 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 5 seconds, Tm for 20 seconds and 72 °C for 20 seconds. The final extension was at 72 °C for 10 minutes. The PCR product was purified and then, along with a pQE30 expression vector, was digested with appropriate restriction enzymes (Table 3.4). The digested PCR products and vector were purified using a E.Z.N.A® Cycle Pure Kit (Omega Bio-Tek) and the gene sequence was then ligated into pQE30 by the method described in Section 3.3.2.4.

Gene	Forward (antisense) primer sequence	Reverse (sense) primer sequence
GPLIN_000313600	CACGGCTTGACTGGTGTATG	TTAATGTGATCCGTGCTCCG
GPLIN_000536400	GTCACGAAGATTGGCAGACC	CCGACATTCTGGTGCCATAC
GPLIN_001111200	TTTCATCGGTGGCCAAATCG	GGCACAAACGGTCAATCGAA
mi-eng-1	GTGCTGCTTTCTGAGTTGCT	GGCAATGATGACCGACTGAC
mi-eng-4	TCAACCTTTGCCAGCTCAAC	TGTGGTGAAGGGGAAGGTTT
mi-xyl	TTGGCGAAGTACTTGGTGAA	TGGTGGTTCTAGTGCTTGGT

Table 3.3- Primer sequences used for *in situ* hybridisation probes.

Gene	Forward primer sequence	Reverse primer sequence	Annealing temperature used (°C)
GPLIN_000313600	ACAGGATCCGCTGCCCCACCATATGGCCA	ACAGTCGACTCATCCGGAGCATCCGCTCG	69
GPLIN_000536400	ACAGGATCCTTGACTGCCACGCCGCCCCC	ACAAAGCTTAGCCCGAACATGCCGTTGC	68
GPLIN_001111200	ACAGGATCCGATCGGCGCTGTCACAGCCC	ACAGTCGACTCAACCGCGGCAACTTACTC	66
mi-eng-1	ACAGGATCCGCTCCTCCATATGGACAATT	ACAAAGCTTCAATGAACGCATTTTCCAT	57
mi-eng-4	ACAGGATCCGCTCCTCCGTATGGGCAATT	ACAAAGCTTCTAAGTGTTCTTTTTGGGAC	56
mi-xyl	ACAGGATCCAATATAGCAAAAATAAATTC	ACAAAGCTTAATTAACGGAAACAAAAG	55

Table 3.4- Primer sequences used to amplify genes of interest for ligation into expression vector pQE-30.

Underlined sequences are restriction sites. GGATCC is BamHI, GTCGAC is SalI and AAGCTT is HindIII.

3.3.4.2 Transformation of recombinant pQE-30 into M15 (pREP4) *E. coli*

A single colony of the expression strain M15 (pREP4) was cultured in 5 ml LB broth supplemented with kanamycin (25 µg/ml) overnight at 37 °C with vigorous shaking. 100 µl culture was removed and used to inoculate 5 ml fresh LB broth which was grown for three hours at 37 °C, also with vigorous shaking. The cells were then cooled on ice for 10 minutes before being aliquoted into 1.5 ml microcentrifuge tubes where they were pelleted by centrifugation at 8,000 rpm for 5 minutes at 4 °C. Pelleted cells were re-suspended in 0.75 ml ice cold, sterile $CaCl_2$/Tris solution (50 mM $CaCl_2$ + 10 mM Tris HCl (pH8)) and placed on ice for a further 15 minutes. Centrifugation was repeated as before to re-pellet the cells which were then re-suspended in 100 µl ice cold $CaCl_2$/Tris solution. An equal volume of sterile glycerol (80 %) was added to the cell solution before it was snap-frozen in liquid nitrogen. Cells were stored at -80 °C until required.

To transform M15 (pREP4) cells, 3 µl pQE-30 ligation reaction was added to thawed *E. coli* cells and chilled on ice for 30 minutes. Cells were transferred to 42 °C for 2 minutes and then immediately placed on ice for a further 5 minutes. 1 ml LB broth (Section 2.4.1) was added to the cells which were incubated at 37 °C for between 30 and 60 minutes with vigorous shaking. 100 µl cell mix was then pipetted directly onto a pre-warmed LB-agar (1 % w/v) plate supplemented with ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The remainder of the transformation mix was then concentrated by brief centrifugation and pipetted onto a separate LB-agar plate supplemented with the same antibiotics. The cells were spread around the plate and left to dry before being incubated overnight at 37 °C. Colony PCR using M13 primers confirmed successful ligation of the gene of interest into the pQE-30 expression vector. Positive clones were sequenced by Sanger sequencing to confirm that the coding sequence was in-frame and that no PCR errors had been introduced.

3.3.4.3 Inducing protein expression in transformed M15 (pREP4) *E. coli*

Positive colonies were cultured in LB broth (5 ml) + ampicillin (100 µg/ml) and kanamycin (25 µg/ml) overnight at 37 °C with vigorous shaking at 200 rpm. 8.75 ml fresh culture media was inoculated with 1.25 ml overnight culture and grown for 30 minutes before isopropylthio-β-galactoside (IPTG; 2 mM) was added to induce protein expression. Cells were grown for a further 3 hours before a 1 ml aliquot was centrifuged at 3,000 rpm for 3 minutes to pellet the cells which were subsequently frozen at -20 °C. A control culture was grown in the same conditions without the addition of IPTG.

3.3.4.4 Cell lysis of transformed M15 (pREP4) *E. coli*

Various approaches were employed in attempts to disrupt transformed *E. coli* cells and release the protein products.

3.3.4.4.1 Incubation with denaturing urea buffer

Pelleted cells were re-suspended in 200 µl Buffer B (8 M Urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl; pH 8.0) and gently vortexed. They were then incubated at room temperature (~20 °C) for 20 minutes.

3.3.4.4.2 Heating in 5 X PAGE buffer

Cells were re-suspended in 5 X polyacrylamide gel electrophoresis (PAGE) sample buffer (10 ml distilled water, 5 ml glycerol, 3.75 ml 2-mercaptoethanol, 1.5 g sodium dodecyl-sulphate (SDS), 0.15 g bromophenol blue (Sigma-Aldrich)) and heated to 95 °C for 7 minutes.

3.3.4.4.3 Sonication

Cells were re-suspended in 300 µl sonication buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8). They were then sonicated for 30 seconds on ice at 30 kHz. This was repeated 3 times for each cell pellet.

3.3.4.4.4 Incubation with BugBuster® Master Mix (EMD Millipore)

Cells were re-suspended in 5 ml/g BugBuster® Master Mix (EMD Millipore) and the mixture was incubated for 20 minutes at room temperature (~20 °C) with rotation.

3.3.4.4.5 Incubation with lysozyme (Sigma-Aldrich)

Cells were re-suspended in 3 ml sonication buffer. 21.4 µl lysozyme (100 mg/ml) (Sigma-Aldrich) was added and the mixture was vortexed before being incubated at 37 °C for 30 minutes.

3.3.4.5 Small-scale purification of protein products from M15 (pREP4)

After cell lysis in denaturing urea buffer, the samples were centrifuged at 15,000 rpm for 10 minutes. 20 µl supernatant was removed and stored on ice for future analysis. 30 µl Ni-NTA resin (Invitrogen) was mixed with the remainder of the supernatant for 30 minutes at room temperature (~20 °C). The mixture was then centrifuged at 15,000 rpm for 10 seconds and the supernatant was discarded. The pelleted resin was washed three times with Buffer C (1 ml) (8 M Urea, 0.1 M Na_4PO_4 and 0.01 M Tris /HCl; pH 6.3). Then, 20 µl Buffer C supplemented with 0.1 M ethylenediaminetetraacetic acid (EDTA) was added to the resin which was gently mixed for 2 minutes at room temperature (~20 °C). The resin was then pelleted at 15,000 rpm for 2 minutes.

5 X PAGE sample buffer was added to the supernatant and it was heated at 95 °C for 7 minutes.

3.3.4.6 Alternative, native purification of protein products from M15 (pREP4)

For non-denaturing lysis methods (mirroring the methods of the small-scale purification), lysed cells were centrifuged and 30 µl Ni-NTA resin (Invitrogen) was mixed with the supernatant for 30 minutes at room temperature (~20 °C). The resin was pelleted by centrifugation, and it was washed five times a wash buffer (1 ml) (50 mM NaH_2PO_4, 500 mM NaCl, pH 6). Following this, 20 µl elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 6) was added, followed by a 30 minute incubation on ice. Again, mirroring the methods of the small-scale purification, the resin was re-pelleted, and 5 X PAGE sample buffer was added to the supernatant. It was then heated at 95 °C for 7 minutes.

3.3.4.7 Visualisation of protein using SDS-PAGE

Samples were electrophoresed on a 12.5 % polyacrylamide gel containing 0.2 % SDS (BioRad) in 1 X running buffer (6 g Tris base, 28.8 g glycine, 2 g SDS in 1 L water) at 100 Volts for 30 minutes. Gels were stained with Coomassie Brilliant Blue (National Diagnostics) and de-stained with a methanol/acetic acid solution (4:1:5; MeOH: ACOOH: H_2O).

3.3.5 Validation of Congo red assay for the assessment of cellulase activity

To validate the efficacy of a Congo red dye-based assay in detecting cellulase activity, homogenates extracted from nematodes with a known ability to breakdown cellulose were examined.

Total nematode homogenate was generated from approximately 10, 100, 500 and 1,000 *G. pallida* and *M. incognita* J2s by grinding them with a micropestle in sterilised tap water (30 µl). The resulting homogenate (30 µl) was applied to 1 % carboxymethylcellulose (Sigma-Aldrich) agar plates that were incubated at 37 °C for 16 hours. After this period, plates were stained with 0.1 % Congo red (Sigma-Aldrich) solution, which binds to cellulose, for 30 minutes. The plates were then washed with 5 M NaCl. The absence of staining in the resultant clearance zones signified cellulose breakdown activity.

3.3.6 Assessment of cellulase activity of putative cellulase genes using Congo red assay

M15 (pREP4) cells containing recombinant pQE-30 plasmids were lysed using BugBuster® Master Mix (EMD Millipore) and pelleted at 15,000 rpm for 10 minutes. 20 µl supernatant was set aside on ice. This cell lysate was then applied to a 1 % carboxymethylcellulose (Sigma-Aldrich) plate and incubated for 16 hours at 37 °C. After this period, plates were incubated with 0.1 % Congo red dye for 30 minutes before they were washed with 5 M NaCl to reveal clearance zones indicating cellulose breakdown activity.

3.4 Results

3.4.1 Identification of genes encoding cell wall-degrading enzymes in *G. pallida* and *M. incognita*

Host-specific gene expression of an endoglucanase gene (*Pc-eng-1*) and an endoxylanase gene (*Pc-xyl*) has previously been reported in *P. coffeae* in response to root exudate detection (Bell *et al*., 2019). It was hypothesised that orthologues of these genes may also show differential expression patterns in other plant parasitic nematodes and so initially, homologous cellulase and endoxylanase-encoding were searched for in *G. pallida* and *M. incognita*.

Through a combination of literature searches and BLASTp sequence similarity searches of annotated genes in genome assemblies, several genes that are similar to those differentially regulated in *P. coffeae* were identified in both species (Tables 3.5 and 3.6).

Thorpe *et al*. (2014) proposed the presence of approximately 40 different cell wall-degrading enzymes in *G. pallida,* of which, 15 were classified as belonging to the GH5 family. Those classified as cellulases are included in Table 3.5. For *M. incognita,* it has been suggested that there are as many as 90 cell wall-degrading enzymes, with 21 of them belonging to the GH5 family (Abad *et al*., 2008, Bozbuga *et al*., 2018). Some of these genes have been characterised further in the literature (Table 3.6) (Huang *et al*., 2004, Mitreva-Dautova *et al*., 2006, Rosso *et al*., 1999) and nine sequences encoding β-1,4-endoglucanases alone have been characterised in *M. incognita* to date (Table 3.6) (Ledger *et al*., 2006).

To identify any additional gene homologous to those described as being up-regulated in *P. coffeae* in response to host detection (Bell *et al*., 2019), BLAST searches, using the amino acid sequences for *Pc-eng-1* and *Pc-xyl* as query sequences, were employed to search the annotated genomes of *G. pallida* and *M. incognita.* The additional genes revealed by these searches are presented in Tables 3.5 and 3.6 and have “BLAST search” listed as their source. The descriptions of all the genes in these tables were derived from the annotations of the genome, gene ontology (GO) terms, or were identified using InterPro Scan.

RNA-Seq data relating to *G. pallida* and *M. incognita* were sourced from WormBase Parasite (Choi *et al*., 2017, Cotton *et al*., 2014). This data allowed relative expression levels of the genes listed in Tables 3.5 and 3.6 to be analysed throughout the different life stages of each nematode species.

In *G. pallida,* seven of the genes of interest presented in Table 3.5 (GPLIN_000552400, GPLIN_000313600, GPLIN_001215600, GPLIN_001111300, GPLIN_001111200,

GPLIN_000933200 and GPLIN_000536400) exhibited their highest expression levels at the J2 stage (Figure 3.2A). In contrast, GPLIN_000755200, GPLIN_000755100 and GPLIN_000293400 demonstrated notably elevated expression during the male stage of the life-cycle (Figure 3.2B). Given the study's focus on genes similar to those found to be up-regulated in *P. coffeae* J2s*,* most of these were excluded from further analysis.

GPLIN_000779200, GPLIN_001185800, GPLIN_000304900, GPLIN_000779000, GPLIN_000827200, GPLIN_001345700, GPLIN_000616300 and GPLIN_000694900 all had very low expression levels when compared to the other selected genes of interest (Figure 3.2C) and so they were omitted from further investigation.

All of the genes of interest in *M. incognita* (listed in Table 3.6) exhibited their highest levels of expression in the J2 stage (Figure 3.3). Whilst gene expression levels varied considerably across these genes, the following exhibited notably higher expression compared to the rest: Minc3s03136g32914 (*Eng-1*), Minc3s00365g11074 (*Eng-2*), Minc3s00139g05823 (*Eng-4*), Minc3s00143g05928 (*Xyl*), Minc3s00139g05824, Minc3s01153g21228, Minc3s02073g28116, Minc3s03138g32920 and Minc3s10875g44433 (Figure 3.3A).

Overall, four genes encoding putative cellulases were selected for further analysis in *G. pallida,* whilst three potential cellulase genes and one potential endoxylanase gene were chosen to be investigated further in *M. incognita* (Table 3.7)*.* Although it was searched for, no endoxylanase orthologue was found in *G. pallida.*

Species	Gene Name	Description	Source
G. pallida	GPLIN_000293400	CBM49 (Carbohydrate binding module) domain-containing protein	BLAST search
G. pallida	GPLIN_000304900	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_000313600	Cellulase domain containing protein	Thorpe *et al*. (2014)
G. pallida	GPLIN_000536400	Endoglucanase	Thorpe *et al*. (2014)
G. pallida	GPLIN_000552400	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_000616300	Endoglucanase	BLAST search
G. pallida	GPLIN_000694900	Endoglucanase	BLAST search
G. pallida	GPLIN_000755100	Cellulase domain containing protein	Thorpe *et al*. (2014)
G. pallida	GPLIN_000755200	Cellulase domain containing protein	Thorpe *et al*. (2014)

Table 3.5- Cell wall-degrading enzymes identified in *G. pallida* through literature and BLAST searches.

Descriptions are annotations in the genome.

G. pallida	GPLIN_000779000	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_000779200	Cellulase	BLAST search
G. pallida	GPLIN_000827200	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_000933200	Cellulase	BLAST search
G. pallida	GPLIN_001111300	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_001111200	Cellulase domain containing protein	Thorpe *et al*. (2014)
G. pallida	GPLIN_001185800	Cellulase domain containing protein	Thorpe *et al*. (2014)
G. pallida	GPLIN_001215600	Cellulase domain containing protein	BLAST search
G. pallida	GPLIN_001345700	Cellulase domain containing protein	BLAST search

Table 3.5 continued- Cell wall-degrading enzymes identified in *G. pallida* through literature and BLAST searches.

Descriptions are annotations in the genome.

Species	Gene Name	Description	Source
M. incognita	Minc3s00139g05823 *(mi-eng-4)*	Cellulase	Ledger *et al*. (2006) / BLAST search
M. Incognita	Minc3s00139g05824	Cellulose binding protein	BLAST search
M. incognita	Minc3s00143g05928 *(mi-xyl)*	Glucosylceramidase	Mitreva-Dautova *et al*. (2006)
M. incognita	Minc3s00202g07474	Cellulase	BLAST search
M. incognita	Minc3s00223g07936 *(mi-eng-8)*	Beta-1,4-endoglucanase	Ledger *et al*. (2006) / BLAST search
M. incognita	Minc3s00365g11074 *(mi-eng-2)*	Beta-1,4-endoglucanase	Ledger *et al*. (2006)
M. incognita	Minc3s00371g11155	Endo-Beta-1,4-glucanase	BLAST search
M. incognita	Minc3s00520g13673 *(mi-eng-3)*	Endoglucanase	Huang *et al*. (2004)
M. incognita	Minc3s00520g13674	Cellulose binding protein	BLAST search
M. incognita	Minc3s00935g19055	Endo-Beta-1,4-glucanase	BLAST search

Table 3.6- Cell wall-degrading enzymes identified in *M. incognita* through literature and BLAST searches.

Descriptions are either the annotation in the genome, a Gene Ontology (GO) annotation or were identified using InterPro Scan.

M. incognita	Minc3s01153g21228	Endo-Beta-1,4-glucanase	BLAST search
M. incognita	Minc3s02073g28116	Endo-Beta-1,4-glucanase	BLAST search
M. incognita	Minc3s02151g28568 *(mi-eng-6)*	Beta-1,4-endoglucanase	Ledger *et al*. (2006) / BLAST search
M. incognita	Minc3s02151g28569 *(mi-eng-5)*	Beta-1,4-endoglucanase	Ledger *et al*. (2006)/ BLAST search
M. incognita	Minc3s03136g32914 *(mi-eng-1)*	Beta-1,4-endoglucanase	Rosso *et al*. (1999)
M. incognita	Minc3s03138g32920	Cellulase	BLAST search
M. incognita	Minc3s03507g34046	Cellulase	BLAST search
M. incognita	Minc3s05188g37760	Cellulase	BLAST search
M. incognita	Minc3s09866g43601	Cellulase	BLAST search
M. incognita	Minc3s10875g44433	Cellulase	BLAST search

Table 3.6 continued- Cell wall-degrading enzymes identified in *M. incognita* through literature and BLAST searches.

Descriptions are either the annotation in the genome, a Gene Ontology (GO) annotation or were identified using InterPro Scan.

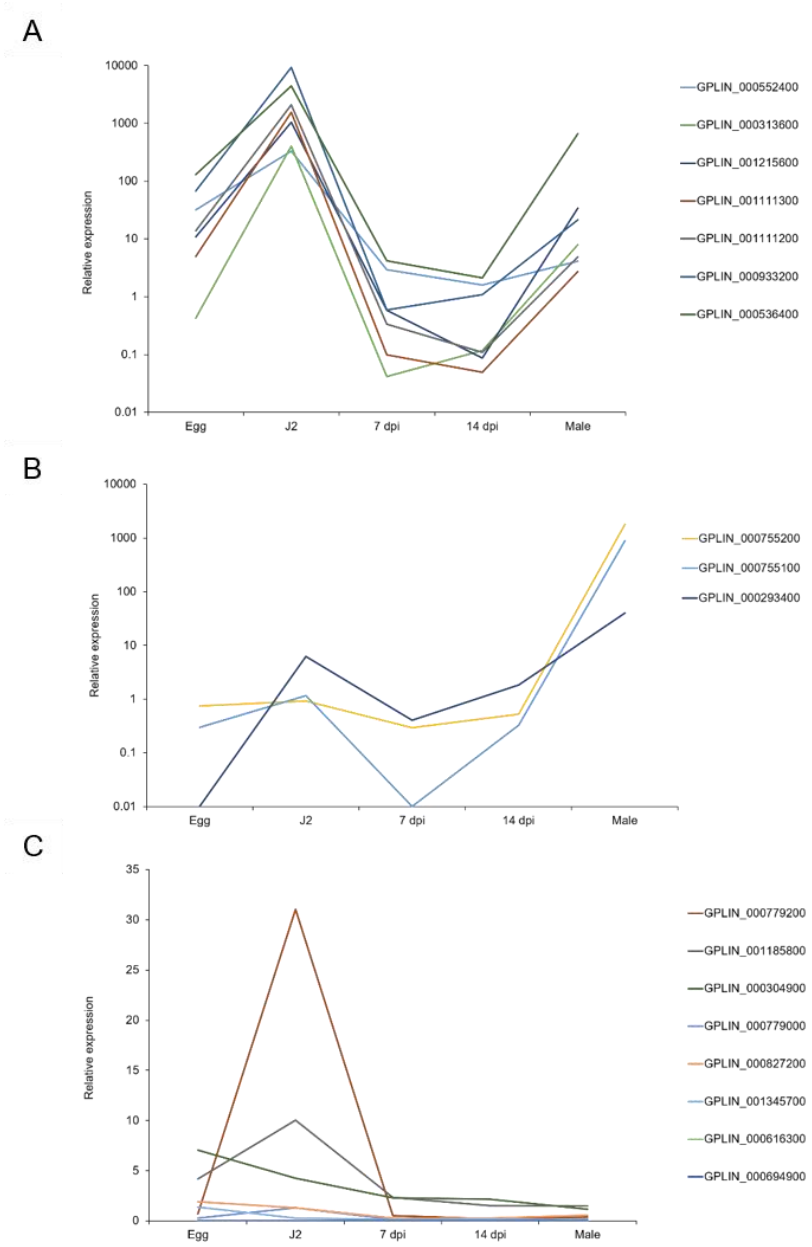


Figure 3.2- RNA-Seq data showing the relative expression of putative cellulase genes in *G. pallida* by life stage.

A) Genes with highest expression in J2s. At the J2 stage the following genes exhibited their peak expression: GPLIN_000552400, GPLIN_000313600, GPLIN_001215600, GPLIN_001111300, GPLIN_001111200, GPLIN_000933200 and GPLIN_000536400 all show the highest expression at the J2 stage. GPLIN_000933200 displayed the highest expression among these genes at this stage.

B) Genes with highest expression in males. GPLIN_000755200, GPLIN_000755100 and GPLIN_000293400 displayed their peak expression in the adult male stage if their life-cycles. GPLIN_000755200 displayed the highest expression among these genes at this stage.

C) Genes with low expression throughout the life-cycle. GPLIN_000779200, GPLIN_001185800, GPLIN_000304900, GPLIN_000779000, GPLIN_000827200, GPLIN_001345700, GPLIN_000616300 and GPLIN_000694900 all showed relatively low expression levels throughout their life-cycles. Although the expression of GPLIN_000779200 and GPLIN_001185800 peaked at the J2 stage, the overall expression is considerably lower than that of other genes.

Lines linking individual points do not convey meaningful relationships.

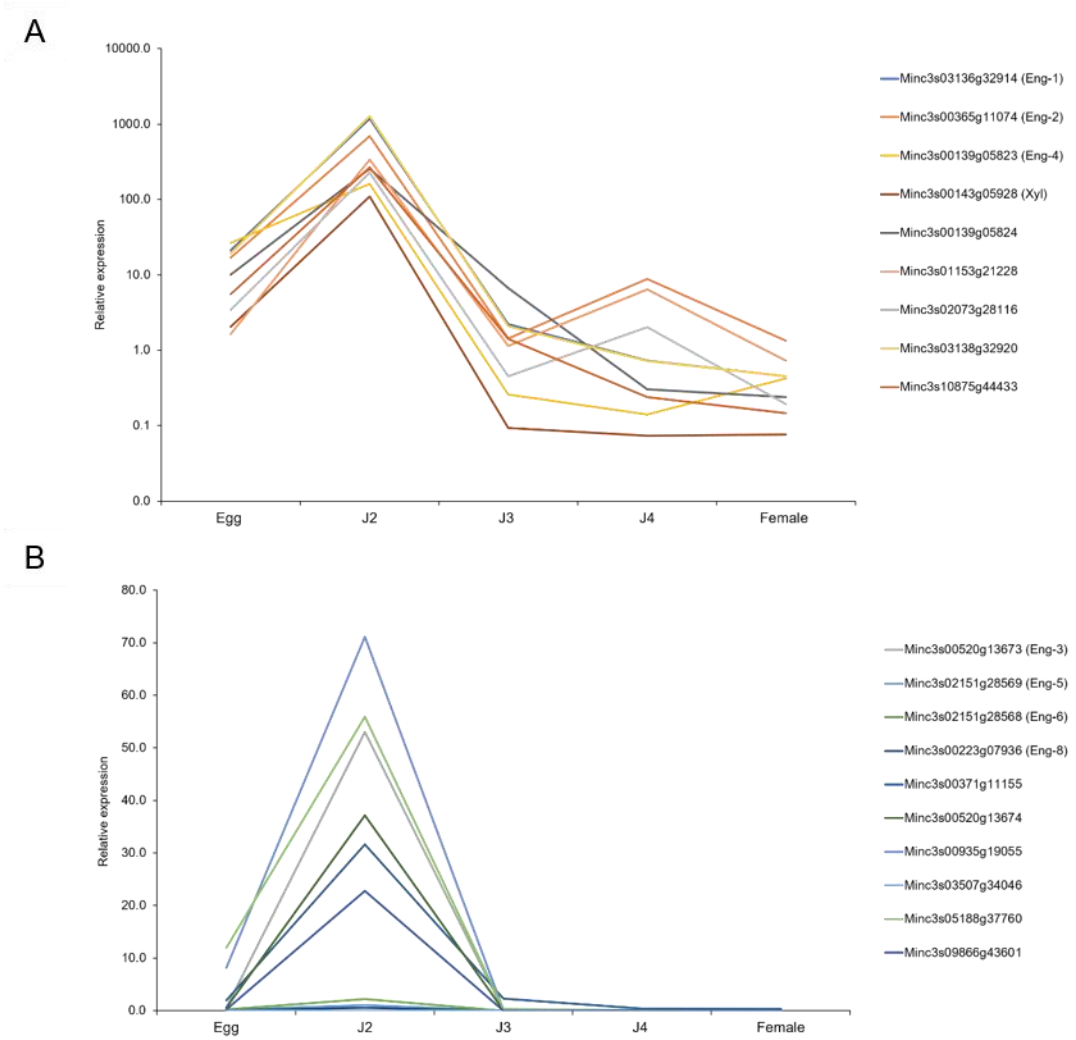


Figure 3.3- RNA-Seq data showing the relative expression of putative cellulase and endoxylanase genes in *M. incognita* by life stage.

A) Genes with comparatively high expression. Among the genes of interest, the following exhibited the highest expression levels throughout their life-cycles: Minc3s03136g32914 (*Mi-eng-1*), Minc3s00365g11074 (*Mi-eng-2*), Minc3s00139g05823 (*Mi-eng-4*), Minc3s00143g05928 (*Mi-xyl*), Minc3s00139g05824, Minc3s01153g21228, Minc3s02073g28116, Minc3s03138g32920 and Minc3s10875g44433. All of these genes displayed the highest expression at the J2 life stage.

B) Genes with comparatively low expression. The following genes exhibited comparatively lower expression levels, leading to their exclusion from further analysis: Minc3s00520g13673 (*Mi-eng-3*), Minc3s02151g28569 (*Mi-eng-5*), Minc3s02151g28568 (*Mi-eng-6*), Minc3s00223g07936 (*Mi-eng-8*), Minc3s00371g11155, Minc3s00520g13674, Minc3s00935g19055, Minc3s03507g34046, Minc3s05188g37760 and Minc3s09866g43601. Similarly, all of these genes displayed the highest expression during the J2 life stage.

Lines linking individual points do not convey meaningful relationships.

Species	Gene name	Description
G. pallida	GPLIN_000313600	Cellulase domain containing protein
G. pallida	GPLIN_000536400	Endoglucanase
G. pallida	GPLIN_000755200	Cellulase domain containing protein
G. pallida	GPLIN_001111200	Cellulase domain containing protein
M. incognita	Minc3s03136g32914 *(mi-eng-1)*	Beta-1,4-endoglucanase
M. incognita	Minc3s00365g11074 *(mi-eng-2)*	Beta-1,4-endoglucanase
M. incognita	Minc3s00139g05823 *(mi-eng-4)*	Cellulase
M. incognita	Minc3s00143g05928 *(mi-xyl)*	Glucosylceramidase

Table 3.7- Cellulase and endoxylanase genes in *G. pallida* and *M. incognita* chosen for further investigation.

Descriptions are either the annotation in the genome, a Gene Ontology (GO) annotation or were identified using InterPro Scan.

Amino acid sequences of the selected putative cell wall-degrading enzymes (Table 3.7) were aligned for each species using the Clustal Omega multiple sequence alignment tool (EMBL-EBI), as shown in Figures 3.4 and 3.5.

Cellulases in plant parasitic nematodes are known to belong to the GH5 family. Analysis of InterPro gene ontology (GO) terms confirmed that each of the selected putative cellulase genes of interest from both species belonged to the GH5 gene family. InterPro scan was also used to confirm the presence of cellulase domains within the amino acid sequence. This analysis confirmed the presence of such domains in all of the proteins encoded by the selected putative cellulase genes in *G. pallida* (Figure 3.4) and *M. incognita* (Figure 3.5). Furthermore, it is noteworthy that GPLIN_000536400 and *mi-eng-1* also encoded a cellulose binding domain (Figures 3.4 and 3.5).

In contrast, although *mi-xyl* is reported to be part of the GH5 family (Mitreva-Dautova *et al*., 2006), it was found to encode a protein with a glucosylceramidase domain. This is characteristic of genes in the GH30 gene family, which endoxylanases belong to (St John *et al*., 2010).

Along with other nematode effectors, cell wall-degrading enzymes are typically secreted through the stylet, into the host to aid in parasitism. Therefore, signal peptides were searched for within the coding sequences of the selected genes of interest using Signal P (Nielsen, 2017) (Figure 3.6). The presence of a signal peptide was confirmed for all of the selected genes of interest in both *G. pallida* and *M. incognita,* except for GPLIN_000536400.

Figure 3.4- Alignment of putative cellulase genes in *G. pallida* using Clustal Omega.

Alignment of GPLIN_000313600, GPLIN_000536400, GPLIN_000755200 and GPLIN_001111200 amino acid sequences. Yellow highlighting shows cellulase domains and green highlighting shows a cellulose binding domain. Asterisks (*) indicate an identical amino acid in all sequences, full stop (.) and colon (:) indicate conservative substitutions of amino acids.

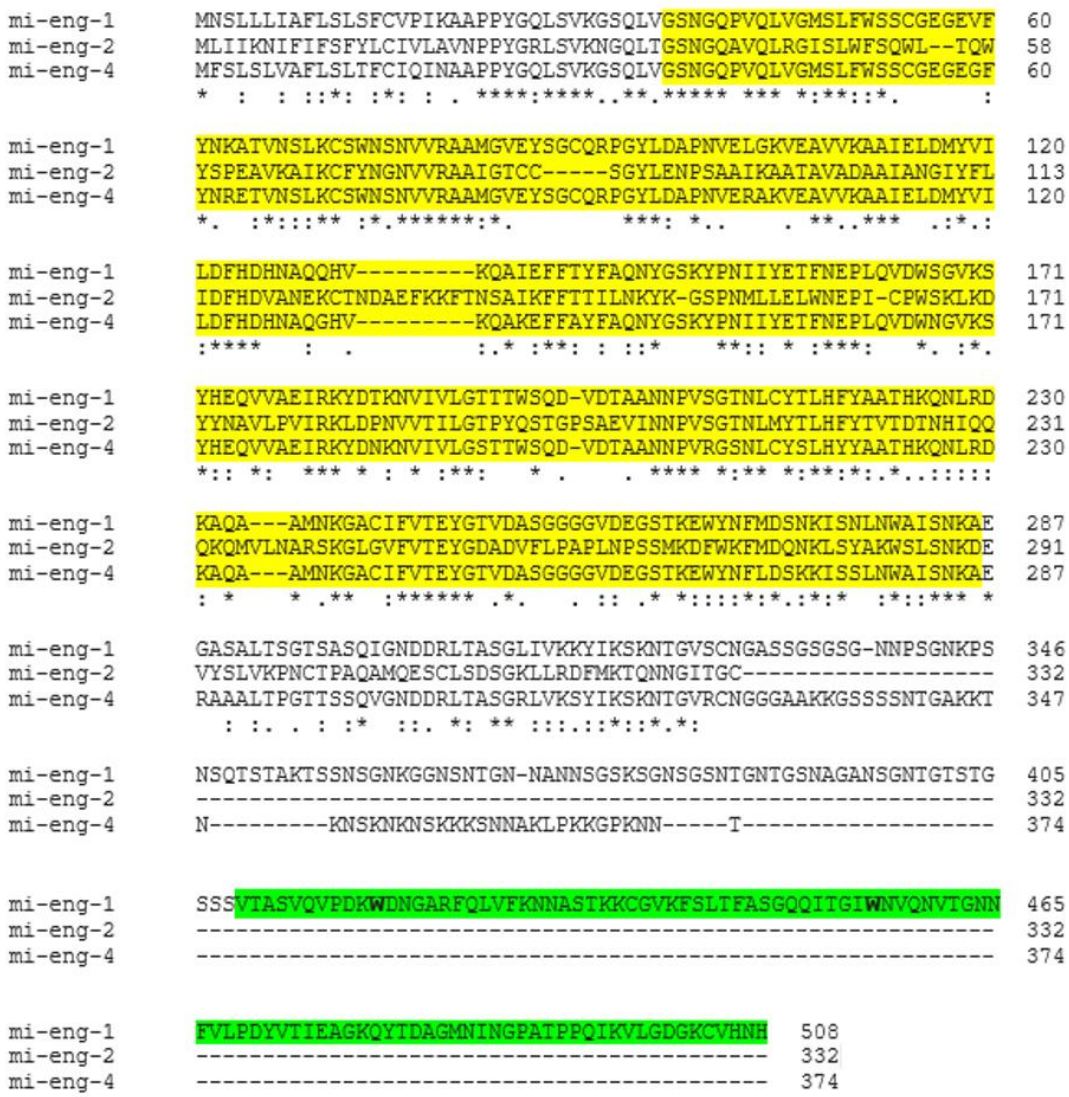


Figure 3.5- Alignment of putative cellulase genes in *M. incognita* using Clustal Omega.

Alignment of *mi-eng-1, mi-eng-2* and *mi-eng-4* amino acid sequences. Yellow highlighting shows cellulase domains and green highlighting shows a cellulose binding domain. Bold tryptophan residues (W) within the cellulose binding domain are conserved in cellulose binding domains (Béra-Maillet *et al*., 2000). Asterisks (*) indicate an identical amino acid in all sequences, full stop (.) and colon (:) indicate conservative substitutions of amino acids.

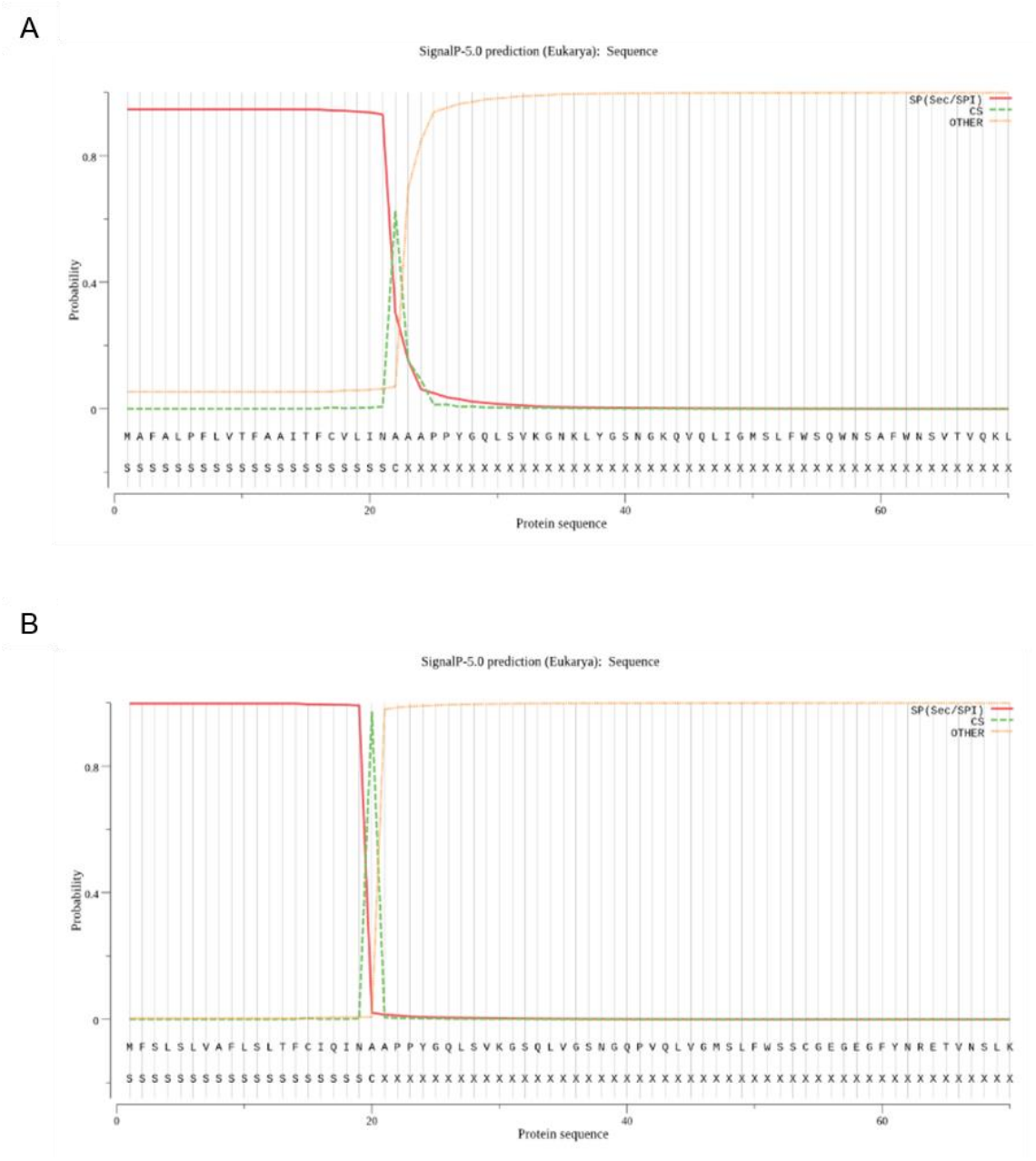


Figure 3.6- Prediction of a signal peptide using Signal P (v5.0).

A) Signal peptide prediction for GPLIN_000313600. "Sec/SPI" refers to a secretory signal peptide, "CS" indicates a cleavage site, and "Other" signifies the absence of a signal peptide within the amino acid sequence. The GPLIN_000313600 gene exhibits a 94.67 % likelihood of encoding a secretory signal peptide (Sec/SPI). Following the signal peptide, a cleavage site (CS) is predicted, with the remaining amino acid sequence expected to lack a signal peptide.

B) Signal peptide prediction for *mi-eng-4.* The *mi-eng-4* gene exhibits a 99.77 % likelihood of encoding a secretory signal peptide (Sec/SPI). A predicted cleavage site (CS) comes after this, followed by the lack a signal peptide in the remaining amino acid sequence. These plots are representative for all of the genes of interest.

3.4.2 Molecular cloning of putative cellulase and endoxylanase genes from *G. pallida* and *M. incognita*

Further investigations were carried out to undertake a comprehensive analysis of the selected putative cellulase and endoxylanase encoding genes in *G. pallida* and *M. incognita.* For both of these species, total RNA was extracted from J2s and this was used to generate cDNA. This life stage was chosen as the genes of interest showed high levels of expression during this life stage (Figures 3.2 and 3.3).

3.4.2.1 *G. pallida*

Initially, each of the genes of interest was amplified from cDNA by PCR using primers designed to encompass the entire coding region. They were then cloned and sequenced using Sanger sequencing.

3.4.2.1.1 GPLIN_000313600

GPLIN_000313600 has an expected size of 1 Kb and although the PCR product for this gene appears slightly bigger when analysing the agarose gel (Figure 3.7A), cloning attempts were still made. Colony PCR confirmed the presence of plasmids harbouring an insert of the appropriate size (Figure 3.7B), which were extracted from cultured cells and digested using EcoRI restriction enzyme (Figure 3.7C). This enzyme cleaves at either side of the cloning site and should therefore excise the complete insert. However, there are two EcoRI sites within the coding sequence of the gene which means the EcoRI cleaves the insert into three distinct fragments of 15, 176 and 817 bp (Figure 3.7C). Clones were sequenced using Sanger sequencing and alignments of resultant sequences confirmed the cloning of GPLIN_000313600 to be successful.

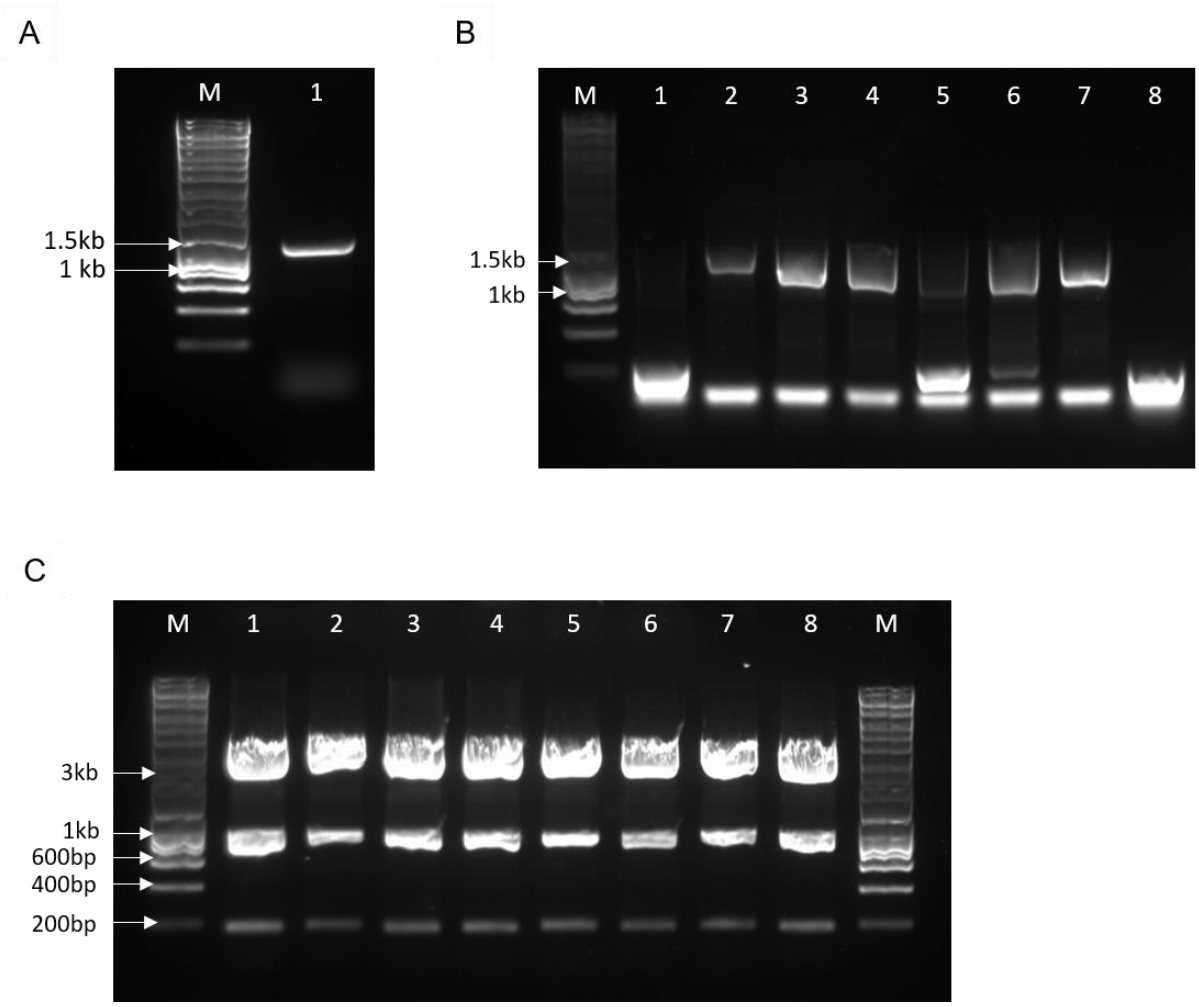


Figure 3.7- Cloning of GPLIN_000313600.

A) Amplification of GPLIN_000313600. Lane 1 contains a PCR product slightly larger than the expected size of GPLIN_000313600 (1 Kb).

B) Colony PCR of *E. coli* harbouring recombinant pGEM® T Easy. PCR products in lanes 1-7 were amplified from white *E. coli* colonies and the PCR product in lane 8 was amplified from a blue *E. coli* colony. Products seen between 1 and 1.5 Kb in lanes 2,3,4,6 and 7 represent the successful ligation of GPLIN_000313600 (1 Kb) into the vector. PCR that produced the products in lanes 1 and 5 amplified only the empty vector region. This colony PCR is representative of colony PCRs carried out throughout the cloning process of putative cellulase genes in *G. pallida.*

C) Digestion of pGEM® T Easy recombinant with GPLIN_000313600 using EcoRI. Lanes 1-8 contain plasmid with cloned PCR products digested with EcoRI. The digestion produced a band at approximately at 3 Kb which corresponds to the size of the pGEM® T Easy vector. GPLIN_000313600 has two EcoRI sites within its sequence which EcoRI has split into three fragments that are 15, 176 and 817 bp in length. While the 15 bp fragment is too small to be resolved in this case, two distinct bands can be seen at 176 and 817 bp. Three of the clones were then sent for sequencing. This digest is representative of digests of successfully cloned putative cellulase genes from *G. pallida.*

3.4.2.1.2 GPLIN_000536400

An initial PCR and subsequent gradient PCRs with Tm ranging between 60 °C and 70 °C all failed to amplify GPLIN_000536400. Further analysis of the genome revealed that upstream of the predicted start site, there is a stretch of NNNs which suggests that the gene model is likely to be incorrect as it is based on incomplete sequence data. An orthologue of this gene was identified in the closely related cyst nematode, *G. rostochiensis* (GROS_g04677.t1), which is longer at the 5' end. As a result, *G. pallida* J2 transcriptome assembly data was analysed and the predicted GPLIN_000536400 sequence was aligned with GROS_g04677.t1 and the *G. pallida* transcript (Figure 3.8A). This revealed that GPLIN_000536400 was truncated at the 5' end (Figure 3.8A). The predicted sequence from the automated gene annotation carried out previously is therefore likely to be incorrect. The sequence alignment with GROS_g04677.t1 and the *G. pallida* transcript reveals the 'true' start site of this gene (Figure 3.8A). The alignment also highlights two likely missing exons in the GPLIN_000536400 gene model (Figure 3.8A). Signal P analysis confirmed that the 'improved' gene model now encodes a protein with a predicted signal peptide.

As a result, two new forward primers were designed which encompassed the newly identified start site of this gene (Table 3.1; GPLIN_000536400 (1) and GPLIN_000536400 (2)). The first forward primer started at the methionine codon corresponding to the start site of GROS_g04677.t1 and the second started at the methionine preceding the start site of GROS_g04677.t1 (Figure 3.8A). Amplification by PCR with both of these primers was successful (Figure 3.8B). The PCR products obtained using primer pair 3.3 were separately ligated into pGEM® T Easy and transformed into ultra-competent *E. coli*. Colony PCR and restriction digest confirmed successful ligation of the gene of interest into the vector. Positive clones were sequenced, and alignments of the resultant sequences confirmed that GPLIN_000536400 had been successfully cloned.

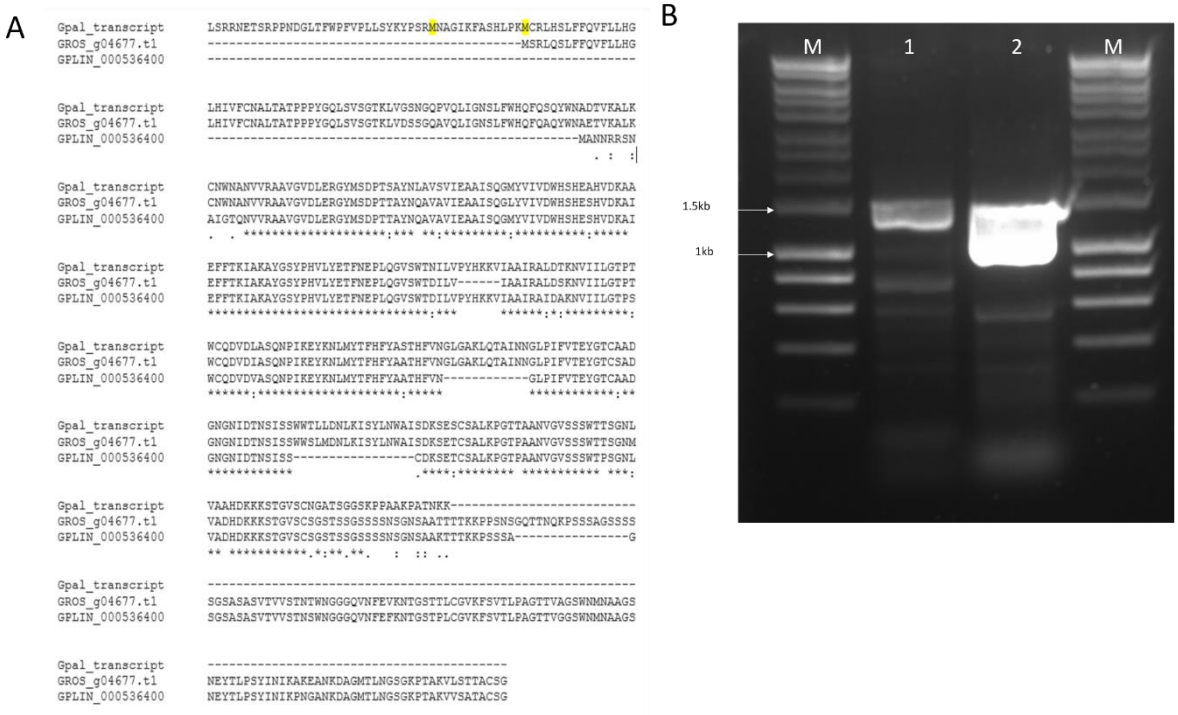


Figure 3.8- Primer design and successful amplification of GPLIN_000536400.

A) Amino acid sequence alignment of GPLIN_000536400 against its orthologue in *G. rostochiensis* (GROS_g04677.t1) and *G. pallida* transcript data. Alignment of these sequences revealed the true start site of GPLIN_000536400. Two new forward primers were designed (Table 3.1) to amplify GPLIN_000536400 starting either at the methionine codon corresponding to the start site of GROS_g04677.t1 (highlighted), or the one preceding this (also highlighted).

B) PCR amplification of GPLIN_000536400 using new forward primers. In lane 1 is the product of the first new forward primer (GPLIN_000536400 (1)) and in lane 2 is the product of the second new forward primer (GPLIN_000536400 (2)). Both new primers, in conjunction with the original reverse primer, amplified products of similar size expected of the gene of interest (1.1 Kb).

3.4.2.1.3 GPLIN_000755200

GPLIN_000755200 was successfully amplified (Figure 3.9A) and cloned into pGEM T Easy® where digestion of the vector confirmed its likely success. Three clones were sent for Sanger sequencing. Of the three, one sequencing reaction failed due to a lack of priming and two sequences were returned which, when translated, did not align fully to the expected amino acid sequence (Figure 3.9B). Although the three sequences align perfectly elsewhere, there is a section starting at the 211 amino acid position of the predicted sequence, where the two cloned sequences start to differ from the gene model (Figure 3.9B). A change in seven amino acids, followed by a stretch of 13 additional amino acids is seen in the cloned sequences before they align again with the predicted sequence (Figure 3.9B). RNA-Seq data from *G. pallida* was therefore analysed using Apollo genome browser which showed transcript coverage through a predicted intron (Figure 3.9C). The observed disparities are believed to be the result of inaccuracies in the gene model prediction, where an additional base has been included, resulting in an intron prediction as the best fit to the sequence data. As the cloned sequences align with the transcript coverage in a continuous manner (Figure 3.9C), there is a high likelihood that the resulting protein sequence is more accurate than the predicted one. This gene was therefore considered successfully cloned.

3.4.2.1.4 GPLIN_001111200

This gene was successfully amplified and cloned. Colony PCR analysis and plasmid digestion suggested that six clones potentially contained GPLIN_001111200 (Figure 3.10A). Four clones were sequenced using Sanger sequencing; however, results showed that all four sequencing attempts failed due to non-specific binding of the M13 primers in the sequencing reactions.

Repeated attempts were made to sequence three of these clones using T7 forward primer and M13 reverse primer (Table 3.2). T7F is an alternative primer that binds to pGEM® T Easy and can be used in combination with M13R for sequencing. These primers worked to sequence these clones and alignments of resultant sequences confirmed the cloning of GPLIN_001111200 to be successful (Figure 3.10B).

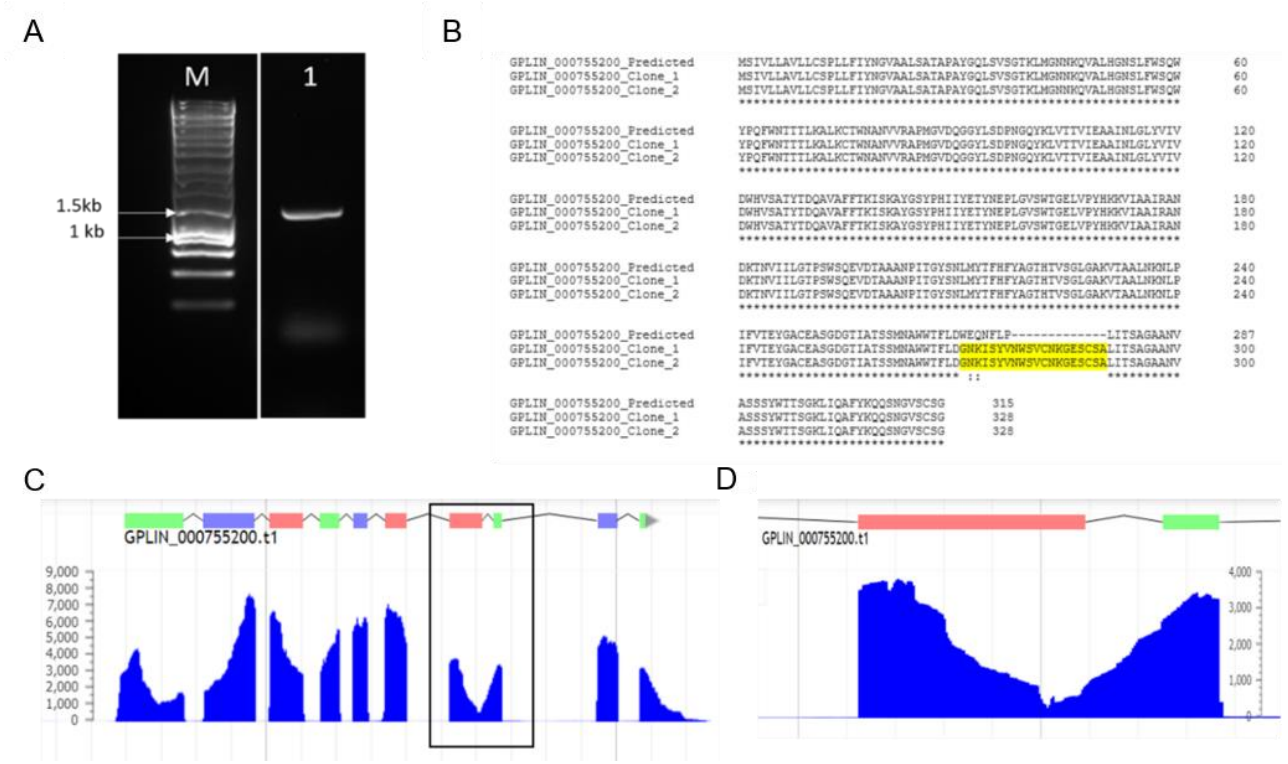


Figure 3.9- Cloning of GPLIN_000755200.

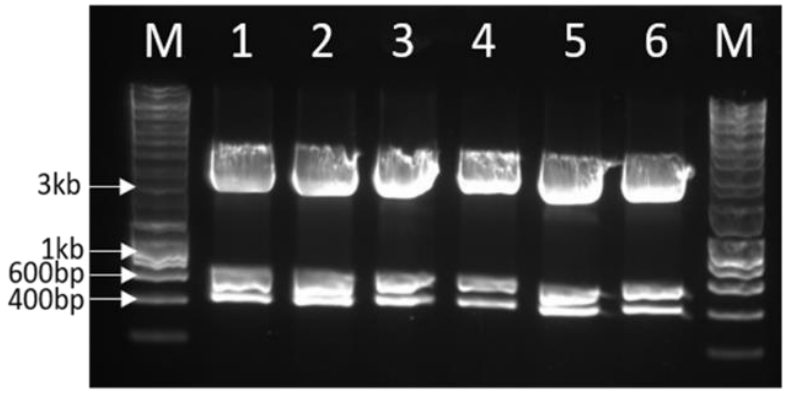
A) Amplification of GPLIN_000755200 by PCR. Lane 1 contains a product amplified by PCR which is slightly larger than the size of GPLIN_000755200 (1 Kb).

B) Alignment of cloned amino acid sequences to the predicted amino acid sequence of GPLIN_000755200. Asterisks (*) indicate an amino acid match, full stop (.) and colon (:) indicate conserved amino acids, and blank spaces indicate gaps in one or more of the sequences. The region of interest is highlighted in yellow for the cloned sequences.

C) Relative expression of GPLIN_000755200. The transcript coverage (blue) aligned to the predicted gene model. The region of interest is highlighted in the black box.

D) Relative expression of GPLIN_000755200 at the region of interest. Transcript coverage (blue) spans the predicted intron.

A



B

```
GPLIN_001111200      MCALICALLPLLFVLMISDHSLIGAVTAPPYGQLAVNGKFLVQKSTKKTVKLHGLSLYWS    60
Clone_1              MCALICALLPLLLVLMISDHSLIGAVTAPPYGQLAVNGKFLVQKSTKKTVKLHGLSLYWS    60
Clone_3              MCALICALLPLLLVLMISDHSLIGAVTAPPYGQLAVNGKFLVQKSTKKTVKLHGLSLYWS    60
Clone_4              MCALICALLPLLLVLMISDHSLIGAVTAPPYGQLAVNGKFLVQKSTKKTVKLHGLSLYWS    60
                     ************:***************************************************

GPLIN_001111200      QWQPRFWVAQTVNRIKCGCNSNVVRAAMGTQKSFGGYVANPSAEYAKMKTIIEAALDQGI    120
Clone_1              QWQPRFWVAQTVNRIKCGCNSNVVRAAMGTQKSFGGYVANPSAEYAKMKTIIEAALDQGI    120
Clone_3              QWQPRFWVAQTVNRIKCGCNSNVVRAAMGTQKSFGGYVANPSAEYAKMKTIIEAALDQGI    120
Clone_4              QWQPRFWVAQTVNRIKCGCNSNVVRAAMGTQKSFGGYVANPSAEYAKMKTIIEAALDQGI    120
                     ************************************************************

GPLIN_001111200      YVIVDWHTGDDLATDEINYAKEFFTKIAQTYGKHPHILYEIWNEPLKQVTWDAVVKPYSK    180
Clone_1              YVIVDWHTGDDLATDEINYAKEFFTKIAQTYGKHPHILYEIWNEPLKQVTWDAVVKPYSK    180
Clone_3              YVIVDWHTGDDLATDEINYAKEFFTKIAQTYGKHPHILYEIWNEPLKQVTWDAVVKPYSK    180
Clone_4              YVIVDWHTGDDLATDEINYAKEFFTKIAQTYGKHPHILYEIWNEPLKQVTWDAVVKPYSK    180
                     ************************************************************

GPLIN_001111200      TMVELIRKYDKNNVIIVGTPNWDQDVEIAARSPLTGFSNIAYTLHFYAGQHNEWLRTRTS    240
Clone_1              TMVELIRKYDKNNVIIVGTPNWDQDVEIAARSPLTGYSNIAYTLHFYAGQHNEWLRTRTS    240
Clone_3              TMVELIRKYDKNNVIIVGTPNWDQDVEIAARSPLTGYSNIAYTLHFYAGQHNEWLRTRTS    240
Clone_4              TMVELIRKYDKNNVIIVGTPNWDQDVEIAARSPLTGYSNIAYTLHFYAGQHNEWLRTRTS    240
                     ************************************:***********************

GPLIN_001111200      TAYKLGLPMFVTEYGIYSEPKNEANNLKELALWYKLLDSLSLSYTAWQVTDINEQHAMMT    300
Clone_1              TAYKLGLPMFVTEYGIYSEPKNEANNLKELAIWYKLLDSLSLSYTAWQVTDINEQHAMMT    300
Clone_3              TAYKLGLPMFVTEYGIYSEPKNEANNLKELAIWYKLLDSLSLSYTAWQVTDINEQHAMMT    300
Clone_4              TAYKLGLPMFVTEYGIYSEPKNEANNLKELAIWYKLLDSLSLSYTAWQVTDINEQHAMMT    300
                     ********************************:***************************

GPLIN_001111200      PGVTINNICNPAYLTTYGKYIYNKLKSQNNGVSCRG 336
Clone_1              PGVTINNICNPAYLTTYGKYIYDKLKSQNNGVSCRG 336
Clone_3              PGVTINNICNPAYLTTYGKYIYDKLKSQNNGVSAAV 336
Clone_4              PGVTINNICNPAYLTTYGKYIYDKLKSQNNGVSCRG 336
                     ********************:**********.
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Figure 3.10- Digestion of pGEM® T Easy vectors containing GPLIN_001111200.

A) Digestion of GPLIN_001111200 out of pGEM® T Easy vectors using EcoRI. Six independent clones of GPLIN_001111200 were digested with EcoRI and they are in lanes 1-6. In all six clones, the product at approximately 3 Kb corresponds with the size of the vector. Two additional bands between 400 and 600 bp in all six clones correspond to the expected size of the gene as GPLIN_001111200 has an EcoRI site within its sequence at 423 bp which EcoRI would split into two fragments 423 bp and 577 bp in length. All six clones were sequenced using T7 and M13 primers.

B) Alignment of cloned amino acid sequences to the predicted amino acid sequence of GPLIN_001111200. Cloned sequences align well with the predicted sequence. Clone 4 has a stop codon two amino acids from the end of the predicted sequence. Asterisks (*) indicate an identical amino acid in all sequences, full stop (.) and colon (:) indicate conservative substitutions of amino acids.

3.4.2.2 *M. incognita*

The putative cellulase and endoxylanase genes in *M. incognita* detailed in Table 3.7 were amplified by PCR using primers designed to encompass the entire coding region. As with the genes of interest in *G. pallida,* these were also cloned and sequenced using Sanger sequencing.

3.4.2.2.1 mi-eng-1

PCR successfully amplified a product of the expected size of *mi-eng-1* (1.6 Kb) (Figure 3.11A). This product was cloned and colony PCR confirmed the presence of clones with likely inserts of the expected size (Figure 3.11B). Plasmids were extracted from cultured cells and digested with EcoRI*. mi-eng-1* has three EcoRI restriction sites within the coding sequence which are 8 bp, 240 bp, and 420 bp after the start codon. These are reflected in the different bands revealed by the agarose gel electrophoresis (Figure 3.11C). Clones were sequenced and the alignments of resultant sequences confirmed the cloning to be successful.

3.4.2.2.2 mi-eng-2

A PCR product of the expected size of *mi-eng-2* (1 Kb) was successfully cloned into pGEM® T Easy. However, alignments with the predicted amino acid sequence showed that the DNA fragment that had been cloned was not *mi-eng-2.* After examination of the cloned sequence, it was apparent that in the original PCR reaction, some residual Oligo-dT primer (used in cDNA synthesis), in combination with one of the gene-specific primers, had led to non-specific annealing and amplification.

To rectify this, the PCR reaction was repeated which failed to produce a product. A gradient PCR was then carried out with annealing temperatures between 40 °C and 60 °C, however this also produced no products. New primers were designed for this gene (Table 3.1; *mi-eng-2* (1)) and repeat amplification was attempted by PCR but this again produced no products. In the interest of time, no further attempts to amplify this gene were made.

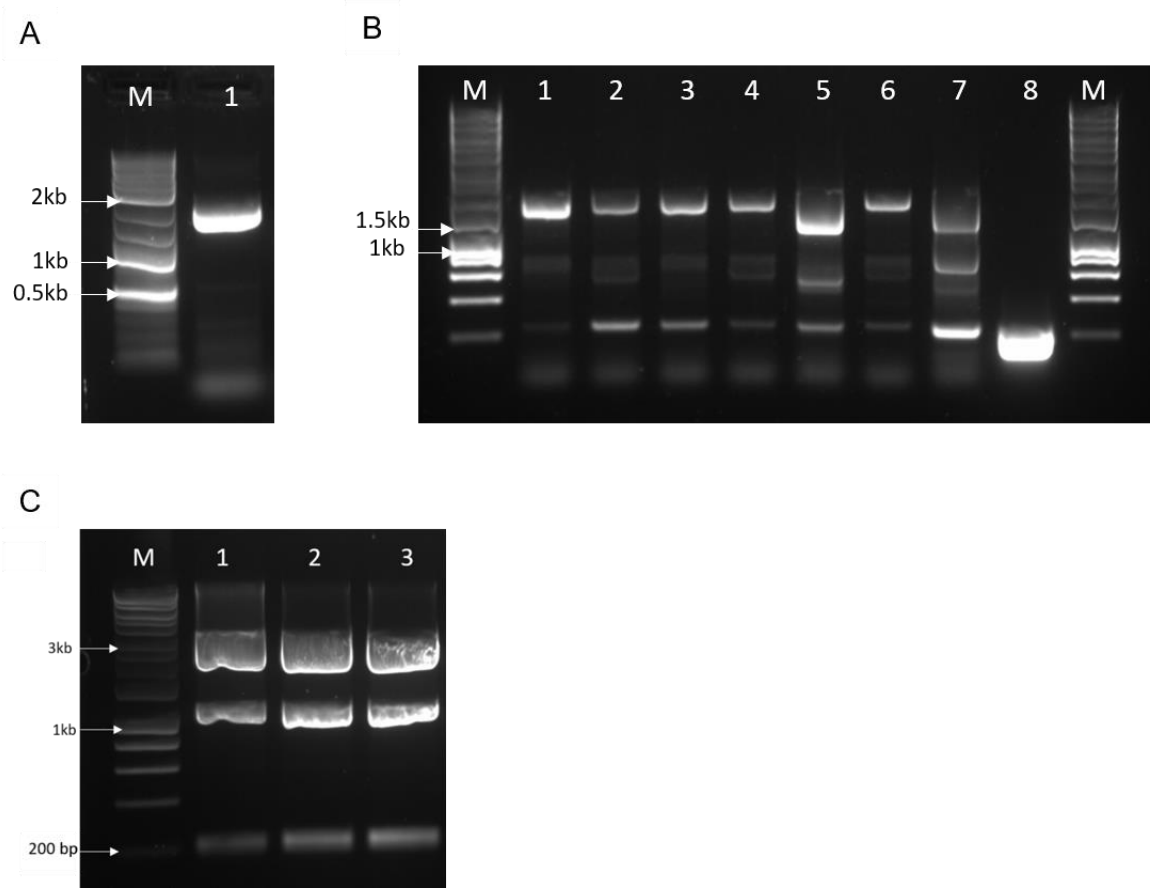

Figure 3.11- Cloning of *mi-eng-1*.

A) Amplification of *mi-eng-1* by PCR. Lane 1 contains an amplified PCR product between 1 and 2 Kb which is of the expected size of *mi-eng-1* (1.6 Kb).

B) Colony PCR of ultra-competent *E.coli* harbouring recombinant pGEM® T Easy. PCR products in lanes 1-7 were amplified from positive white *E. coli* colonies and the PCR product in lane 8 was amplified from a blue *E. coli* colony as a negative control. Products seen above 1.5 Kb in lanes 1-7 represent the successful ligation of *mi-eng-1* (1.6 Kb) into the vector. This colony PCR is representative of colony PCRs carried out throughout the cloning process of putative cellulase genes in *M. incognita.*

C) Digestion of pGEM® T Easy recombinant with *mi-eng-1* using EcoRI. Lanes 1-3 contain three independent clones of *mi-eng-1* that were digested with EcoRI. All of the digestions produced products of 3 Kb which corresponds to the size of the pGEM® T Easy vector. The *mi-eng-1* gene is cut into four fragments of 8 bp (not seen), 180 bp, 232 bp, and 1180 bp in size due to the EcoRI sites within the coding sequence. The 180 bp and 232 bp fragments are not separately resolved under these electrophoresis conditions. This restriction digestion is representative of those carried out throughout the cloning process of putative cellulase and endoxylanase genes in *M. incognita.* All three clones were sequenced.

3.4.2.2.3 mi-eng-4

The *mi-eng-4* coding region was successfully amplified by PCR (Figure 3.12A) and cloned into pGEM® T Easy vector. Plasmids containing *mi-eng-4* were digested with EcoRI which is also expected to cut the gene at 218 bp (Figure 3.12B). The agarose gel electrophoresis analysis of the digestion revealed two distinct bands of approximately this size. This observation deviates from the anticipated outcome based on the predicted sequence. However, further investigation revealed an additional EcoRI site within the cloned sequences at 408 bp, explaining the presence of two bands, each approximately 200 bp in size (Figure 3.12B). Subsequent sequencing of these clones and aligning their sequences with the gene model confirmed that this gene was also successfully cloned.

3.4.2.2.4 mi-xyl

The initial primers designed to amplify *mi-xyl* (Table 3.1) included the entire coding region but failed to produce any amplified products. Altering the annealing temperatures by using gradient PCR (60-50 °C) did not resolve the problem. As a result, a new forward primer was designed to amplify the sequence for the mature *Xyl* protein (Table 3.1; *mi-xyl* (1)), lacking the N-terminal signal peptide. The PCR was repeated with the new forward primer with a Tm of 55 ºC and amplification of the gene was then observed by agarose gel electrophoresis (Figure 3.13A). The PCR product was ligated into pGEM® T Easy vector. Plasmids were extracted from cultured cells and digested using EcoRI (Figure 3.13B) before being sequenced and aligned to the predicted amino acid sequence. Amino acid sequences of cloned genes matched those of gene predictions suggesting this gene has been successfully cloned.

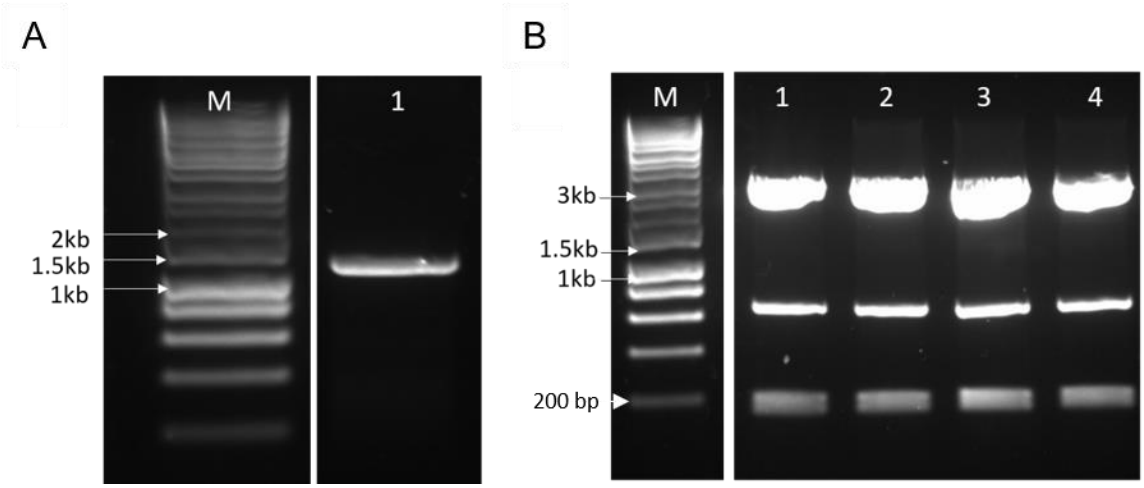

Figure 3.12- Cloning of *mi-eng-4*.

A) Amplification of *mi-eng-4* by PCR. Lane 1 contains the successfully amplified *mi-eng-4* (1.2 Kb).

B) Digestion of pGEM® T Easy recombinant with *mi-eng-4* using EcoRI. Lanes 1-4 contain four independent clones of *mi-eng-1* that were digested with EcoRI. All of which produced a product of 3 Kb which corresponds to the size of the pGEM® T Easy vector. The remaining products correspond to the *mi-eng-4* gene, which is split into three fragments as it contains two EcoRI sites at 218 bp and 408 bp, respectively. All four clones were sequenced.

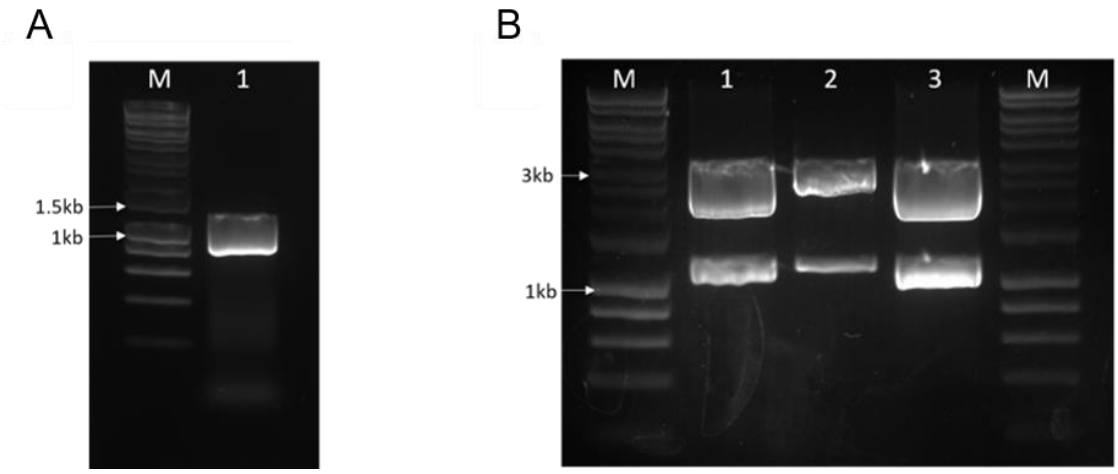

Figure 3.13- Cloning of *mi-xyl.*

A) PCR using new forward primer successfully amplified *mi-xyl.* Lane 1 contains the PCR product of *mi-xyl* (1.2 Kb) which was successfully amplified using the new *mi-xyl* (1) forward primer.

B) Digestion of pGEM® T Easy recombinant with *mi-xyl* using EcoRI. Lanes 1-3 contain three independent clones of *mi-xyl* which were digested with EcoRI. All of the digests produced a product of just larger than 1 Kb *(mi-xyl)* and one of approximately 3 Kb (pGEM® T Easy vector). All three clones were sequenced.

3.4.3 *In situ* hybridisation of putative cellulase and endoxylanase genes in *G. pallida* and *M. incognita*

Nematode effectors such as the genes of interest in *G. pallida* and *M. incognita* are generally synthesised in the dorsal and sub-ventral pharyngeal glands before being secreted into the host through the stylet. It was therefore expected that the putative cellulase and endoxylanase genes identified in *M. incognita* and *G. pallida* would be expressed in the same glands if they were *bona fide* effectors.

Once the genes had been successfully cloned from both nematode species, *in situ* hybridisation was carried out to determine their spatial expression. To verify expression as being in the gland cells, the location of the resultant staining was compared to that of numerous previously documented genes expressed in the gland cells of *G. pallida* (Blanchard *et al*., 2007, Jones *et al*., 2009, Kud *et al*., 2019, Thorpe *et al*., 2014) and *M. incognita* (Bellafiore *et al*., 2008, Jaouannet *et al*., 2018, Mitchum *et al*., 2023). The distance of the sub-ventral gland cells from the head of a J2 was calculated to be approximately 180 µm in 10 published images.

3.4.3.1 GPLIN_000536400

In situ hybridisation confirmed that the spatial expression of GPLIN_000536400 was in the sub-ventral pharyngeal glands (Figure 3.14C). The staining is distinctly localised to the two sub-ventral gland cells with minimal staining observed elsewhere within the nematode.

3.4.3.2 GPLIN_000313600

Although the precise location of the staining associated with GPLIN_000313600 is indistinct, there is clearly hybridisation in the region of the gland cells (Figure 3.14E), implying potential expression of this gene in the dorsal or sub-ventral pharyngeal gland cells.

3.4.3.3 GPLIN_001111200

The *in-situ* hybridisation analysis also failed to reveal the exact spatial domain of GPLIN_001111200 expression. Similar to GPLIN_000313600, staining can be seen in the region of the location of the gland cells (Figure 3.14G) which indicates that this gene could also be expressed here.

3.4.3.4 *mi-eng-1*

The expression of *Mi-eng-1* was confirmed to be in the pharyngeal glands (Figure 3.15C). The staining associated with this gene is seen to be confined to these cells. The position of the staining suggests that it is most likely to be in the sub-ventral glands (Figure 3.15C).

3.4.3.5 *mi-eng-4*

Mi-eng-4 was confirmed to be expressed in the sub-ventral pharyngeal glands (Figure 3.15E). Again, the staining associated with this gene distinctly localised to the sub-ventral glands, with minimal staining observed elsewhere.

3.4.3.6 *mi-xyl*

In situ hybridisation analysis of *Mi-xyl* was unable to reveal the exact spatial domain of its expression (Figure 3.15G). Although there is some staining within the general gland cell region, it does not provide sufficient grounds to definitively affirm specific gland cell expression of the gene.

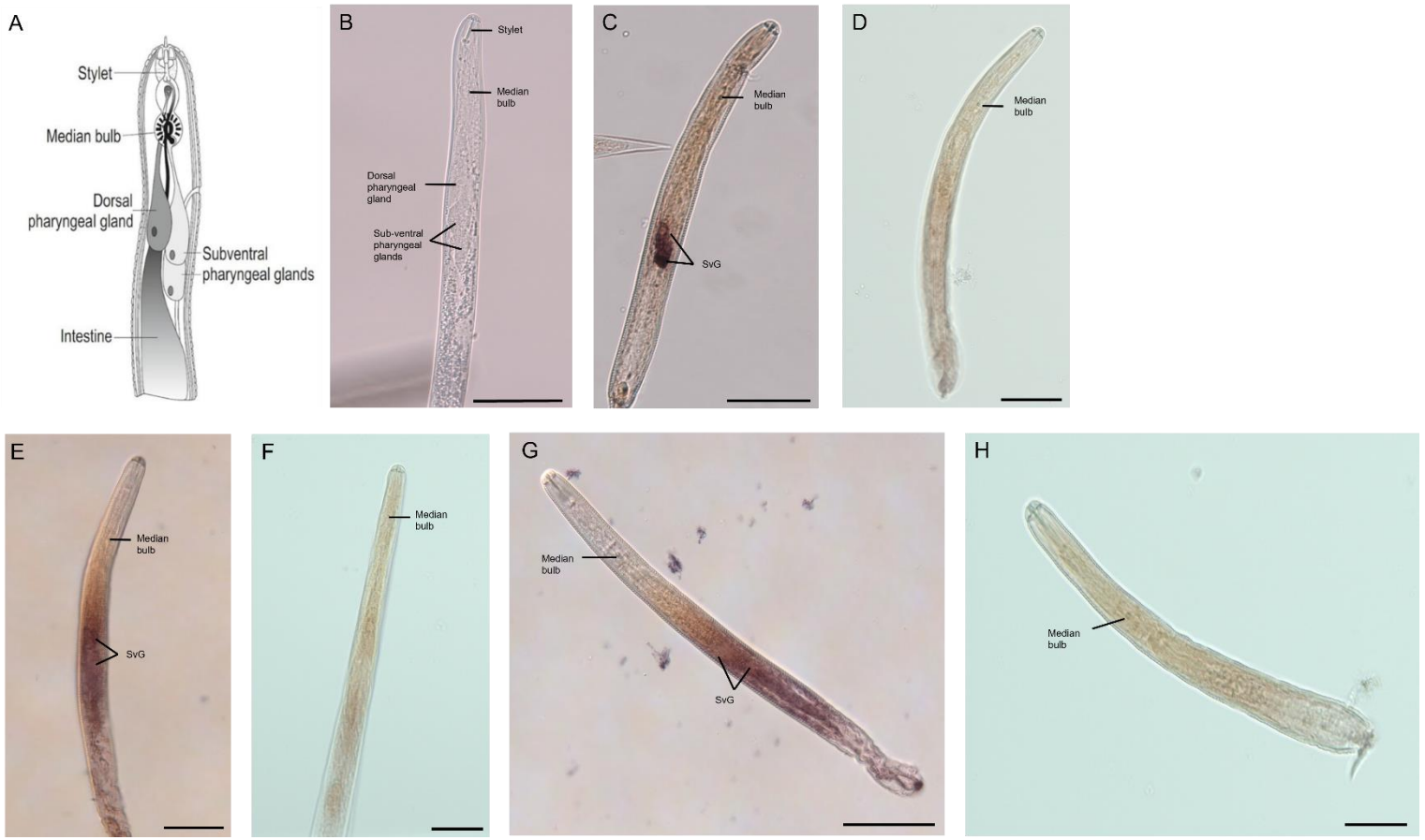

Figure 3.14- *In situ* hybridisation of putative cellulase genes in *G. pallida.*

A) Anatomy of a plant parasitic nematode. **B)** Microscope image of a J2 with the dorsal and sub-ventral pharyngeal glands highlighted **C)** *In situ* hybridisation of **GPLIN_000536400** using an antisense probe. **D)** *In situ* hybridisation of **GPLIN_000536400** using a sense probe. **E)** *In situ* hybridisation of **GPLIN_000313600** using an antisense probe. **F)** *In situ* hybridisation of **GPLIN_000313600** using a sense probe. **G)** *In situ* hybridisation of **GPLIN_001111200** using an antisense probe. **H)** *In situ* hybridisation of **GPLIN_001111200** using a sense probe. Brown/purple staining highlights the spatial expression of the gene of interest. Size markers are 50 µm.

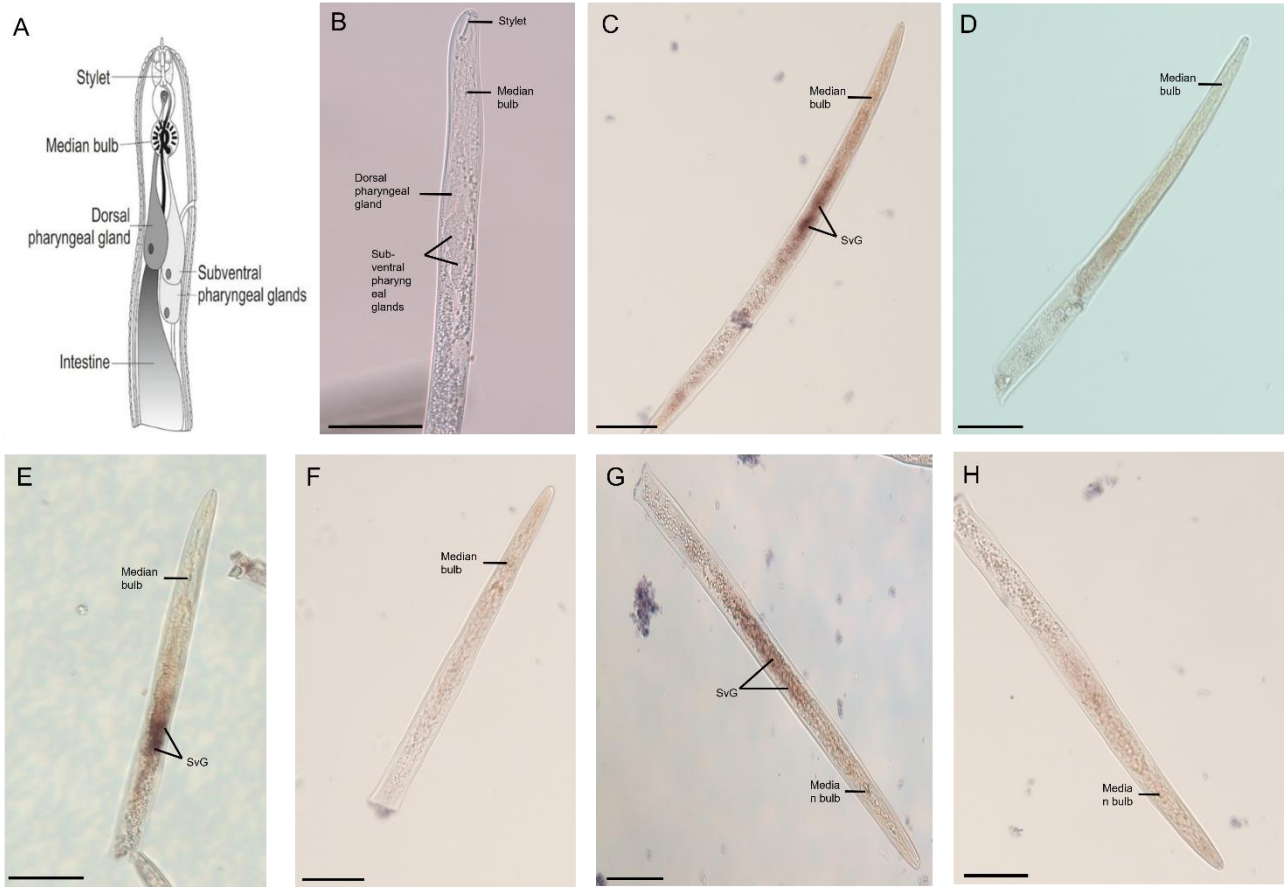

Figure 3.15- *In situ* hybridisation of putative cellulase and endoxylanase genes in *M. incognita.*

A) Anatomy of a plant parasitic nematode. **B)** Microscope image of a J2 with the dorsal and sub-ventral pharyngeal glands highlighted. **C)** *In situ* hybridisation of ***mi-eng-1*** using an antisense probe. **D)** *In situ* hybridisation of ***mi-eng-1*** using a sense probe. **E)** *In situ* hybridisation of ***mi-eng-4*** using an antisense probe. **F)** *In situ* hybridisation of ***mi-eng-4*** using a sense probe. **G)** *In situ* hybridisation of ***mi-xyl*** using an antisense probe. **H)** *In situ* hybridisation of ***mi-xyl*** using a sense probe. Brown/purple staining highlights the spatial expression of the gene of interest. Size markers are 50 µm.

3.4.4 Confirmation of enzymatic activity of putative cellulase genes in *G. pallida and M. incognita*

The extent of the up-regulation observed for the cellulase and endoxylanase genes in *P. coffeae* exhibited a correlation with the specific concentration of substrate present in the root exudates they were exposed to. This may also extend to the genes of interest identified in *G. pallida* and *M. incognita.* Therefore, attempts were made to express and purify the proteins they encode with the aim of assessing their enzymatic activity.

3.4.4.1 Cloning *G. pallida* genes of interest into an expression vector, pQE-30

Genes of interest in *G. pallida* (GPLIN_000313600, GPLIN_000536400 and GPLIN_001111200) were successfully amplified by PCR (Figure 3.16) using primers that encompassed the coding sequence, without the signal peptide. The forward primers incorporated BamHI and the reverse primers incorporated either HindIII or SalI restriction sites (Table 3.3).

pQE-30 expression vectors and PCR products were both successfully digested with either BamHI and HindIII or BamHI and SalI (Figure 3.16). Digested PCR products were ligated into appropriately digested expression vectors and the recombinant plasmids were transformed into M15 (pREP4) *E. coli*. Colony PCR was used to confirm that the genes of interest had been successfully ligated into the expression vector and transformed into M15 (pREP4).

Recombinant plasmids were digested with their appropriate restriction enzymes to confirm ligation of the correct PCR product (Figure 3.17). Clones that harboured DNA fragments of the expected size were sequenced by Sanger sequencing and the resultant sequences were aligned to the expected sequences using Clustal Omega (EMBL-EBI) to confirm cloning success, as described previously. These alignments confirmed that GPLIN_000313600, GPLIN_000536400 and GPLIN_001111200 had been successfully cloned.

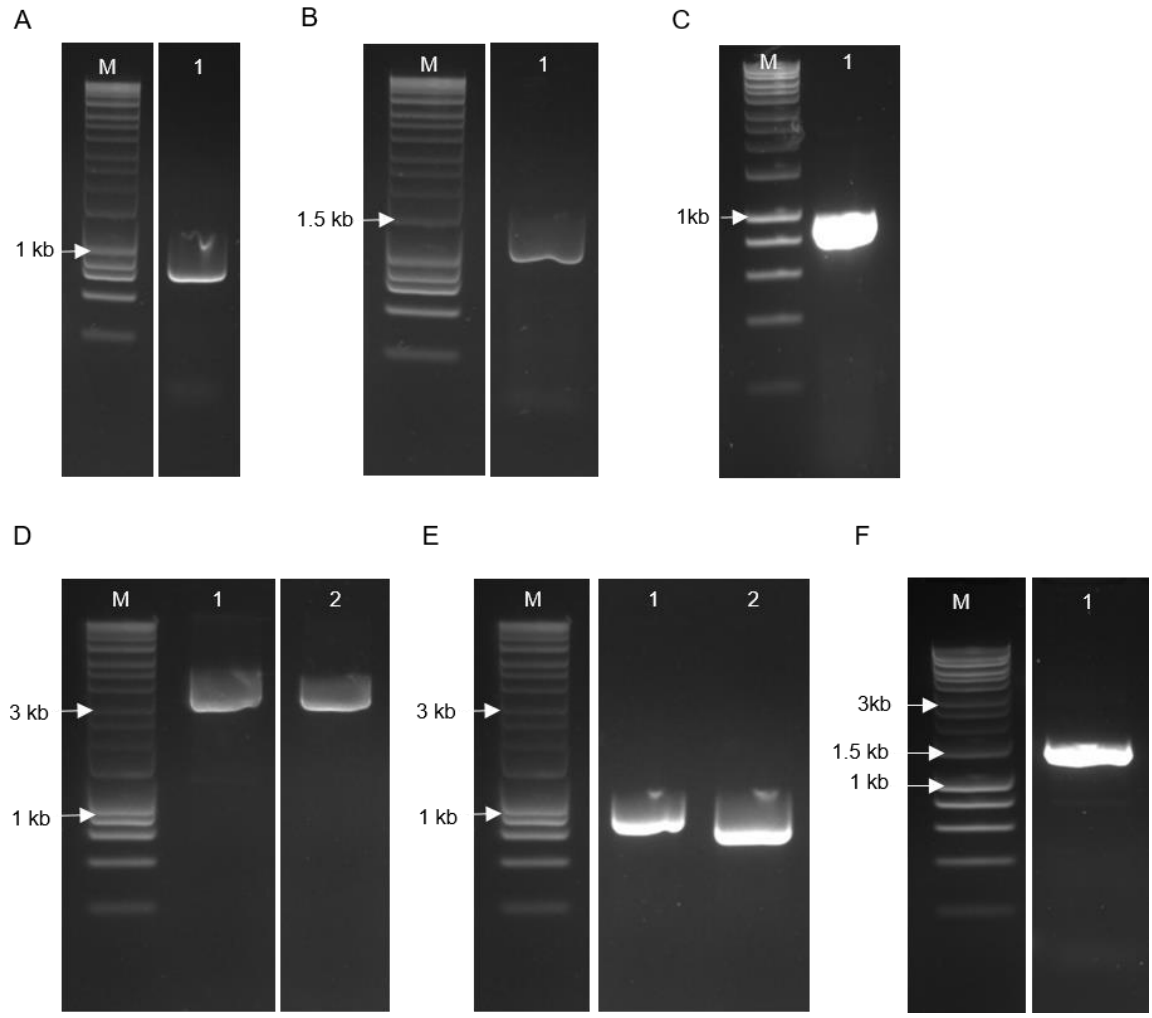

Figure 3.16- Cloning of *G. pallida* putative cellulase genes into expression vector, pQE-30.

A) PCR amplification of GPLIN_000313600. Lane 1 is a PCR product corresponding to the expected size of GPLIN_000313600 (1 Kb).

B) PCR amplification of GPLIN_000536400. Lane 1 is a PCR product corresponding to the expected size of GPLIN_000536400 (1.3 Kb).

C) PCR amplification of GPLIN_001111200. Lane 1 is a PCR product corresponding to the expected size of GPLIN_001111200 (1 Kb).

D) Digestion of pQE-30 with restriction enzymes. Lane 1 is pQE-30 (3.4 Kb) linearised with BamHI and HindIII. Lane 2 is pQE30 (3.4 Kb) linearised with BamHI and SalI.

E) Digestion of GPLIN_001111200 and GPLIN_000313600 with restriction enzymes. Lane 1 is GPLIN_001111200 (1 Kb) digested with BamHI and SalI. Lane 2 is GPLIN_000313600 (1 Kb) digested with BamHI and SalI.

F) Digestion of GPLIN_000536400 with restriction enzymes. Lane 1 is GPLIN_000536400 (1 Kb) digested with BamHI and HindIII.

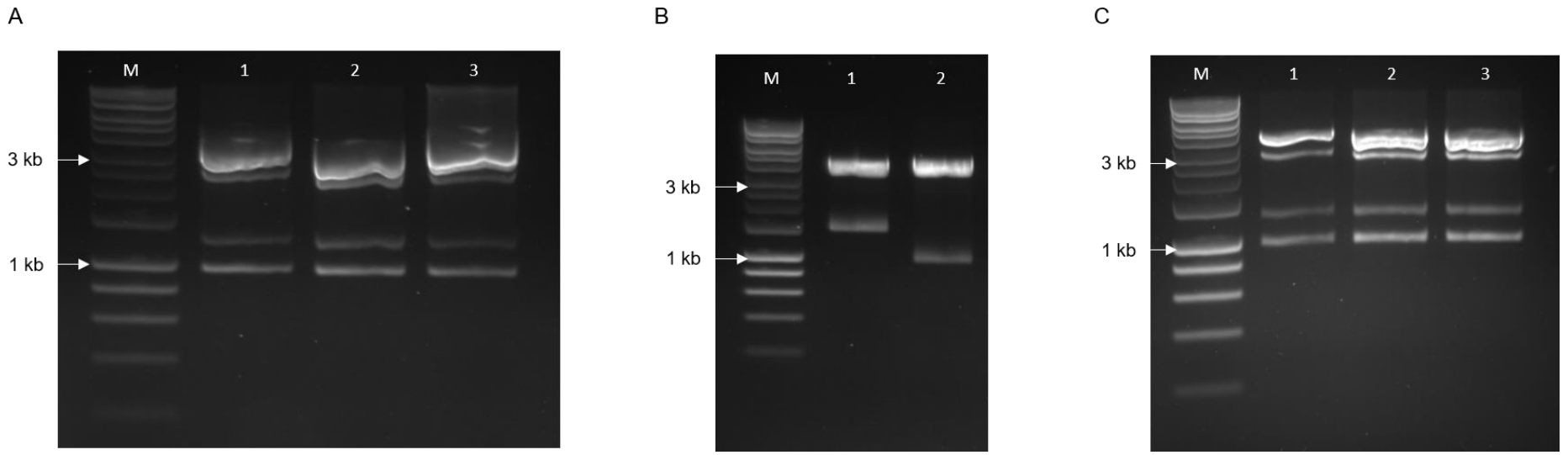

Figure 3.17- Digestion of positive *G. pallida* clones from expression vector, pQE-30.

A) Digestion of GPLIN_000313600 clones with restriction enzymes. Lanes 1-3 contain three independent pQE-30 vectors containing GPLIN_000313600 (1 Kb) digested with BamHI and SalI. Product at ~3.5 Kb corresponds to the pQE-30 vector (3.4 Kb).

B) Digestion of GPLIN_000536400 clones with restriction enzymes. Lanes 1 and 2 contain two independent clones of GPLIN_000536400 (1 Kb) digested with BamHI and HindIII. Product at ~3.5 Kb corresponds to the pQE-30 vector (3.4 Kb).

C) Digestion of GPLIN_001111200 clones with restriction enzymes. Lanes 1-3 contain three independent clones of GPLIN_001111200 (1 Kb) digested with BamHI and SalII. Product at ~3.5 Kb corresponds to the pQE-30 vector (3.4 Kb).

Additional bands seen in A and C are due to the presence of the pREP4 plasmid in M15(pREP4).

3.4.4.2 Cloning *M. incognita* genes of interest into an expression vector, pQE-30

Primers to amplify the coding sequence for each gene, without the signal peptide, were designed to incorporate BamHI and HindIII restriction sites. They successfully amplified genes of interest in *M. incognita* (*mi-eng-1, mi-eng-4* and *mi-xyl*) by PCR (Figure 3.18).

pQE-30 vector, and PCR products were both successfully digested with BamHI and HindIII (Figure 3.18). Digested PCR products were ligated into appropriately digested expression vectors and the recombinant plasmids were transformed into M15 (pREP4) *E. coli*. As for *G. pallida,* successful cloning was confirmed by colony PCR and digestion of recombinant plasmids with BamHI and HindIII to confirm the insertion of a DNA fragment of the expected size (Figure 3.19). Sanger sequencing of these confirmed the successful cloning of *mi-eng-4* and *mi-xyl* into the expression vector, pQE30. It was confirmed that *mi-eng-1* was not successfully cloned and would not be investigated beyond this point.

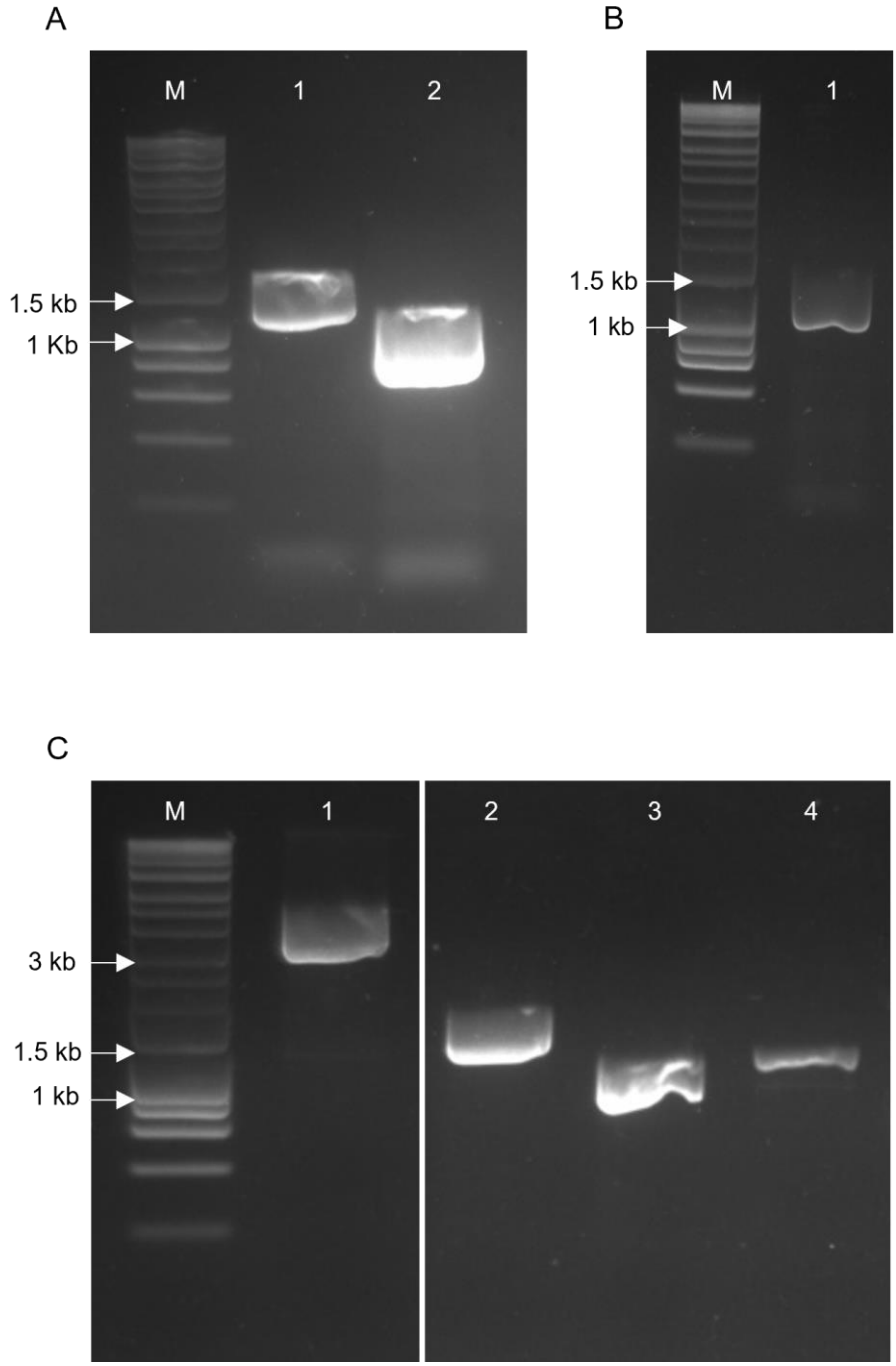

Figure 3.18- Cloning of *M. incognita* putative cellulase and endoxylanase genes into expression vector, pQE-30.

A) PCR amplification of *mi-eng-1* and *mi-eng-4.* Genes were amplified using primers that incorporated BamHI and HindIII restriction sites. Lane 1 is the PCR product for *mi-eng-1* (1.6 Kb) and Lane 2 is the PCR product for *mi-eng-4* (1.2 Kb).

B) PCR amplification of *mi-xyl*. *mi-xyl* (1.2 Kb) was also amplified using primers that incorporated BamHI and HindIII restriction sites. Lane 1 is the PCR product.

C) Digestion of pQE-30 and *mi-eng-1, mi-eng-4* and *mi-xyl* PCR products with restriction enzymes. Lane 1 is the expression vector (pQE-30) digested with BamHI and HindIII. Lane 2 is *mi-eng-1* digested with BamHI and HindIII. Lane 3 is *mi-eng-4* digested with BamHI and HindIII. Lane 4 is *mi-xyl* digested with BamHI and HindIII.

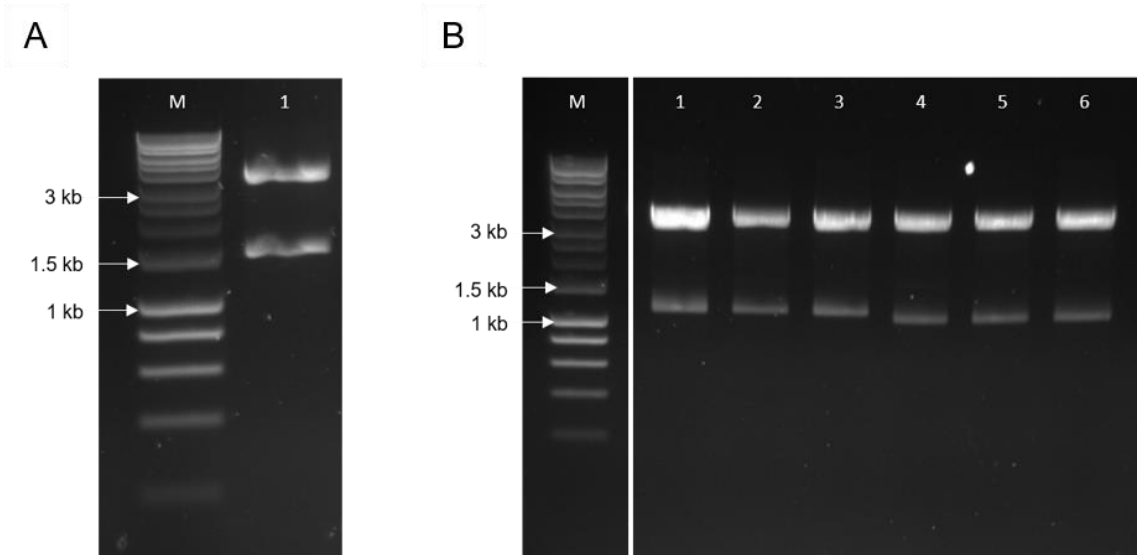

Figure 3.19- Digestion of positive *M. incognita* clones from expression vector, pQE-30.

A) Digestion of *mi-eng-1* clone with restriction enzymes. Lane 1 is a pQE-30 vector (3.4 Kb) containing what is assumed to be *mi-eng-1* (1.6 Kb) digested with BamHI and HindIII.

B) Digestion of *mi-eng-4* and *mi-xyl* clones with restriction enzymes. Lanes 1-3 contain three independent clones of *mi-eng-4* (1.2 Kb) digested with BamHI and HindIII. Lanes 4-6 contain three independent clones of *mi-xyl* (1.2 Kb) digested with BamHI and HindIII.

3.4.4.3 Analysing protein expression in transformed M15 (pREP4) cultures

Once the putative cellulase genes from *G. pallida* and *M. incognita* and the endoxylanase gene from *M. incognita* had been cloned, protein expression was induced in M15 (pREP4) cultures harbouring the recombinant plasmids by the addition of IPTG (2 mM). Cultures were then grown for a further three hours before being pelleted. Control cultures were produced without the addition of IPTG.

To test for successful protein expression in a small-scale experiment, and purify the proteins encoded by the genes of interest in *G. pallida* and *M. incognita,* cells were lysed in a strongly denaturing buffer (Buffer B) to both release cellular components and solubilise any protein that had been sequestered into inclusion bodies by the bacteria. Initially, protein induction was attempted for GPLIN_000313600, GPLIN_000536400, *mi-eng-4* and *mi-xyl* genes, but the induction could not be visualised because cell lysis using the denaturing buffer proved to be unsuccessful. This was repeated multiple times for multiple different cell pellets, but all attempts proved to be unsuccessful.

It was evident from the many unsuccessful cell lysis attempts using denaturing buffer for cell lysis, that an alternative lysis approach was necessary. Initially, an aliquot of cells from Mi-ENG-4 and Mi-XYL samples were extracted prior to pelleting and then incubated directly with 5 X PAGE buffer at 95 °C (Figure 3.20). This approach effectively lysed the cells and also revealed the presence of induced proteins in the Mi-ENG-4 sample (Figure 3.20). One closely matched the expected size of MI-ENG-4 (40.06 kDa), whereas the other is between 50 and 60 kDa in size and its identity remains unknown (Figure 3.20). The same procedure was repeated for GPLIN_000313600 and GPLIN_000536400, but no protein induction was seen in those cases. As a result, future efforts to refine the method of protein purification from cell cultures were primarily focussed on Mi-ENG-4.

While subjecting the samples to heating with 5 X PAGE buffer effectively induced cell lysis, the aim was to purify the active protein to assess its activity. Consequently, further native cell lysis techniques including sonication, incubation in BugBuster®, and lysozyme addition were trialled. All of these methods successfully lysed bacterial cells and yielded visible proteins, although the lysozyme treatment was the least effective (Figure 3.21).

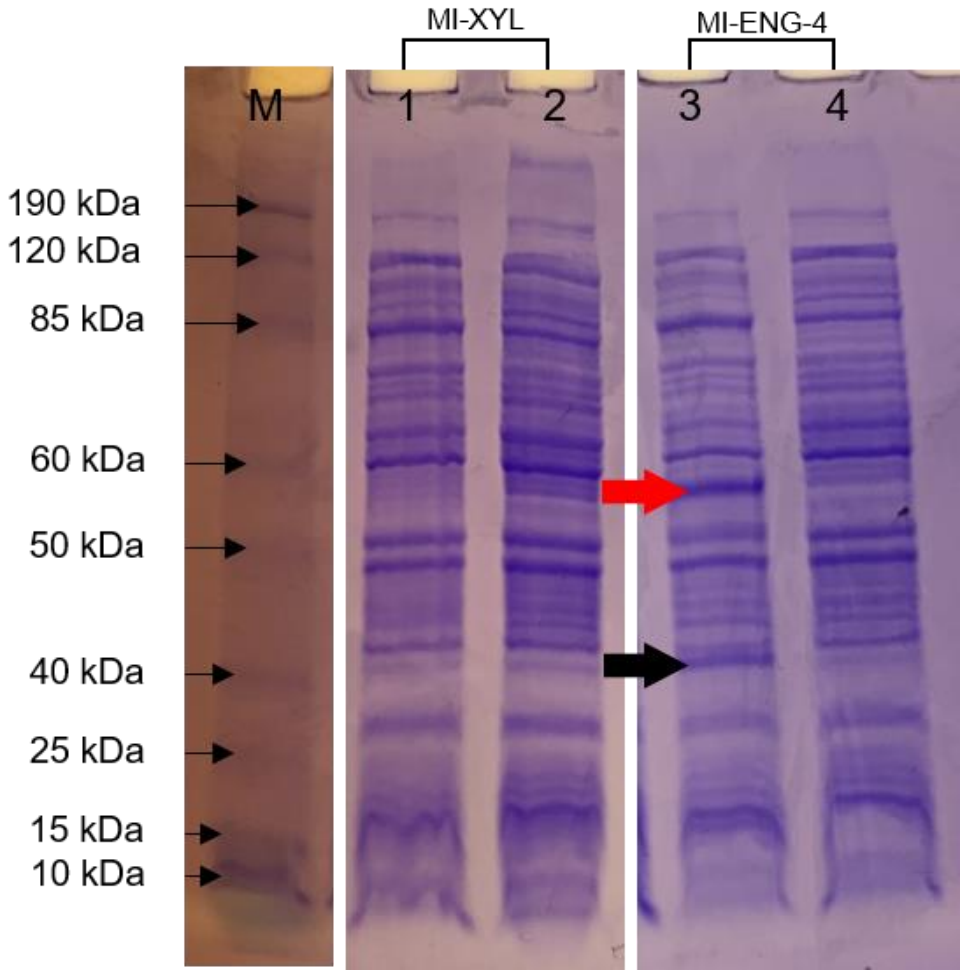

Figure 3.20- Lysis of M15 (pREP4) cells containing recombinant pQE-30 by boiling with 5 X PAGE buffer.

Lane 1 contains cells lysed by boiling with 5 X PAGE buffer where the expression of Mi-XYL is induced by the addition of IPTG.

Lane 2 contains cells lysed in the same way where the expression of Mi-XYL is not induced (control).

Lane 3 contains lysed cells where the expression of Mi-ENG-4 is induced by the addition of IPTG.

Lane 4 contains lysed cells where the expression of Mi-ENG-4 is not induced (control).

Cells are successfully lysed with 5 X PAGE buffer and proteins can be visualised using this method. The black arrow highlights a protein of the expected size of Mi-ENG-4 (40.06 kDa) that is expressed in the induced cell culture but not in the control culture and therefore indicates the potential induction of the Mi-ENG-4 protein.

The red arrow highlights an unknown protein of between 50 and 60 kDa that is also induced.

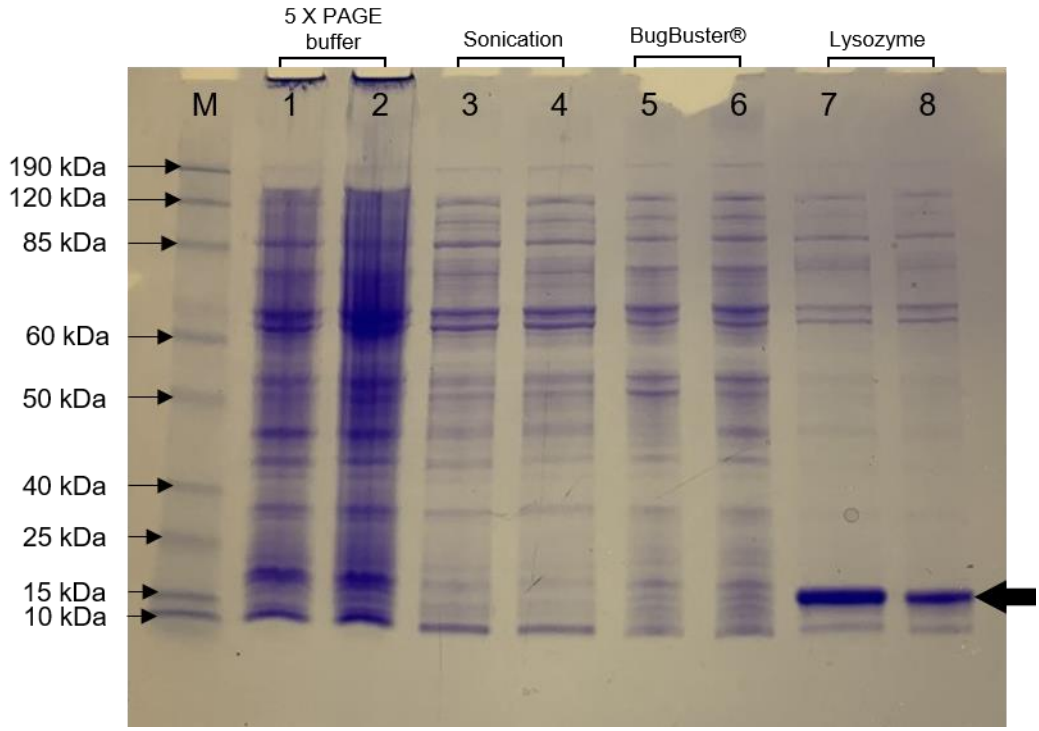

Figure 3.21- Analysis of M15 (pREP4) *E. coli* cell lysis methods.

MI-ENG-4 protein expression was induced by adding IPTG to a cell culture harbouring recombinant pQE-30 plasmids. A control culture was created without the addition of IPTG. MI-ENG-4 was not induced in this experiment, shown by the lack of differences between the induced (odd lanes) and control (even lanes).

Lanes 1 and 2 contain cells lysed by boiling with 5 X PAGE buffer.

Lanes 3 and 4 contain cells lysed by sonication.

Lanes 5 and 6 contain cells lysed by BugBuster® (EMD Millipore).

Lanes 7 and 8 contain cells lysed by the addition of lysozyme. The arrow indicates the lysozyme protein (~15 kDa).

Lysis by BugBuster® produced the most distinct protein bands and so this method was used to lyse cells in subsequent attempts at protein purification.

BugBuster® was selected as the lysis method for native purification attempts of Mi-ENG-4. Induction of the protein was repeated and subsequent cell lysis using this method revealed the induction of a protein, presumed to be Mi-ENG-4, in the cell culture subjected to the addition of IPTG (Figure 3.22). Notably, the unknown protein of between 50 and 60 kDa induced previously (Figure 3.20) was not evident in this case (Figure 3.22).

Analysis of an aliquot of the sample supernatant after addition of the Ni-NTA resin on a polyacrylamide gel confirmed its absence due to the successful binding of the protein of interest to the resin (Figure 3.22). However, the elution fraction, obtained by adding an imidazole-based elution buffer (as described in Section 3.3.4.6), did not exhibit detectable levels of the purified protein when visualised on the gel (Figure 3.22). The elution fractions of both the induced and control samples did, however, contain an unknown protein slightly larger than 60 kDa (Figure 3.22). To ensure that the protein of interest was not lost during the resin wash steps, aliquots from each wash were retained and analysed on the polyacrylamide gel. The protein of interest was not seen to be contained in the washes (Figure 3.22).

At this stage, it was apparent that the protein had been successfully induced and bound to the resin, but the challenge was eluting the purified protein. As a result, Buffer C was supplemented with 0.1 M EDTA from the small-scale purification method (Section 3.3.4.5). This was then tested as a more aggressive chemical agent capable of stripping the nickel ions from the Ni-NTA resin and solubilising any protein that may have precipitated (Figure 3.23). Two separate elution steps using EDTA were performed, but they both failed to elute the purified protein (Figure 3.23). Due to time constraints, protein purification attempts were discontinued at this point.

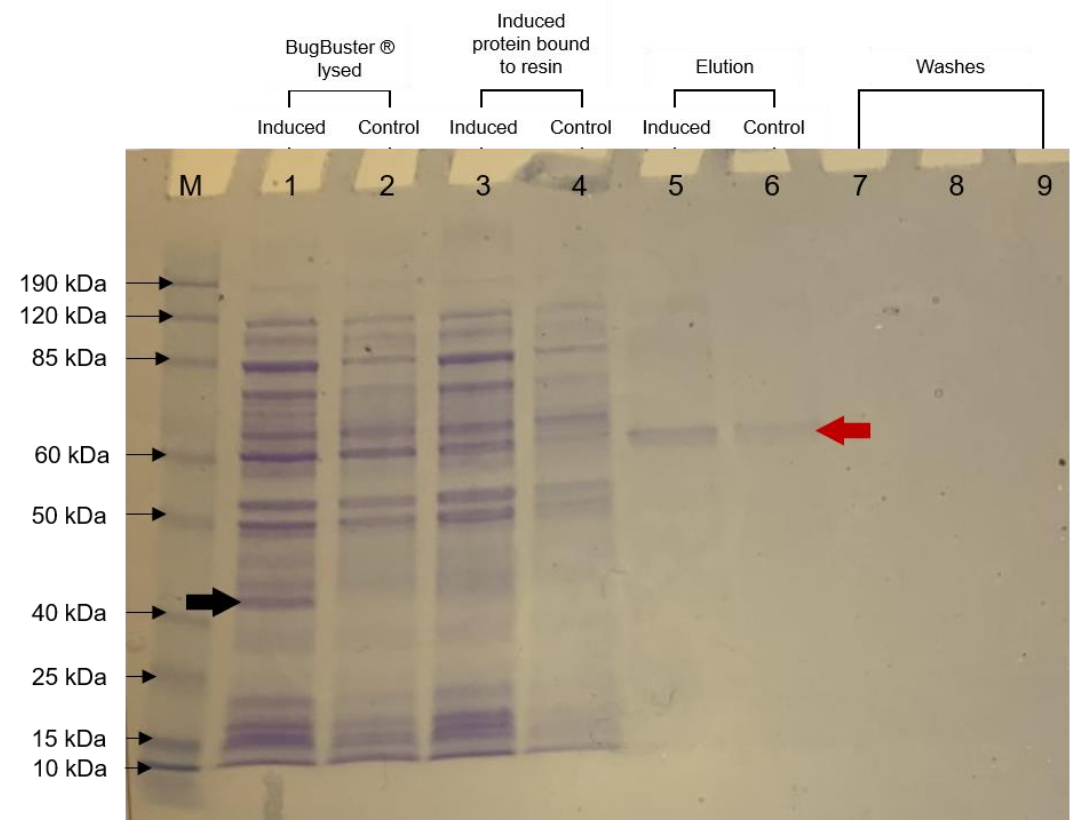

Figure 3.22- Attempted protein purification of Mi-ENG-4.

Mi-ENG-4 protein expression was induced in M15 (pREP4) *E. coli* cell cultures by the addition of IPTG. Control cultures were grown without the addition of IPTG.

Lane 1 contains cells lysed by BugBuster® where the expression of Mi-ENG-4 has been induced. The black arrow highlights a protein of the expected size of Mi-ENG-4 (40.06 kDa) that is expressed in the induced cell culture but not in the control culture and therefore indicates the successful induction of the Mi-ENG-4 protein.

Lane 2 contains control cells lysed by BugBuster®.

Lane 3 contains an aliquot of the induced sample after the addition of Ni-NTA resin. The lack of protein at ~40 kDa suggests it has successfully bound to the resin.

Lane 4 contains an aliquot of the control sample after the addition of Ni-NTA resin.

Lane 5 contains an aliquot of the elution of the induced sample. The protein of interest has not been eluted in this case.

Lane 6 contains an aliquot of the elution of the control sample. The protein present in lanes 5 and 6 highlighted by a red arrow is a non-target protein (~60 kDa) eluted in both the induced and control samples.

Lanes 7, 8 and 9 contain aliquots from the first three wash steps of the induced sample which show that the protein was not lost in the washes.

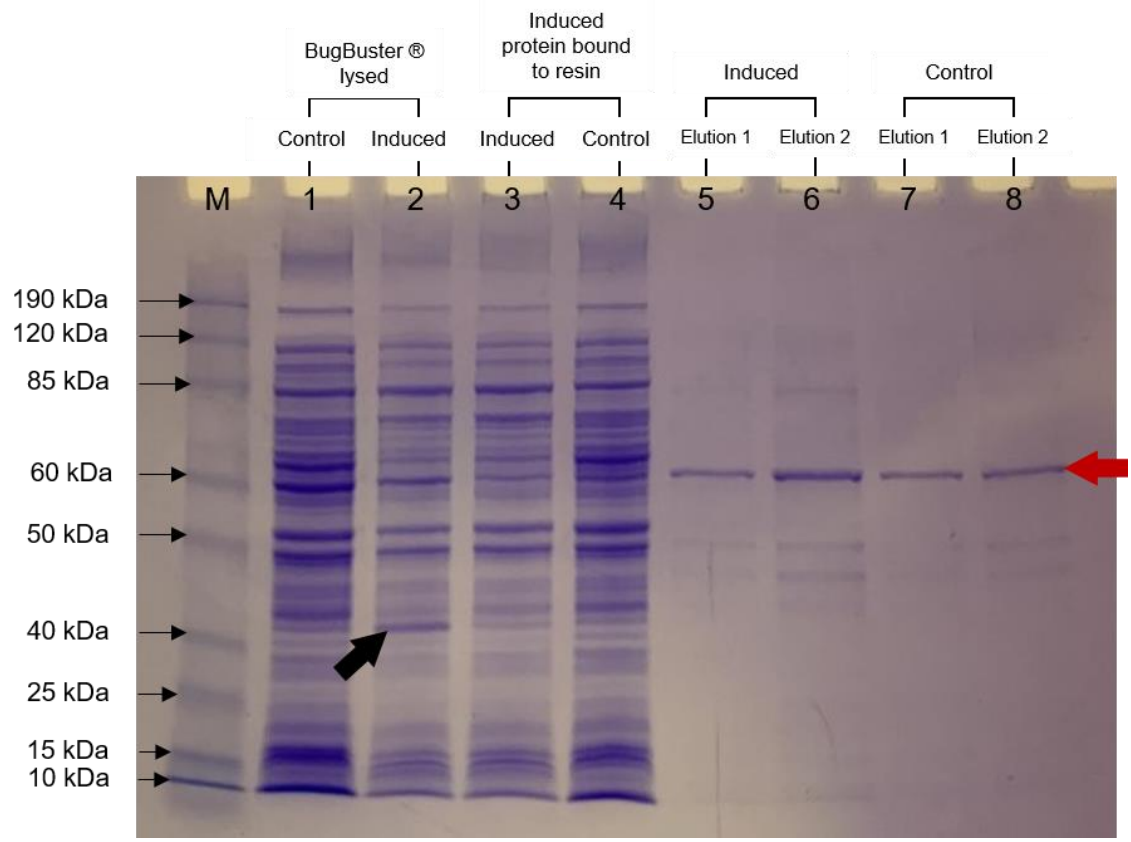

Figure 3.23- Final attempt to purify Mi-ENG-4 protein using an EDTA-based elution buffer.

Mi-ENG-4 protein expression was induced in M15 (pREP4) *E. coli* cell cultures by the addition of IPTG. Control cultures were grown without the addition of IPTG.

Lane 1 contains control cells lysed by BugBuster®.

Lane 2 contains induced cells lysed by BugBuster®. The black arrow highlights a protein of the expected size of Mi-ENG-4 (40.06 kDa) that is expressed in the induced cell culture but not in the control culture.

Lane 3 contains an aliquot of the induced sample after the addition of Ni-NTA resin. The protein (~40 kDa) has bound to the resin.

Lane 4 contains an aliquot of the control sample after the addition of Ni-NTA resin.

Lane 5 contains an aliquot of the first elution of the induced sample.

Lane 6 contains an aliquot of the second elution of the induced sample.

Lane 7 contains an aliquot of the first elution of the control sample.

Lane 8 contains an aliquot of the second elution of the control sample.

Protein present in lanes 5,6,7 and 8 at approximately 60 kDa (highlighted by the red arrow) is a non-target protein.

3.4.5 Confirmation of the efficacy of a Congo red based assay for the assessment of cellulase activity

The effectiveness of the Congo red assay in detecting cellulase activity was evaluated through its application to nematode homogenate derived from *G. pallida* and *M. incognita* J2s.

Although the cellulase activity of homogenate from 10 J2s nematodes was below the detection limit of this assay (Figure 3.24), it effectively revealed the cellulase activity of pools of 100, 500 and 1,000 *G. pallida* and *M. incognita* J2s, as evidenced by the appearance of clearance zones (Figure 3.24). Notably, in both nematode species, the intensity of the cellulase activity was seen to be correlated with the quantity of nematodes utilised to prepare the nematode homogenate, shown by the progressively expanding clearance zones observed (Figure 3.24).

The noticeably increased clearance zones observed for *M. incognita* (Figure 3.24) suggests that cellulase activity is stronger than that exhibited by *G. pallida*.

3.4.6 Assessment of cellulase activity of *M. incognita* Mi-ENG-4

Despite the unsuccessful protein purification of the putative cellulase and endoxylanase genes in *G. pallida* and *M. incognita,* the cellulase activity of the Mi-ENG-4 protein from *M. incognita* could still be evaluated using a simple cellulose plate assay. The successful induction of protein expression upon IPTG addition had been confirmed through polyacrylamide gel analysis. Cells lysed with BugBuster were applied to 1 % carboxymethylcellulose plates. This process facilitated the visualisation of clearance zones where the cell lysate containing the presumed Mi-ENG-4 enzyme had digested the cellulose substrate (Figure 3.25), confirming successful expression of soluble protein and the cellulase activity of this enzyme.

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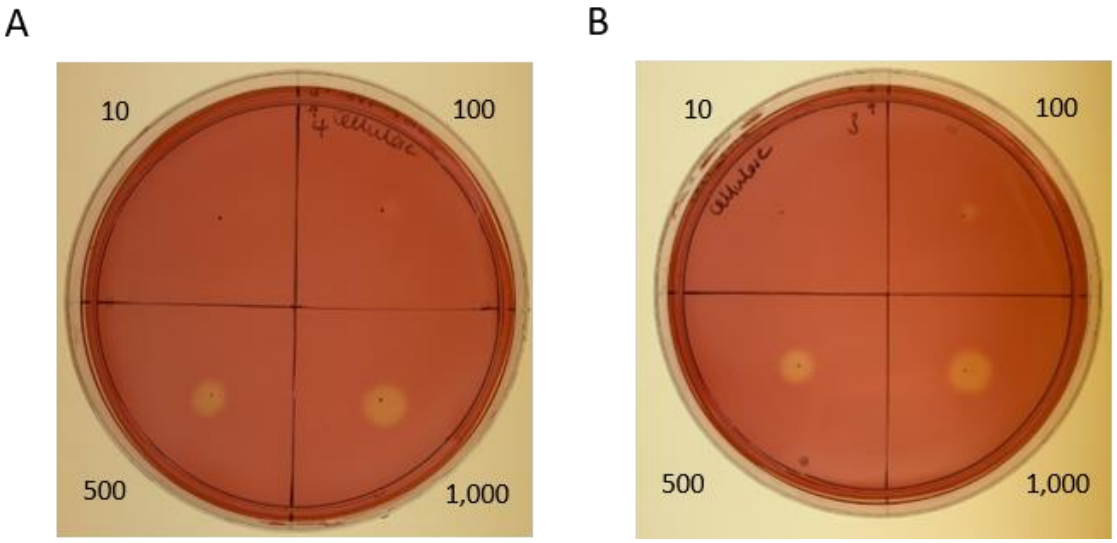


Figure 3.24- Cellulase activity of nematode homogenate.

A) Cellulase activity of *G. pallida* J2 homogenate. *G. pallida* J2 nematode homogenate exhibits cellulase activity which increases with the number of nematodes the homogenate was prepared from. Cellulose is stained with Congo Red and clearance zones show areas of cellulose digestion. Numbers next to each quarter of the cellulose plate represent the number of nematodes utilised in the generation of homogenate.

B) Cellulase activity of *M. incognita* homogenate. *M. incognita* J2 nematode homogenate also exhibits cellulase activity which also increases with the number of nematodes the homogenate was prepared from. Numbers next to each quarter of the cellulose plate represent the number of nematodes utilised in the generation of homogenate.

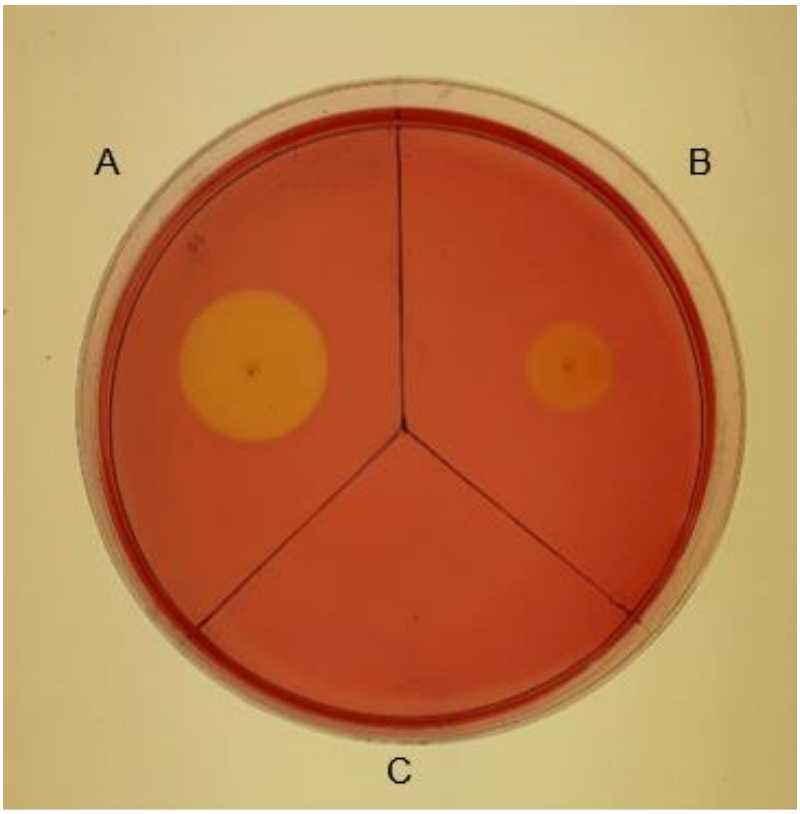


Figure 3.25- Cellulase activity of the protein encoded by *mi-eng-4*.

IPTG was added to M15 (pREP4) cells that contained recombinant pQE-30 to induce the expression of the protein encoded by *mi-eng-4.* Control cultures were generated without the addition of IPTG. **A)** 30 µl macerozyme (0.1 g/ml in water) (positive control); **B)** cell lysate from induced; **C)** or un-induced cell cultures was carefully applied to the centre of designated sections of a carboxymethylcellulose plate (marked). Cell lysate containing induced expression of MI-ENG-4 created clearance zones which indicates cellulase activity.

3.5 Discussion

3.5.1 The role of cell wall-degrading enzymes

The findings of this chapter identified genes encoding cellulases and endoxylanases in *G. pallida* and *M. incognita.* These enzymes are responsible for digesting and modifying cellulose and xylan within the plant cell wall. It is important for *G. pallida* and *M. incognita,* as endoparasitic nematodes, to possess cell wall-degrading enzymes in order to successfully parasitise their host plants. The secretion of these enzymes into the host, through the stylet, is known to facilitate root penetration and subsequent migration towards the vasculature where the nematodes form their permanent feeding sites. The inability to degrade cellulose or any other cell wall component may result in the failure of the nematode to penetrate the root and/or migrate within the root tissue, ultimately leading to the death of the nematode.

G. pallida and *M. incognita* possess a range of cell wall-degrading enzymes. Approximately 40 have been suggested in the genome of *G. pallida* (Thorpe *et al*., 2014) and there is evidence for up to 90 in *M. incognita* (Abad *et al*., 2008, Bozbuga *et al*., 2018). The genes encoding these belong to varying gene families and the numbers in each of these families varies between the nematode species (Thorpe *et al*., 2014). This suggests that these nematodes possess unique repertoires of cell wall-degrading enzymes.

A diverse range of cell wall-degrading enzymes gives plant parasitic nematodes the ability to degrade cellulose, xylan, pectin, and arabinan (Abad *et al*., 2008, Bozbuga *et al*., 2018). RNA-Seq data showed that the majority of genes encoding cellulases and endoxylanases in *G. pallida* and *M. incognita* are significantly up-regulated in J2 stage nematodes. Although the specific roles of these genes are not well reported, due to their expression profiles, it is speculated that they could contribute to a wide range of processes during early parasitism, considering that other genes expressed in these glands in the same nematode species are recognised for their roles in this context.

Cell wall-degrading enzymes have a similar role in the interactions of phytophagous insects with their host plants. Beetles and termites, for example, also utilise a diverse range of cell wall-degrading enzymes which contributes to their feeding diversity (Kirsch *et al*., 2016, Pauchet *et al*., 2010, Watanabe and Tokuda, 2009). Like plant parasitic nematodes, they are known to employ cellulases, pectinases, and endoxylanases in order to gain access to essential nutrients from their plant hosts

(Antony *et al*., 2017, Kirsch *et al*., 2016, Pauchet and Heckel, 2013, Pauchet *et al*., 2010, Watanabe and Tokuda, 2009). Similarly, a large number of plant pathogenic fungi species also employ various different cell wall-degrading enzymes to degrade the plant cell walls of their hosts including cellulases, hemicellulases and pectate lyases (Kubicek *et al*., 2014). Like for parasitic nematodes, these enzymes play a crucial role in host invasion They are especially important for fungi that lack specialised penetration structures (Kubicek *et al*., 2014).

Despite the identification of a substantial number of genes encoding cell wall-degrading enzymes in *G. pallida* and *M. incognita,* their roles in parasitism remain largely unclear. To investigate the significance of these enzymes in host-parasite interactions, a subset of genes presumed to encode cellulases and endoxylanases were identified and cloned in *G. pallida* and *M. incognita.* Further characterisation was carried out through the application of *in situ* hybridisation.

3.5.2 Putative cellulase genes in *G. pallida* and *M. incognita*

Cellulase genes usually encode a signal peptide at the beginning of the coding sequence followed by a catalytic domain (Ali *et al*., 2017). In plant parasitic nematodes, the signal peptide ensures that the protein that is produced is secreted through the stylet into the plant cells. The catalytic domain encodes the cellulase activity of the enzyme. Sometimes, cellulase genes can also have a cellulose/carbohydrate binding domain which facilitates stronger binding of the enzyme to the substrate which in turn, activates the catalytic domain (Linder and Teeri, 1997).

All of the putative cellulase genes of interest both *G. pallida* and *M. incognita* encode a signal peptide and a cellulase catalytic domain. In addition, one putative cellulase in *G. pallida* (GPLIN_000536400) and another in *M. incognita* (*mi-eng-1*) were found to have a cellulose binding domain within their coding sequence, which could help the protein bind to the substrate (Boraston *et al*., 2004). This domain is common in cellulase genes in other plant parasitic nematode species and the cellulose binding domains identified in GPLIN_000536400 and *mi-eng-1* share homology with those in cellulases of *G. rostochiensis* and *H. glycines* (Béra-Maillet *et al*., 2000). Notably, the conserved domains are also present in the cellulases of plant-feeding bacterial species such as *Thermomonospora fusca* and *Streptomyces halstedii,* which share common conserved tryptophan residues with those in plant parasitic nematodes (Béra-Maillet *et al*., 2000). These domains are suggested to allow the cellulase enzymes in *G. rostochiensis* and *H. glycines* to bind to, and degrade “crystalline cellulose” (Smant *et al*., 1998). It is therefore hypothesised that GPLIN_000536400 and *mi-eng-1* also play a role in degrading “crystalline cellulose” in the cell walls of the hosts of *G. pallida* and

M. incognita. The presence of these specific domains supports the authenticity of these enzymes as cellulases. The other putative cellulases, without a cellulose binding domain, are hypothesised to degrade soluble polysaccharides formed during the breakdown of this 'crystalline cellulose' as well as some hemicelluloses including xyloglucan (Smant *et al*., 1998). This facilitates efficient weakening of plant cell walls and aids in successful penetration of, and migration through the root tissue.

A rich cellulase repertoire in plant parasitic nematodes is beneficial as it could give them the ability to target multiple forms of cellulose at varying stages of degradation. Much like other organisms, where three classes of cellulases: endoglucanases, cellobiohydrolases and β-glucosidases, work simultaneously to catalyse the breakdown of cellulose (Patel *et al*., 2019), a similar occurrence could be happening in plant parasitic nematodes. The speculation that different cellulase genes encode enzymes that have differing activity against different forms of cellulose could explain why nematodes with broad host ranges, such as *M. incognita*, have several different cellulase genes. This allows them to target the different cellulose forms that they could be presented with in the wide range of hosts they attempt to infect.

Although the broad host range of *M. incognita* can explain the requirement for numerous different cellulase genes, this does not account for the large number found in *G. pallida.* Analysis of normalised expression data for eight different life stages revealed that the majority of the genes of interest were most highly expressed at the J2 stage. GPLIN_000755100, however was most highly expressed in adult males and expression of GPLIN_000536400 was also relatively high in adult males. Males are also motile and must respond to environmental cues in order to migrate through host tissue and navigate within the rhizosphere, which will depend upon the secretion of cellulases and other cell wall-degrading enzymes (Eisenback and Hunt, 2010). It may be, therefore, that *G. pallida* employs distinct sets of cellulase genes to facilitate different stages of its parasitic life-cycle. Specifically, one set could be involved in enabling host entry, another set could be responsible for facilitating formation of the feeding site, and another set could be dedicated to enabling host exit.

3.5.3 Lack of an endoxylanase gene in *G. pallida*

After a combination of extensive literature searches and BLAST sequence similarity searches of genome assemblies, it was evident that *G. pallida* lacked a gene encoding an endoxylanase. This could be due to the narrow host range of this parasite. *G. pallida* only infects plants in the *Solanaceae* family and xylan is not a prominent component of the primary cell walls of these particular plants (Ebringerová and Heinze, 2000). Considering the preferred infection site of *G. pallida* is the root tip, where the roots cells

are still in the process of maturation, the absence of an endoxylanase encoding gene can be justified. The presence of an endoxylanase would likely be redundant since the need for xylan degradation by this species is diminished.

In contrast, a gene encoding an endoxylanase was identified in *M. incognita* (*mi-xyl*) which encoded a signal peptide in addition to a glycosyl hydrolase domain. This finding could reflect the fact that this parasite has a very broad host range (Moens *et al*., 2010, Abad *et al*., 2008). The diverse range of host plants necessitates the possession of a comprehensive repertoire of cell wall-degrading enzymes to effectively target a range of cell wall polysaccharides as the cell wall composition varies significantly between plant species. Given that xylan is abundantly present in the cell walls of woody plants and grasses (Ebringerová and Heinze, 2000), the inclusion of an endoxylanase in *M. incognita's* cell wall-degrading enzyme collection is essential in allowing this nematode to maintain such a broad host range.

The contrasting observations regarding the presence or absence of an endoxylanase gene in *M. incognita* and *G. pallida,* respectively, highlights the interplay between host range and the repertoire of cell wall-degrading enzymes in plant parasitic nematodes. This, along with the contrasting numbers of genes belonging to different cell wall-degrading gene families in these species (Thorpe *et al*., 2014) suggests that nematodes with different host ranges seem to employ different combinations of cell wall-degrading enzymes to successfully infect their hosts of choice. Similar observations have been made in plant pathogenic fungi. Those that employ different modes of pathogenesis are known to utilise distinct combinations of cell wall-degrading enzymes tailored to the specific cell wall compositions of their hosts in order to successfully infect them (Kubicek *et al*., 2014). The differences could be a result of co-evolutionary adaptations between nematodes and fungi with their respective host plants.

Understanding the differences in repertoires of cell wall-degrading enzymes between nematode species contributes to our knowledge of their adaptation strategies and host interactions. It provides valuable insights into the molecular mechanisms underlying host specificity of *G. pallida* and the ability of *M. incognita* to exploit a wide range of host plants.

3.5.4 Functional characterisation of putative cellulase and endoxylanase genes in *G. pallida* and *M. incognita*

After successfully identifying and cloning genes that potentially encode cellulase and endoxylanases in *G. pallida* and *M. incognita*, supplementary experiments were carried out to characterise them further. *In situ* hybridisation was employed to investigate the spatial expression patterns of the genes of interest. The findings revealed that

GPLIN_000536400, GPLIN_000313600, *mi-eng-1* and *mi-eng-4* all exhibited notable expression in the pharyngeal glands of the nematodes. This spatial distribution is of significance because authentic nematode effectors, including cell wall-degrading enzymes, are typically expressed in the dorsal and sub-ventral pharyngeal glands, where they are primed for secretion through the stylet into the host. The expression of the genes of interest in the pharyngeal glands, together with the presence of signal peptides encoded by these genes strengthens the evidence to suggest that their corresponding proteins indeed are secreted through the stylet into the host root. This reinforces their role in manipulating the host to the nematode's advantage.

To assess the post-secretion activity of the genes of interest, attempts were made to express and purify their corresponding proteins. Although multiple attempts to purify the proteins were made, they were all unsuccessful. If time constraints had allowed, alternative expression systems could have been trialled. Nevertheless, it was possible to assess the proteins' enzymatic activity using a simple assay. Protein expression was successfully induced in *E. coli* cells carrying recombinant plasmids harbouring the coding sequence of *mi-eng-4*. These cells were subsequently lysed and applied to cellulose plates where cellulose digestion could be visualised. As this assay confirmed that the protein encoded by *mi-eng-4* does have cellulase activity, it can be reasonably inferred that the remaining cellulase genes of interest, which share the same cellulase domain, likely exhibit similar enzymatic activity.

By revealing the expression patterns of the genes of interest and the probable enzymatic activity of the corresponding proteins, the results provide a foundation for understanding the molecular basis of host manipulation more clearly.

3.5.5 Conclusions

Along with various other cell wall-degrading enzymes, multiple cellulase genes were identified within the genomes of *G. pallida* and *M. incognita* which likely serve distinct stages in the parasitic life-cycle. Further investigation of a subset of these genes revealed that the putative cellulases encode signal peptides, cellulase domains, and in some cases, cellulose binding domains. The presence of these specific domains supports the authenticity of these enzymes as cellulases. A putative endoxylanase gene was included in the subset of genes of interest in *M. incognita* but an orthologue of this gene was absent in *G. pallida,* potentially reflecting their host ranges and preferences. Spatial expression of the genes of interest in the pharyngeal glands supports the role of these genes as effectors that are secreted through the stylet. Cellulase activity was confirmed for *mi-eng-4* and inferred for the other putative cellulase genes, suggesting

that these enzymes play a role in cellulose degradation or modification, which is essential for the nematodes' effective infection of their hosts.

Understanding the presence, expression and activity of genes encoding cell wall-degrading enzymes is important in understanding the mechanisms of host invasion employed by *G. pallida* and *M. incognita*.

3.6 Summary

- Putative cellulase genes in *G. pallida* and *M. incognita* encode signal peptides and cellulase catalytic domains. In some cases, they also encode a cellulose binding domain.

- The need for different cellulase genes could be explained by the hypothesis that they are utilised during the different life-cycle stages of *G. pallida*.

- *M. incognita* possesses a gene encoding an endoxylanase, but *G. pallida* does not. This could be related to host range and/or host-preference.

- A subset of putative cellulase and endoxylanase genes identified in each *G. pallida* and *M. incognita* are expressed in the sub-ventral pharyngeal glands, suggesting they encode effectors.

- The protein encoded by *mi-eng-4* shows cellulase activity. Other putative cellulase genes in both *G. pallida* and *M. incognita,* also encoding a cellulase domain, likely exhibit similar enzymatic activity.

Chapter 4
Behavioural and transcriptional responses of plant parasitic nematodes following host detection

4.1 Introduction

Root exudates play an important role in host-pathogen interactions and plant parasitic nematodes must recognise and respond to them in order to locate the roots of their hosts. Living plants naturally release root exudates into the surrounding environment (Koo *et al*., 2005). They are a complex mixture of primary metabolites, including sugars, carbohydrates, and amino acids, which constitute a substantial proportion, up to 20 percent, of a plants photosynthetically fixed carbon (Canarini *et al*., 2019). When these metabolites are released into the soil, they mix with water, creating a concentration gradient that extends away from the root surface (Cooper *et al*., 2018). The exact composition of root exudate varies between plant species (Barber and Martin, 1976, Bertin *et al*., 2003).

Nematodes of various modes of parasitism detect and respond to the presence of a nearby host through sensing of root exudates in the soil. Once they have orientated to the root, thrusting of the stylet physically damages the root, which is the essential first step of host root invasion. The mechanical action of the stylet is combined with the injection of effectors, including cell wall-degrading enzymes which also play an important role in root penetration (Rai *et al*., 2015b, Macharia *et al*., 2023). All plant parasitic nematodes, irrespective of their parasitic lifestyle, possess cell wall-degrading enzymes. In Chapter 3, several genes encoding these enzymes were identified in *G. pallida* and *M. incognita,* and it was speculated that different cell wall-degrading enzymes may be involved at different life-cycle stages. Some genes may function to facilitate the initial root penetration of the nematode during the J2 stage, whilst others could assist in the subsequent migration within the root. Additionally, specific genes might play a role in enabling adult males to exit the root successfully. It was considered that the presence of a large number of genes encoding cell wall-degrading enzymes in *M. incognita* may facilitate its ability to infect a wide array of host species. This is an intriguing area for further exploration as the expression of these genes and the activity of the encoded enzymes could be host-specific and this could extend to other plant parasitic nematodes with relatively broad host ranges, including *R. similis.* It would be interesting to investigate whether or not certain genes exhibit selective activation in response to

specific host species or if they become activated to facilitate infection across a diverse range of hosts.

In contrast, individual cyst nematode species have relatively narrow host ranges and some, such as potato cyst nematodes, are known to hatch only in response to specific cues from their respective hosts (Masler and Perry, 2018). As these nematodes rely on specific signals from their preferred hosts to initiate their life-cycle, it is reasonable to suggest that transcriptional responses to root exudates may also display host specificity and that gene expression patterns are finely tuned to respond to the unique chemical composition of root exudates of their specific hosts. It would therefore be interesting to determine the genes activated in response to preferred hosts in comparison to non-host species.

Recent findings from Bell *et al*. (2019) provide evidence that differential gene expression in a plant parasitic nematode in response to host detection varies with the host plant and that it could be influenced by the concentrations of particular substrates present in root exudates. Two genes encoding cell wall-degrading enzymes, namely an endoglucanase (*Pc-eng1*) and a xylanase (*Pc-xyl*) in the broad host range, root-lesion nematode, *P. coffeae* exhibited unique expression patterns in response to different root exudate treatments (Bell *et al*., 2019). Each gene was expressed most highly in response to root exudates with the highest concentration of its respective substrate, cellulose or xylan. These enzymes are important in breaking down plant cell walls, enabling the nematodes to efficiently invade and migrate through root tissue.

Previous studies have documented differential gene expression in response to host signals in *G. pallida* and *M. incognita,* even if not in the context of cell wall-degrading enzymes. For example, when *G. pallida* encounters the roots of a susceptible host, it exhibits distinct gene expression compared to when it encounters those of a resistant host (Kooliyottil *et al*., 2019). Similarly, differential gene expression has been observed in this species when hatched in the presence of root exudate from susceptible vs resistant potato varieties (Kud *et al*., 2021). However, there is currently a lack of gene-specific data regarding transcriptional changes in *G. pallida* when faced with differing host and non-host plants. Comparably, *M. incognita* has been shown to differentially express genes in response to *A. thaliana* root exudates (Teillet *et al*., 2013), however, the impact of varying host plants on gene expression remains unexplored. In general, limited research has been conducted to elucidate the differential expression of genes in response to host signals among plant parasitic nematodes including *G. pallida, M. incognita* and *R. similis.*

With the knowledge that the expression of endoglucanase and endoxylanase genes in *P. coffeae* is host-regulated, and that root exudate composition varies between plant species (Barber and Martin, 1976, Bertin *et al*., 2003), a hypothesis was drawn that genes in these families might also exhibit distinct expression patterns in other plant parasitic nematodes. To test this hypothesis, qRT-PCR analysis was initially conducted to investigate whether or not the putative cell wall-degrading enzymes, identified in *G. pallida* and *M. incognita* in Chapter 3, exhibit differential, host-specific expression in response to the detection of root exudates from differing host species.

Gene expression analysis provides a powerful tool to investigate how nematodes respond to host signals, adapt to different environments and exploit host resources. By studying changes in gene expression, insights can be gained into the strategies employed by plant parasitic nematodes to locate, migrate through, and feed from their host roots.

It would be interesting to examine whether or not genes encoding effectors also exhibit host-dependent expression. Extending the analysis beyond individual genes and identifying the entire complement of genes that exhibit differential expression in response to root exudates could be achieved using RNA-Seq analysis. By identifying differentially expressed genes, exploring potential host-specific expression patterns beyond known cell wall-degrading enzymes and effectors, a comprehensive understanding of the complex molecular mechanisms underlying host-parasite interactions could be gained for these plant parasitic nematodes.

This broader analysis will not only shed light on the adaptive strategies of plant parasitic nematodes but could also uncover new targets for control by opening new avenues for effective management strategies in the field. This could involve developing novel nematicides tailored towards targeting specific signalling pathways or metabolic processes involved in the infection process. Additionally, innovative approaches such as engineering crop varieties to naturally produce specialised chemicals that target these pathways could also be explored.

4.2 Aims

- To verify whether or not *G. pallida* and *M. incognita* respond to root exudate detection from various host and non-host plants using a stylet thrusting assay.

- To investigate whether or not cell wall-degrading enzymes such as cellulases are differentially regulated in response to root exudate detection in *G. pallida* and *M. incognita.*

- To use RNA-Seq to obtain a comprehensive gene expression analysis of *G. pallida* and *M. incognita* exposed to root exudates from varying host and non-host plants.

- To determine whether or not behavioural and/or transcriptional modifications in *G. pallida* and *M. incognita* in response to root exudates are host-specific.

4.3 Materials and Methods

4.3.1 Stylet thrusting rates in response to root exudates

Two hundred J2s each of *M. incognita* and *G. pallida* were incubated in either 40 µl sterile tap water or 40 µl root exudate (generated as previously described (Section 2.1.2)) from plants from the host spectrum of *M. incognita* and which represented both hosts and non-hosts of *G. pallida* (cabbage, carrot, maize, potato or tomato) roots, for one hour. As a positive control, nematodes were incubated in 40 µl 5-hydroxytryptamine (5-HT) (10 mM), also for one hour.

Nematodes were observed at a magnification of x10 (Zeiss AXIO Scope A1) and stylet thrusts (a single movement of the stylet forwards and then backwards to its original position) per minute were recorded for 30 nematodes in each treatment. The proportion of nematodes exhibiting a response was recorded, and the mean stylet thrusts per minute were calculated from those that were responding. A comparison of the response rate among different root exudate treatments was also conducted.

Data were analysed using One-way ANOVA and post-hoc Tukey's post hoc test in SPSS (SPSS v.25; IBM Corporation Armonk, New York, USA).

4.3.2 Quantification of cellulose in root exudates

To investigate whether or not different responses of the nematodes to root exudates were linked to cellulose detection, concentrations of this polysaccharide were quantified using an Anthrone assay (Updegraff, 1969). Acetic/nitric reagent (8:2:1 acetic acid: nitric acid: water) (0.3 ml) was added to centrifuged root exudate samples in triplicate from cabbage, carrot, maize, potato and tomato plants.

Samples were incubated at 90 ºC for 30 minutes to remove lignin, hemicelluloses, and xylan. The samples were re-centrifuged and washed with sterile tap water. They were then re-suspended in sulphuric acid (67 %) (0.5 ml) and vortexed to dissolve the cellulose.

Anthrone reagent (0.2 %) (Sigma-Aldrich) was mixed with the samples and they were incubated at 90 ºC for 16 minutes before being left to stand at room temperature (~20 ºC) for a further ten minutes. This allowed Anthrone reagent to bind to the dissolved cellulose. Absorbance was read at 595 nm on an ELx800 microplate reader (BioTek instruments). Absorbance at this wavelength was used as a proxy for relative cellulose concentration with greater absorbance values indicating greater cellulose content.

4.3.3 Determining relative gene expression of cellulase genes in response to root exudates

Between 15,000-20,000 *M. incognita* or *G. pallida* J2s were exposed to either sterile tap water or potato or maize root exudate (generated as described in Section 2.1.2) for four hours. Nematodes were flash frozen and total RNA was extracted using a E.Z.N.A® Plant RNA Kit (Omega Bio-Tek), including DNase digestion steps (Section 2.2.1).

Gene expression analysis of GPLIN_000536400, GPLIN_000536400, *Mi-eng-1* and *Mi-eng-4* was carried out by qRT-PCR, (Section 2.2.6), using primers detailed in Table 4.1. Each primer pair had an amplification efficiency between 95 and 105 percent. The cycling parameters of the reactions were as follows: 10 minute denaturing phase at 95 ºC followed by 40 cycles of 30 seconds at 95 ºC and 10 seconds at 60 ºC.

The $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) was followed to calculate relative gene expression values (Section 2.2.6) which were calculated from means of three technical replicates for each of three biological replicates. Reference genes used for *G. pallida* were Elongation factor 1A (*Gp-EIF1*) and Initiation factor 1 (*Gp-EF1-A*) and for *M. incognita,* they were Actin 2 (*Mi-Act-2*) and PTP (*Mi-PTP*).

Data were analysed using One-way ANOVA and post-hoc Tukey's posh hoc test in SPSS (SPSS v.25; IBM Corporation Armonk, New York, USA).

4.3.4 RNA extraction and sequencing

G. pallida and *M. incognita* J2s were collected and washed extensively in tap water before being maintained in tap water for three days prior to the experiment. Seven replicate pools of approximately 15,000 *G. pallida* J2s were then exposed to either tap water, or carrot, maize or potato root exudate (Section 2.1.2) for four hours. Similarly, seven replicate pools of approximately 20,000 *M. incognita* J2s were exposed to either tap water, or cabbage, carrot, maize, potato or tomato root exudate for four hours. Nematodes were snap-frozen with liquid nitrogen and total RNA was extracted from the seven biological replicates for each root exudate exposure using E.Z.N.A® Plant RNA Kit (Omega Bio-Tek) (including a DNase steps) (Section 2.2.1).

RNA was eluted in nuclease free water and the concentration and quality of each sample was assessed using spectrophotometry (Thermo Scientific Nanodrop™ 1000) and a bioanalyser (Agilent 2100). Only RNA samples with an RNA integrity number (RIN) of greater than six were selected for further analysis.

RNA sequencing was performed using the NextSeq 2000™ platform by the Next Generation Sequencing Facility at the University of Leeds (Figure 4.1). This included poly-A selection, addition of adapter sequences, library preparation and paired-end

100 bp sequencing, with 25-30 million reads per sample. Between three and five replicate samples were sequenced for each root exudate treatment.

4.3.5 RNA-Seq analysis

Resultant transcriptomic data from *G. pallida* and *M. incognita,* alongside transcriptomic data obtained from mixed-stage *R. similis* exposed to either sterile tap water, coffee, maize or potato root exudate, were analysed using OmicsBox v.2.2.4.

Default options and settings were used for each tool unless stated otherwise. FASTQ files were trimmed, and adapter and contamination sequences were removed using the Preprocessing tool which uses Trimmomatic software (Bolger *et al*., 2014). The quality of the trimmed FASTQ files was then checked using the FastQC Quality Check tool which is based on FastQC software (Andrews, 2010).

Surviving reads were mapped onto either the *G. pallida* "Lindley" genome (PRJEB123) (Cotton *et al*., 2014) or the *M. incognita* genome (PRJEB8714) (Blanc-Mathieu *et al*., 2017) (downloaded from WormBase ParaSite (Howe *et al*., 2017)) using the RNA-Seq Alignment tool. Similarly, surviving reads were mapped onto a *de novo* assembly of the *R. similis* genome using the same tool. A Count Table was created for each nematode species which allowed the number of reads and associated expression to be quantified for each gene.

Differential gene expression analysis (based on edgeR software package (Robinson *et al*., 2010)) was then carried out to identify genes that were differentially expressed in each of the root exudate treatments compared to the control using the Pairwise Differential Gene Expression Analysis tool. Identification of genes that were differentially expressed between root exudate treatment groups was carried out in the same way. The Gene Expression Analysis tool compares normalised gene counts for each gene (in each condition) and tests for statistical significance using a Fisher's exact test.

Genes were considered to be significantly differentially expressed if the false discovery rate (FDR) was ≤ 0.05. Further to the FDR threshold, only genes that showed a fold change (FC) of ≥ 1.5 or ≤ -1.5 were considered for further investigation. A 1.5-fold change cut-off was chosen to highlight more substantial and biologically relevant differences in gene expression, surpassing the comparably minimal 1-fold change cut-off enforced by OmicsBox v.2.2.4.

Gene Ontology (GO) IDs, names and terms were mapped to either the *G. pallida,* or *M. incognita* coding region sequences (CDS) (obtained from WormBase ParaSite (Howe*et al*., 2017)), or the or *R. similis* CDS from the *de novo* assembly, using the

Blast2Go and InterPro Scan tools (Götz *et al*., 2008). Over- and under-represented GO terms in the root exudate treatments compared to the control were identified by enrichment analysis using the Enrichment Analysis tool (Fisher's exact test (FDR ≤ 0.05)).

Nematode species	Gene name	Description	Forward primer	Reverse primer
G. pallida	EIF1	Initiation factor- Housekeeping gene	GCCTAAAGATGCCTTCGAGC	GGTGATAGTCTTCCTCCCCG
G. pallida	EF1-A	Elongation factor- Housekeeping gene	AATGACCCGGCAAAGGAGAG	GTAGCCGGCTGAGATCTGTC
G. pallida	GPLIN_000536400	Endoglucanase	CCTACGGCTCATACCCACAC	CGAGGCCACATCCACATCTT
G. pallida	GPLIN_000313600	Cellulase domain-containing protein	GGAGCCAGTGGAATTCAGCT	CCACGGCTTGACTGGTGTAT
M. incognita	Actin 2	Actin- Housekeeping gene	GATGGCTACAGCTGCTTCGT	GGACAGTGTTGGCGTAAAGG
M. incognita	PTP	Protein tyrosine phosphatase- Housekeeping gene	GGAAAGCATTTTCACCTGCGA	ACGGGTTGCCAACAGATGAA
M. incognita	*mi-eng-1*	Endoglucanase	CCAGCAACTCACAAACCAGC	TGCCTGTACTGGTTCCCGTA
M. incognita	*mi-eng-4*	Cellulase	AATGCTGCTCCTCCGTATGG	TAACCTGGTCGTTGGCATCC

Table 4.1- qRT-PCR primers for *G. pallida* and *M. incognita* cellulase genes.

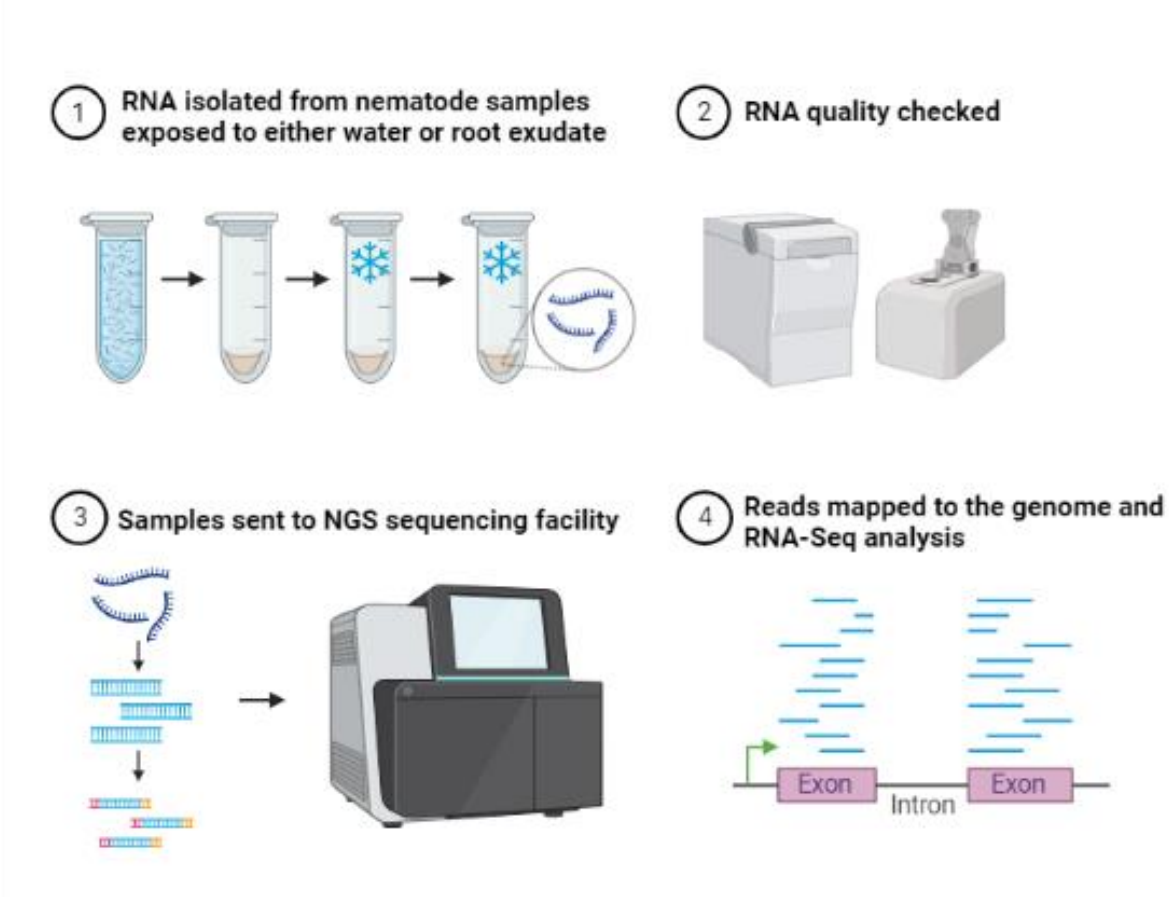


Figure 4.1- RNA sequencing and analysis work flow.

1) Nematodes were exposed to either water (control) or root exudate for four hours before being pelleted and snap frozen with liquid nitrogen. Total RNA was extracted from nematode pools.

2) The quality and concentration of RNA was analysed using spectrophotometry (Thermo Scientific Nanodrop™ 1000) and a bioanalyser (Agilent 2100).

3) Samples with an RNA integrity number (RIN) greater than six were sent to the Next Generation Sequencing Facility at the University of Leeds where poly-A selection was performed, libraries were prepared and adapter sequences were added before samples were sequenced using the NextSeq 2000™ platform.

4) Analysis of the data was performed using OmicsBox v.2.2.4. Sequencing reads were quality checked using the Preprocessing tool and surviving reads were mapped to the appropriate nematode genome using the RNA-Seq Alignment tool. Differential expression analysis and gene ontology (GO) and enrichment analysis was performed using the Pairwise Differential Gene Expression Analysis tool, and the Blast2Go and InterPro Scan tools. Statistical significance was measured using Fisher's exact test in both the differential gene expression analysis and the GO enrichment analysis.

4.4 Results

4.4.1 Cellulose concentration varies in root exudates from different host species

As cellulose content varies between plant species (Albersheim *et al*., 2010), an Anthrone assay (Updegraff, 1969) was carried out on root exudate samples generated from cabbage, carrot, maize, potato and tomato plants to determine whether or not these differences are reflected in root exudates.

Relative absorbance (595 nm) was used as a proxy for cellulose concentration, and it was found that root exudates from different plants did contain different concentrations of cellulose (Figure 4.2).

Potato root exudates contained the lowest concentration of cellulose, followed by carrot, tomato and then cabbage. Maize root exudates contained the highest concentrations of cellulose (Figure 4.2). Significant differences in cellulose concentrations were seen when comparing maize and cabbage root exudates to the remaining three (Figure 4.2).

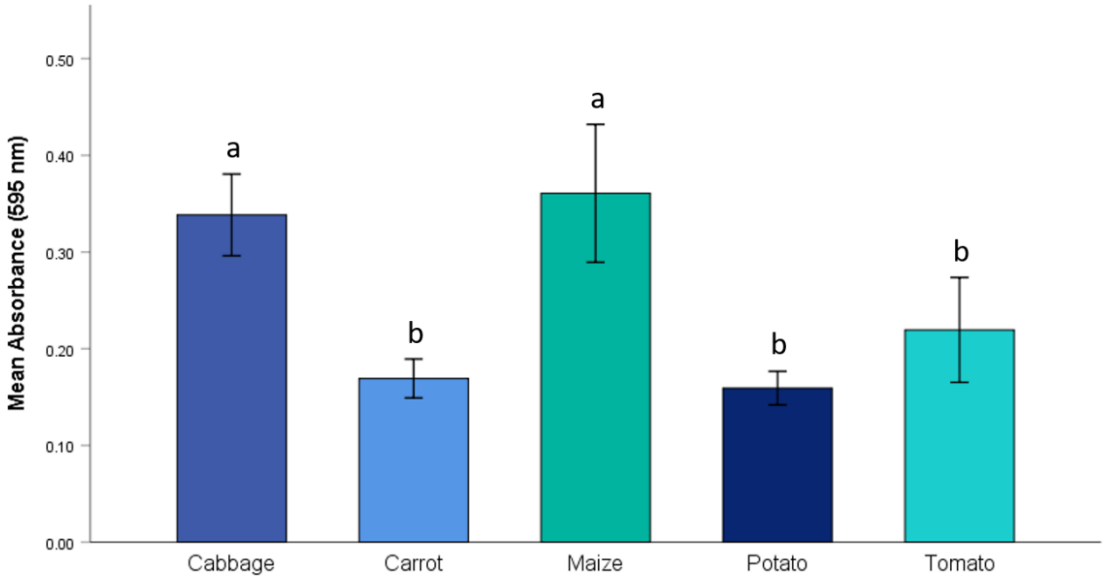


Figure 4.2- Cellulose content of root exudates differs between plant species.

Cellulose content of cabbage (n=5), carrot (n=3), maize (n=4), potato (n=6) and tomato (n=3) root exudate samples was determined using an Anthrone-based assay. Relative concentration of cellulose in each root exudate was observed as an average of absorbance values (595 nm). Maize root exudate produced the highest absorbance values. This signifies the highest cellulose concentration. Error bars are +/- SEM. Letters represent homogeneous subsets (One-way ANOVA, Tukey's post hoc test).

4.4.2 Responses of nematodes to different plant root exudates

4.4.2.1 Stylet thrusting responses

Sedentary plant parasitic nematodes, such as *G. pallida* and *M. incognita,* respond with appropriate behaviours to the detection of nearby roots. J2 nematodes were exposed to root exudates from varying plant species to identify any effects this may have on nematode stylet thrusting rate.

It has previously been shown that the model plant species, *A. thaliana,* stimulates stylet thrusting in *M. incognita* (Teillet *et al*., 2013), but their response to exudates from other plant roots has not been reported. The impact of host detection on stylet thrusting in *G. pallida* also remains elusive.

To determine whether or not *G. pallida* and *M. incognita* respond to root exudates from varying hosts, stylet thrusting assays were carried out. Nematodes were exposed to either sterile tap water (negative control), cabbage, carrot, maize, potato or tomato root exudate, or 5-hydroxytryptamine (5-HT) (10 mM) (positive control) for one hour and stylet thrusts were counted for 30 individual nematodes for each treatment. 5-HT was used as a positive control because it has previously been shown that plant parasitic nematodes respond with rapid stylet thrusting upon exposure to this chemical (Crisford *et al*., 2020, Masler, 2007).

It was found that exposure to root exudates stimulates a stylet thrusting response in J2s of both *M. incognita* and *G. pallida.* Pools of J2s from both nematode species showed significantly higher rates of stylet thrusting in all root exudate treatments compared to water controls (Figure 4.3). The proportion of responding nematodes was also higher in pools exposed to root exudates compared to the water exposed control (Figure 4.3).

In *G. pallida,* differences in stylet thrusting rate were also observed between some root exudate treatment groups. A significantly higher number of stylet thrusts were seen in *G. pallida* J2s that had been exposed to potato and cabbage root exudates compared to maize root exudates (Figure 4.3A). The proportion of responding nematodes was also considerably higher for those exposed to potato root exudate compared to the other root exudate exposures (Figure 4.3A).

Although differences in stylet thrusting rate were seen between *M. incognita* J2s exposed to root exudates and those exposed to the water control, no significant differences were seen between root exudate treatments (Figure 4.3B).

The differences observed in stylet thrusting rates between root exudate treatments in *G. pallida* did not seem to correlate with their differing cellulose concentrations that had previously been quantified (Figure 4.2).

4.4.2.2 Transcriptional responses

Since stylet thrusting activity is essential for host root invasion in both *G. pallida* and *M. incognita* it was questioned whether or not exposure to varying root exudates could also affect the expression of genes that are likely to be involved in root invasion.

It has previously been shown that *P. coffeae* differentially regulates expression of an endoglucanase and an endoxylanase gene in a host-specific manner (Bell *et al*., 2019) and in Chapter 3, putative cellulase genes were identified in *G. pallida* and *M. incognita.* The spatial expression of some of these genes was confirmed to be in the pharyngeal glands by *in situ* hybridisation which reinforces the designation of their role as putative nematode effectors (Chapter 3).

Two genes from each of *G. pallida* (GPLIN_000536400 and GPLIN_000313600) and *M. incognita* (*Mi-Eng-1* and *Mi-Eng-4*) were selected for initial gene expression analysis to determine transcriptional responses to root exudates in these nematode species. J2 nematodes of *G. pallida* and *M. incognita* were separately exposed to either sterile tap water, or root exudates from maize or potato plants for 4 hours and the gene expression was analysed by qRT-PCR.

GPLIN_000313600 was significantly up-regulated in nematodes exposed to potato root exudate compared to the negative control (Figure 4.4A). Exposure to potato root exudate also caused a significant up-regulation of the same gene compared to maize root exudate which did not increase gene expression compared to the control (Figure 4.4A). No significant differences in expression of GPLIN_000536400 were seen in response to either potato or maize root exudate compared to the control, or between root exudate treatments (Figure 4.4B).

In *M. incognita,* although *Mi-eng-1* was not differentially expressed in response to maize root exudate compared to the control, it showed significant up-regulation in response to potato root exudate compared to the control (Figure 4.5A). Differential expression of this gene was also seen between root exudate treatments, with it being significantly up-regulated in response to potato root exudate compared to maize (Figure 4.5A). *Mi-eng-4* was up-regulated in nematodes that had been exposed to both maize and potato root exudate compared to the control (Figure 4.5B). This gene also showed the same expression pattern as *mi-eng-1* between root exudate treatments, having

significantly higher expression in response to potato root exudate than maize (Figure 4.5B).

The relative expression of both cellulase-encoding genes studied in *G. pallida* and *M. incognita* did not correlate with the concentrations of cellulose in the root exudate (Figure 4.2).

4.4.3 Sequencing of RNA from *G. pallida* following root exudate exposure

Following the initial proof of principle demonstration of differential root exudate effects on gene expression, RNA-Seq was carried out for *G. pallida* J2s exposed to root exudates to gain a more comprehensive understanding of the transcriptional responses of these nematode species to root exudate exposure.

Approximately 15,000 J2s were exposed to either sterile tap water, or root exudates from carrot, maize or potato plants for four hours and total RNA was extracted from flash-frozen nematode pellets. Seven biological replicates for each condition were generated and RNA quality was checked using the Agilent 2100 bioanalyser for each sample (Figure 4.6).

Five samples (Table 4.2) with the best quality RNA (Figure 4.6) were chosen to be sequenced using the NextSeq2000™ platform. This generated FASTQ files which contained the reads for each sample. Each sample yielded over 20 million reads and over 6,000 million bases (Table 4.3). The quality of the reads was assessed using OmicsBox v.2.2.4 using the Preprocessing and FastQC Quality Check tools. As a result of this, one sample was discounted due to the FASTQ file being truncated. The removed sample was a water exposed control for *G. pallida,* which left four repeats in this group for further analysis (Table 4.2). All five repeats for the other conditions were deemed to be of good quality and therefore were all included in further analysis (Table 4.2).

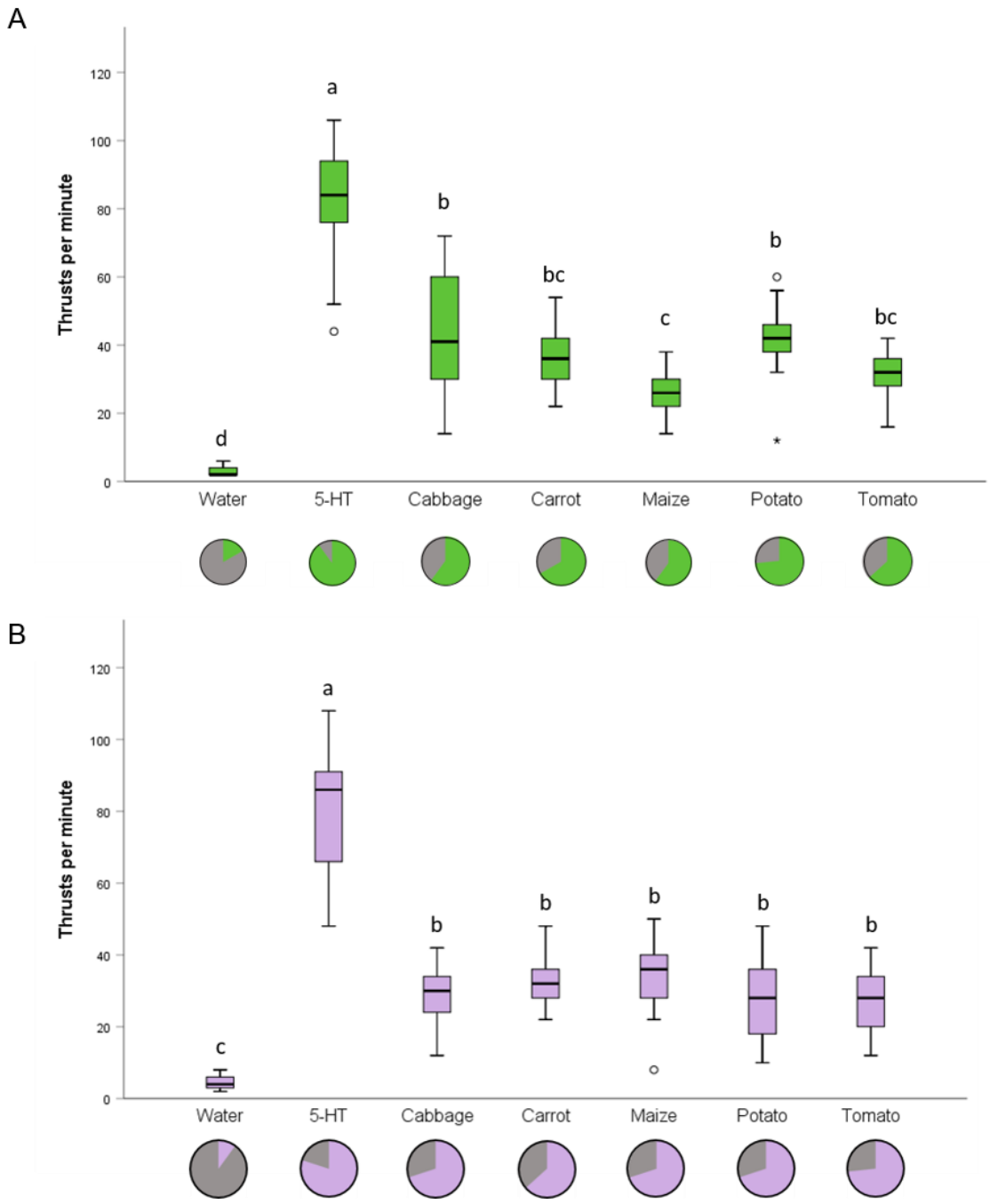


Figure 4.3- Root exudates from different host plants stimulate stylet thrusting in *G. pallida* and *M. incognita*.

A) ***G. pallida*** or **B)** ***M. incognita*** J2s (n=30) were exposed to various root exudates and stylet thrusts per minute were counted for nematodes that were visually responding to the root exudate treatment. The dark line in the centre of the boxplot represents the median number of stylet thrusts per minute and the coloured box illustrates the interquartile range of the data set. The ends of the whiskers show the minimum and maximum number of recorded stylet thrusts per minute, while the circles (o) and asterisks (*) represent outliers. Letters represent homogeneous subsets (One-way ANOVA, Tukey's post hoc test). Pie charts below each condition indicate the proportion of nematodes visually responding to the root exudate treatment (coloured) compared to those not responding. ie zero stylet thrusts during observation (grey).

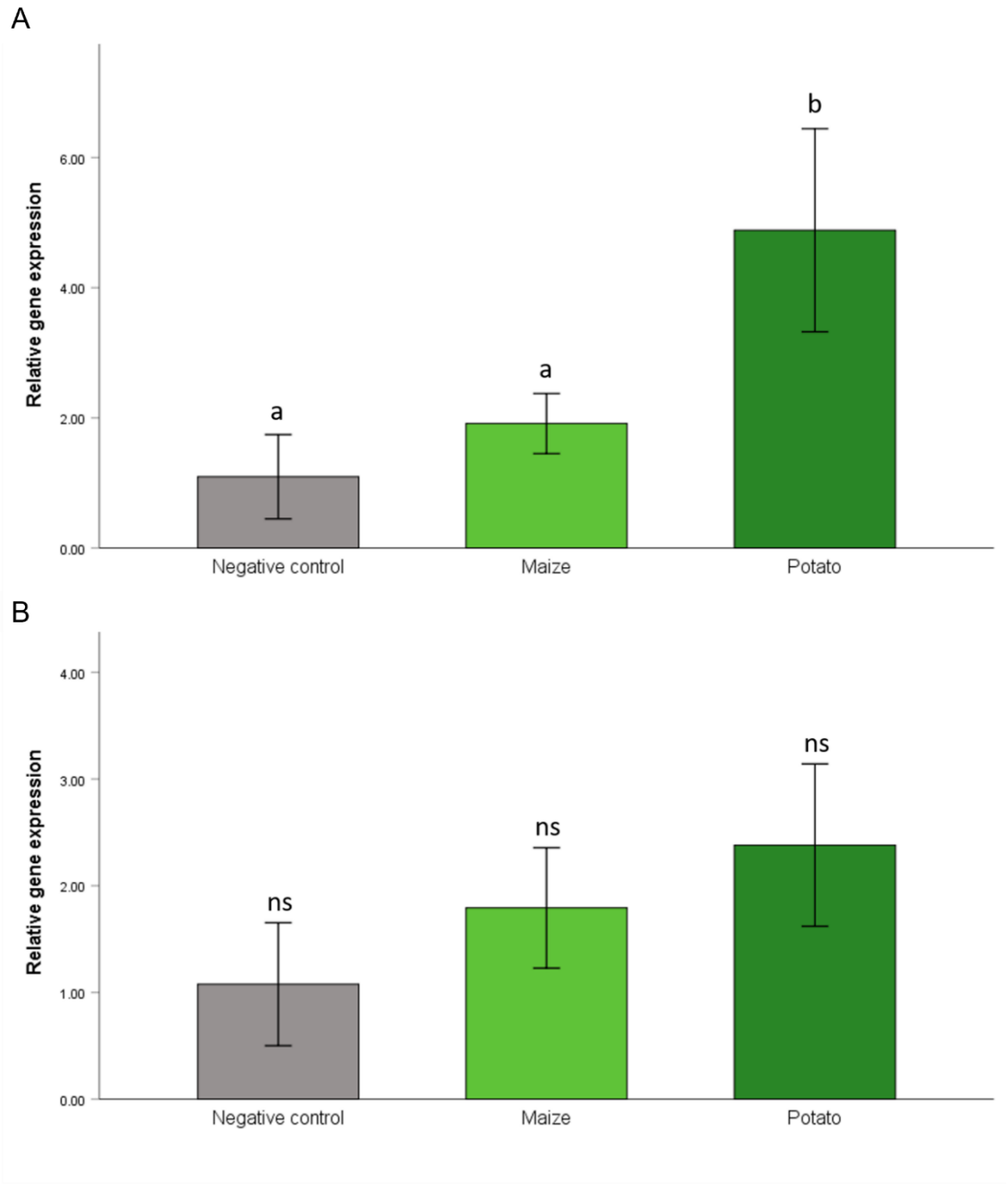


Figure 4.4- Putative cellulase genes GPLIN_000313600 and GPLIN_000536400 are differentially expressed in response to maize and potato root exudates in *G. pallida*.

A) Relative expression of GPLIN_000313600. *G. pallida* J2s were exposed to either sterile tap water or potato or maize root exudate and gene expression was analysed using qRT-PCR. Mean expression of 3 biological repeats. Significant differences in expression were seen between root exudate treatment groups. Error bars are +/- SEM. Letters represent homogeneous subsets (One-way ANOVA, Tukey's post hoc test).

B) Relative expression of GPLIN_000536400. *G. pallida* J2s were exposed to either sterile tap water or potato or maize root exudates and gene expression was analysed using qRT-PCR. Mean expression of 3 biological repeats. Error bars are +/- SEM. No significant differences were seen between treatment groups (One-way ANOVA, Tukey's post hoc test).

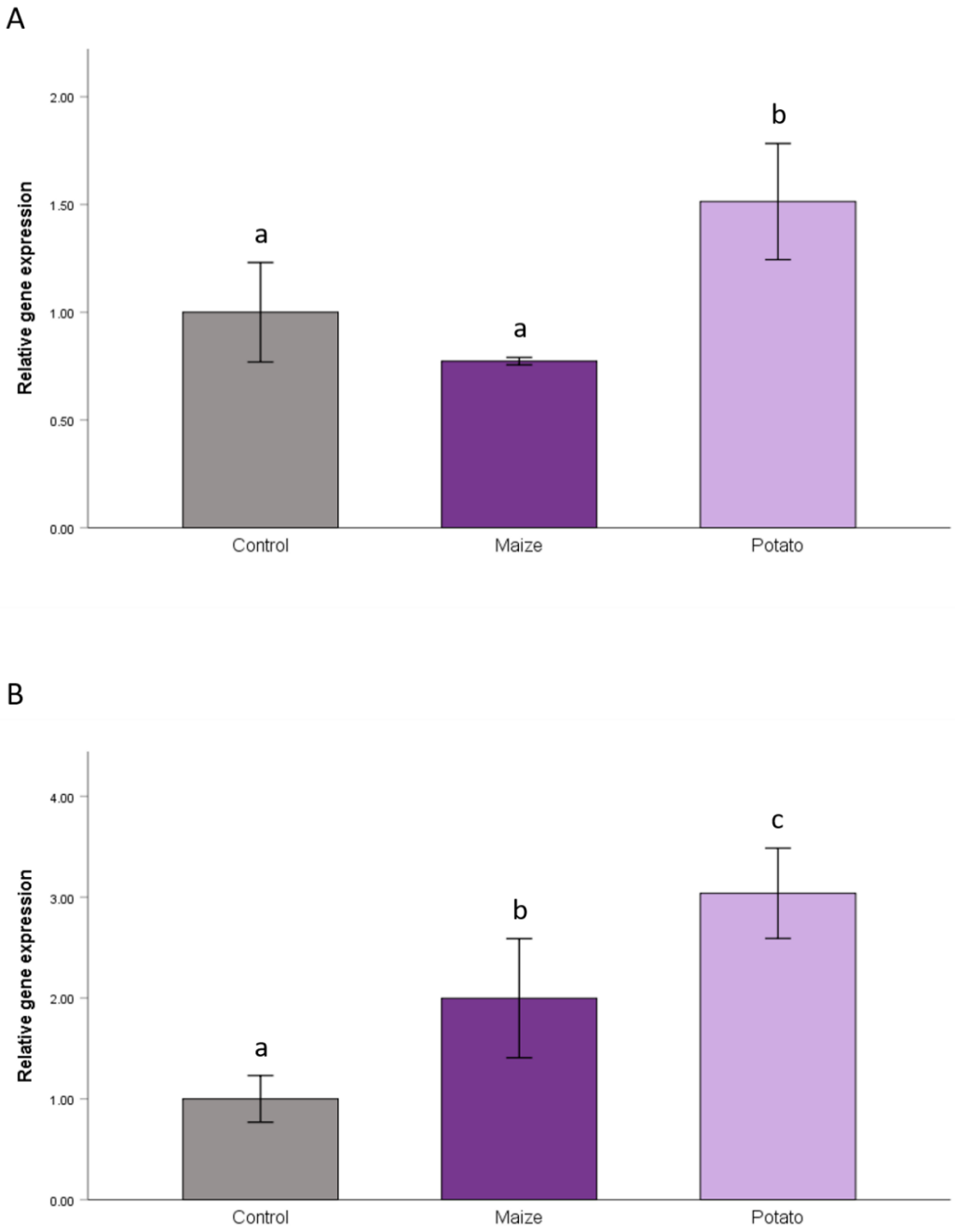


Figure 4.5- Putative cellulase genes *mi-eng-1* and *mi-eng-4* are differentially expressed in response to maize and potato root exudates in *M. incognita.*

A) Relative expression of *mi-eng-1*. *M. incognita* J2s were exposed to either sterile tap water or potato or maize root exudates and gene expression was analysed using qRT-PCR. Mean expression of 3 biological repeats. Significant differences in expression were seen between root exudate treatment groups. Error bars are +/- SEM. Letters represent homogeneous. (One-way ANOVA, Tukey's post hoc test).

B) Relative expression of *mi-eng-4*. *M. incognita* J2s were exposed to either sterile tap water or potato or maize root exudates and gene expression was analysed using qRT-PCR. Mean expression of 3 biological repeats. Significant differences in expression were seen between root exudate treatment groups. Error bars are +/- SEM. Letters represent homogeneous subsets (One-way ANOVA, Tukey's post hoc test).

Nematode species	Condition	Total number of samples sequenced by RNA-Seq (n)	Total number of samples utilised in RNA-Seq analysis (n)
G. pallida	Water exposure (control)	5	4
G. pallida	Carrot root exudate exposure	5	5
G. pallida	Maize root exudate exposure	5	5
G. pallida	Potato root exudate exposure	5	5

Table 4.2- Total number of *G. pallida* samples used for RNA-Seq.

Five samples from each treatment group were sequenced by RNA-Seq and all five were utilised in the analysis for carrot, maize and potato root exudate exposure groups. Due to low quality reads in one of the samples from the water exposure group, four samples were utilised in the subsequent RNA-Seq analysis.

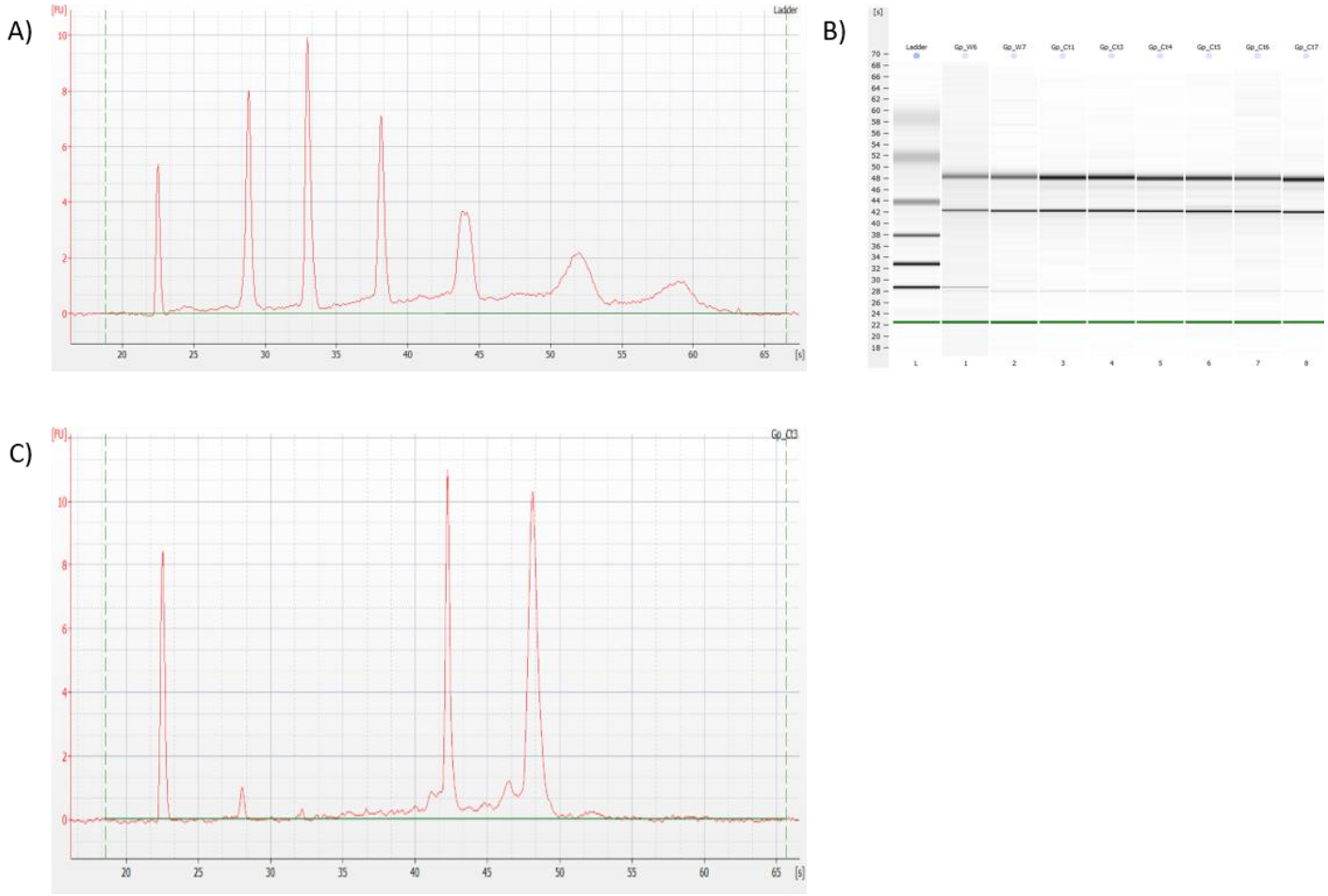


Figure 4.6- *G. pallida* RNA quality.

A) Electropherogram of RNA ladder. Comparison for RNA integrity for *G. pallida* RNA samples.
B) Gel electrophoresis of a variety of *G. pallida* RNA samples. RNA shows good integrity compared to the RNA ladder. Clear, sharp bands represent 28S and 18S rRNA.
C) Electropherogram of representative *G. pallida* RNA sample. Integrity was compared to the RNA ladder.

Treatment	Replicate number	Total number of reads	Yield (Million bases)	Uniquely mapped reads (%)
Water control	1	24,378,948	7,229,974	68.847
	2	25,697,400	7,626,892	68.785
	4	21,968,988	6,407,441	67.798
	5	25,028,434	7,437,891	68.041
Maize root exudate	1	28,299,784	7,901,436	64.889
	2	27,821,922	7,965,137	66.197
	3	30,008,291	8,621,204	66.262
	4	26,363,177	7,655,294	66.995
	5	24,339,965	6,879,378	65.754
Carrot root exudate	1	24,687,327	7,511,589	69.077
	2	26,141,185	8,001,950	69.753
	3	28,222,421	8,615,240	69.581
	4	25,365,810	7,382,363	68.219
	5	29,539,765	8,814,035	69.78
Potato root exudate	1	24,580,083	7,332,619	69.569
	2	32,399,821	9,622,152	69.651
	3	25,990,701	7,788,605	69.614
	4	28,214,010	8,523,902	70.543
	5	23,816,981	7,197,394	70.214

Table 4.3- Sequencing and alignment of RNA from *G. pallida* exposed to root exudates.

G. pallida J2s were exposed to either sterile tap water, or carrot, maize or potato root exudate and RNA-Seq was performed using the NextSeq2000™ platform. Reads in these files were aligned to the *G. pallida* “Lindley” genome (Cotton *et al*., 2014) using Omics Box v2.2.4.

4.4.3.1 Differential gene expression in *G. pallida* J2s in response to varying root exudates

Differential gene expression analysis of the RNA reads confirmed that exposure to root exudates from different plants causes varying degrees of transcriptional alterations in *G. pallida* J2s (Figure 4.7).

A number of genes were found to exhibit significant differential expression (FC ≥ 1.5 or ≤ -1.5) in response to each root exudate treatment relative to the water control. Genes that showed the highest fold changes (FC) (FDR ≤ 0.05) in response to root exudate exposure vary between treatments and these are listed in Tables 4.4-4.8. Notably, potato root exudate induced the highest number of differentially expressed genes in *G. pallida* J2s, having a much greater impact on gene expression than both carrot and maize root exudates, combined (Figure 4.7; Table 4.9).

Exposure to potato root exudate led to a total of 1,412 genes being differentially expressed in *G. pallida* J2s (Table 4.9), with 775 being up-regulated and 638 down-regulated. In comparison, the impact of maize and carrot root exudate exposure on gene expression was almost negligible (Figure 4.7). Upon exposure to maize root exudate, the majority of the differentially expressed genes were up-regulated, with ten out of 12 genes showing this pattern. Two genes were down-regulated in this treatment group. Exposure to carrot root exudate resulted in the differential expression of just four genes in *G. pallida* J2s, all of which were up-regulated.

This work initially focussed on assessing the differential expression of cellulase encoding genes in *G. pallida* and *M. incognita,* so it is noteworthy that a cell wall-associated hydrolase, GPLIN_001463700, consistently exhibits up-regulation in response to root exudates from carrot and maize and ranks among the genes displaying the greatest degree of up-regulation in response to potato root exudate (Tables 4.4-4.8). This was also observed for another cell wall-associated hydrolase, GPLIN_001116500, which exhibits the highest fold change in response to potato root exudate and is also up-regulated in response to maize root exudate.

Interestingly, among the top three down-regulated genes in *G. pallida* in response to potato root exudates is a gene annotated as a beta-1,4-endoglucanase-2 precursor and one annotated as a gland-specific protein (Table 4.5). The descriptions of these genes suggest both are probable effectors.

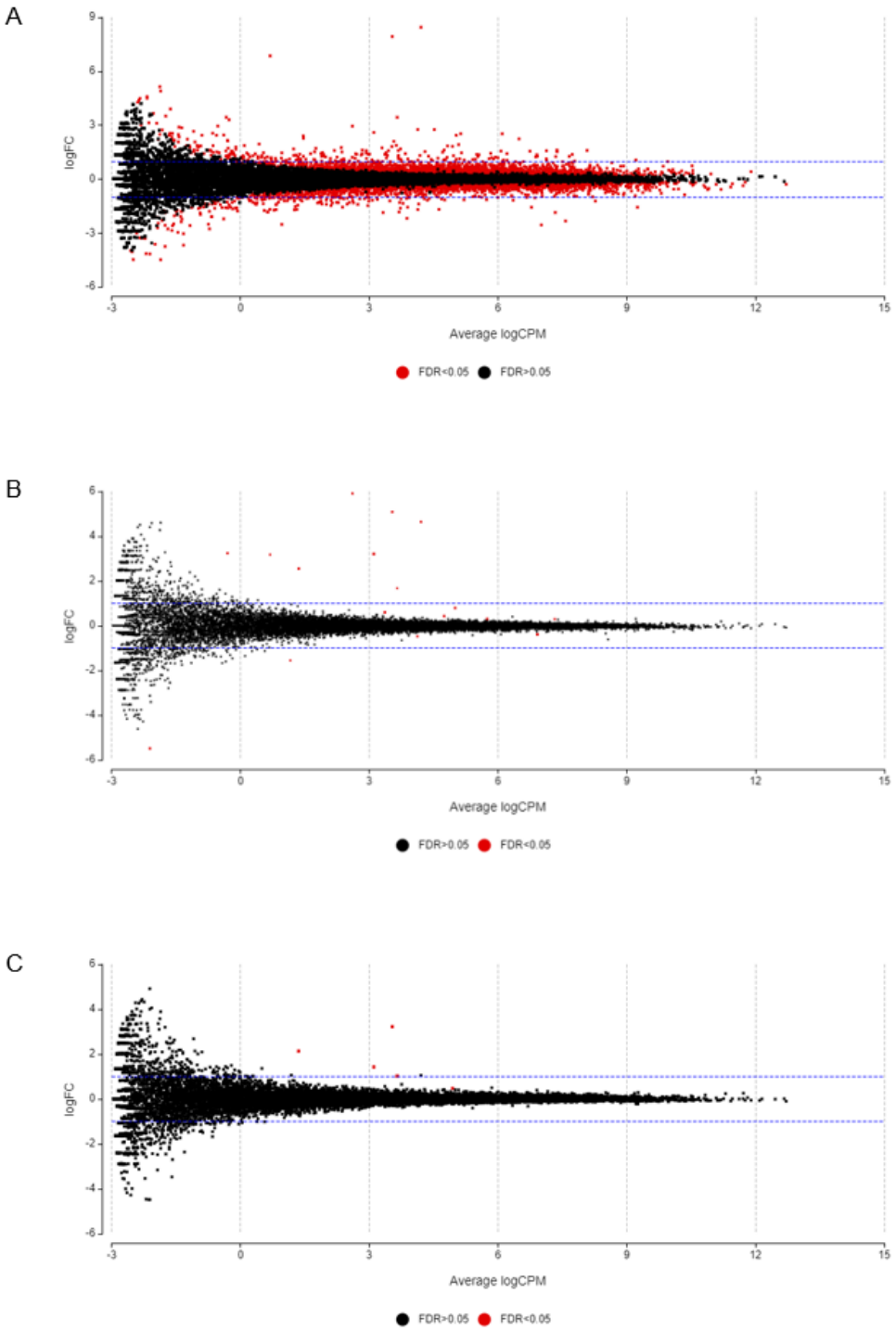


Figure 4.7- Differentially expressed genes in *G. pallida* in response to varying root exudates.

A) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *G. pallida* J2s exposed to **potato root exudate** and *G. pallida* J2s exposed to the water control.
B) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *G. pallida* J2s exposed to **maize root exudate** and *G. pallida* J2s exposed to the water control.
C) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *G. pallida* J2s exposed to **carrot root exudate** and *G. pallida* J2s exposed to the water control. Significantly differentially regulated genes are coloured red. Those that are above 0 are up-regulated and those that are below 0 are down-regulated. Dotted blue lines indicate the implemented fold change (FC) cut offs.

Gene ID	Fold change (FC)	Description
GPLIN_001116500	348.80	Cell wall-associated hydrolase
GPLIN_001116400	243.20	Unnamed protein product
GPLIN_001507200	115.74	4-hydroxybenzoyl-CoA thioesterase
GPLIN_001253100	35.14	Unnamed protein
GPLIN_001639200	29.86	Titin
GPLIN_000178300	24.01	Trehalase
GPLIN_000761800	22.60	Col_cuticle_N domain-containing protein
GPLIN_000241500	22.42	Hypothetical protein GPALN_002326
GPLIN_000836600	20.86	Hypothetical protein GPALN_004924
GPLIN_000905200	19.34	Hypothetical protein GPALN_009816
GPLIN_001634900	14.90	Zinc finger protein
GPLIN_001427400	12.53	FAD binding domain-containing protein
GPLIN_001532300	11.33	Chitin-binding, type 1 domain and glycosyl hydrolase family 18,
GPLIN_000798000	10.83	Major facilitator superfamily domain-containing protein
GPLIN_001463700	10.80	Cell wall-associated hydrolase
GPLIN_000067200	9.82	Transmembrane protein
GPLIN_001498800	8.74	Hypothetical protein GPALN_004181
GPLIN_001492400	8.58	Phosphoenolpyruvate carboxykinase
GPLIN_001631000	8.09	G-PROTEIN-RECEP-F1-2 domain-containing protein
GPLIN_001571000	7.70	Major facilitator superfamily domain-containing protein 5

Table 4.4- Up-regulated genes in *G. pallida* exposed to potato root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *G. pallida* when exposed to potato root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
GPLIN_000363100	-22.46	CPSF_A domain-containing protein
GPLIN_000031200	-22.35	Beta-1,4-endoglucanase-2 precursor
GPLIN_000667500	-17.99	Gland-specific protein g4g05
GPLIN_000368200	-17.82	WH2 domain-containing protein
GPLIN_000245000	-16.56	Hypothetical protein GPALN_014882
GPLIN_001578700	-15.90	Seca DEAD-like domain-containing protein
GPLIN_001179600	-13.47	AFG3-like protein 2
GPLIN_001521000	-12.83	Annexin 4C10
GPLIN_000204700	-12.37	Kinesin motor domain-containing protein
GPLIN_001526000	-9.91	Core-2/I-Branching enzyme domain-containing protein
GPLIN_000836700	-9.80	HORMA domain-containing protein
GPLIN_000572100	-9.69	DUF19 domain-containing protein
GPLIN_001109100	-8.25	Hypothetical protein GPALN_014670
GPLIN_001162900	-8.11	Adenylate/guanylate cyclase catalytic domain protein
GPLIN_000445100	-7.81	Homeobox domain-containing protein
GPLIN_001209200	-7.29	Unnamed protein
GPLIN_000112000	-6.87	Pept_C1 domain-containing protein
GPLIN_001633300	-6.86	Hypothetical protein GPALN_014752
GPLIN_001351500	-6.59	Phorbol ester/diacylglycerol-binding protein unc-13
GPLIN_000973400	-6.08	RING-type domain-containing protein

Table 4.5- Down-regulated genes in *G. pallida* exposed to potato root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *G. pallida* when exposed to potato root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
GPLIN_000255800	60.31	Fibronectin type-III domain-containing protein
GPLIN_001116400	33.96	Unnamed protein product
GPLIN_001116500	24.99	Cell wall-associated hydrolase
GPLIN_000641700	9.41	NADH-ubiquinone oxidoreductase
GPLIN_001469500	9.22	Unnamed protein product
GPLIN_001507200	9.02	4-hydroxybenzoyl-CoA thioesterase
GPLIN_000298000	5.84	Unnamed protein product
GPLIN_001463700	3.19	Cell wall-associated hydrolase
GPLIN_000596200	1.73	Guanylate cyclase
GPLIN_000431800	1.51	FRG1 protein homolog, putative

Table 4.6- Up-regulated genes in *G. pallida* exposed to maize root exudate.

Up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *G. pallida* when exposed to maize root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
GPLIN_000667500	-44.85	Gland-specific protein g4g05
GPLIN_000804500	-2.93	Guanylate cyclase

Table 4.7- Down-regulated genes in *G. pallida* exposed to maize root exudate.

Down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *G. pallida* when exposed to maize root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
GPLIN_001116400	9.32	Unnamed protein product
GPLIN_000298000	4.40	Unnamed protein product
GPLIN_001469500	2.69	Unnamed protein product
GPLIN_001463700	2.03	Cell wall-associated hydrolase

Table 4.8- Up-regulated genes in *G. pallida* exposed to carrot root exudate.

Up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *G. pallida* when exposed to carrot root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

	Water (control)	**Carrot root exudate**	**Maize root exudate**	**Potato root exudate**
Water (control)				
Carrot root exudate	4			
Maize root exudate	12	19		
Potato root exudate	1,412	1,290	1,607	

Table 4.9- Total number of differentially expressed genes in *G. pallida* J2s in response to varying root exudates.

G. pallida J2s were exposed to either sterile tap water, or carrot, maize or potato root exudate and RNA-Seq was performed using the NextSeq 2000™ platform. Differential gene expression analysis revealed numbers of genes that were significantly differentially expressed between treatment groups compared to the water control, as well as each of the treatment groups compared to each other (FDR ≤ 0.05, FC ≥ 1.5 or ≤ -1.5).

4.4.3.2 Differential gene expression is host-specific in *G. pallida*

Differential gene expression analysis showed different magnitudes of responses in *G. pallida* J2s in response to different root exudate treatments (Figure 4.8). The majority of genes up- or down-regulated in response to potato root exudate are unique to this treatment (Figure 4.8). In contrast, only a very small proportion of genes exhibited differential regulation, resulting in a minimal response to carrot and maize root exudate treatments. The genes that are differentially in response to these treatments are also differentially expressed in response to potato root exudate (Figure 4.8).

Overall, four genes were commonly up-regulated in response to all three root exudate treatments (Figure 4.8A). Mapping of GO IDs, terms and names to the *G. pallida* genome confirmed that one of these was a cell wall-associated hydrolase, however the remaining three were described as “unnamed protein products”. As mentioned previously, the commonly up-regulated cell wall-associated hydrolase was among the top 20 most highly up-regulated genes in *G. pallida* in response to potato root exudate (Table 4.4).

Although no genes were down-regulated in *G. pallida* J2s in response to carrot root exudate, the two genes displaying down-regulation in response to maize root exudate exhibited the same response to potato root exudate (Figure 4.8B). GO annotations suggest that one of these genes is a gland-specific protein and the other is a guanylate cyclase.

To confirm the host-specific expression of the putative cellulase gene, GPLIN_000313600, as previously reported in qRT-PCR results (Figure 4.4), a more comprehensive analysis was undertaken as part of the RNA-Seq investigation. Specifically, this gene showed significant up-regulation in response to potato root exudate compared to the water control, whereas its expression remained unaltered in response to maize or carrot root exudate exposure (Figure 4.9A). Additional cellulase encoding genes, which were previously identified and cloned in Chapter 3, also displayed host-specific gene expression patterns. Notably, GPLIN_001111200 exhibited significant up-regulation in response to potato root exudate but showed no increase in expression in response to maize or carrot root exudates (Figure 4.9B). Furthermore, GPLIN_000755200 was significantly up-regulated in response to carrot and potato root exudate, but not in response to maize root exudate (Figure 4.9C). Consistent with the previous qRT-PCR findings, GPLIN00036400 displayed no significant up-regulation in response to any of the root exudate exposures (Figure 4.9D). These putative cellulase genes displayed specificity in their expression with respect to the host root exudate the nematode was exposed to.

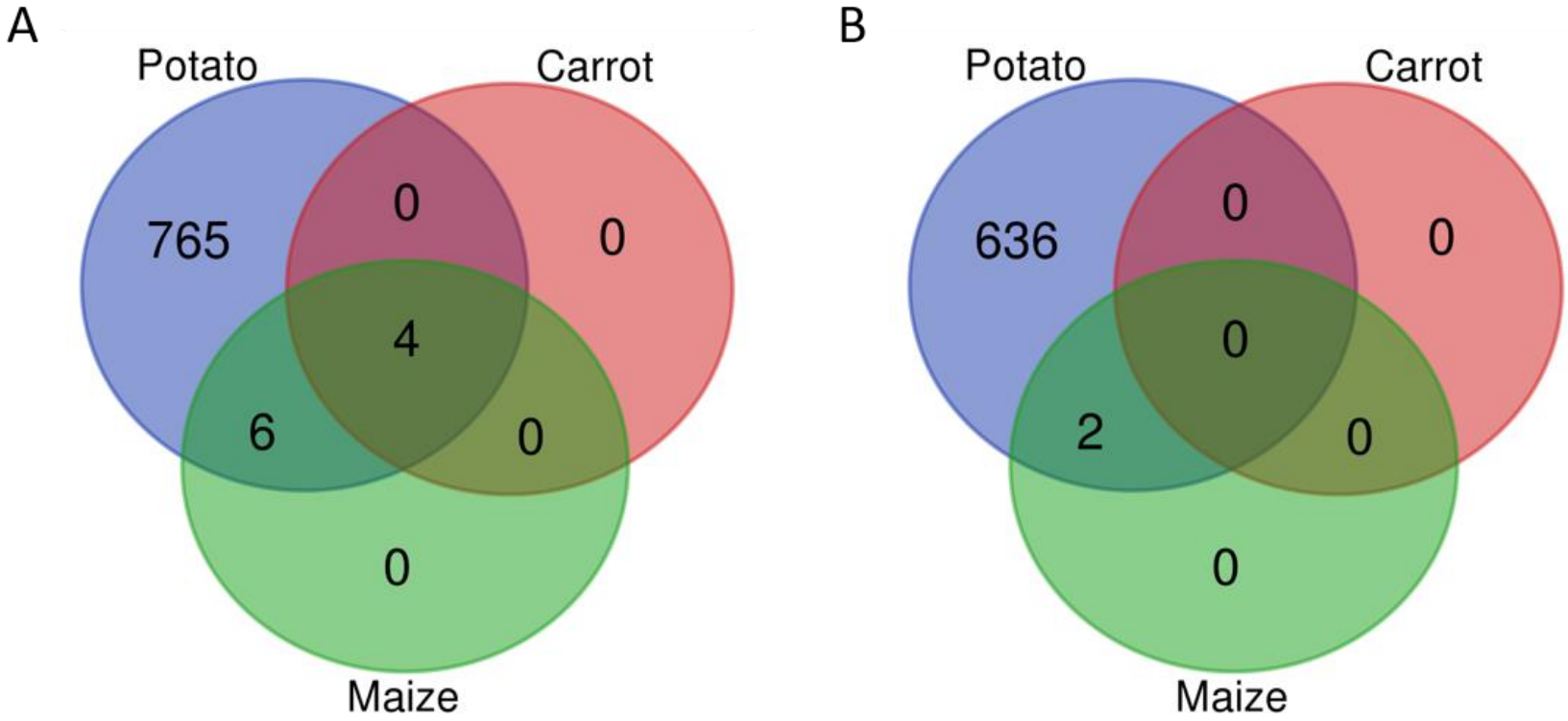


Figure 4.8- Comparisons of genes up- and down-regulated in *G. pallida* J2s in response to root exudates from carrot, maize and potato plants.

RNA-Seq was performed for *G. pallida* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate. Differential gene expression analysis revealed the different numbers of up- and down-regulated genes compared to the control. Venn diagrams display numbers of **A) up-regulated** (FDR ≤ 0.05, FC ≥ 1.5) and **B) down-regulated** (FDR ≤ 0.05, FC ≤ -1.5) genes in each root exudate treatment and indicate common up- or down-regulated genes across root exudate treatment groups compared to the control.

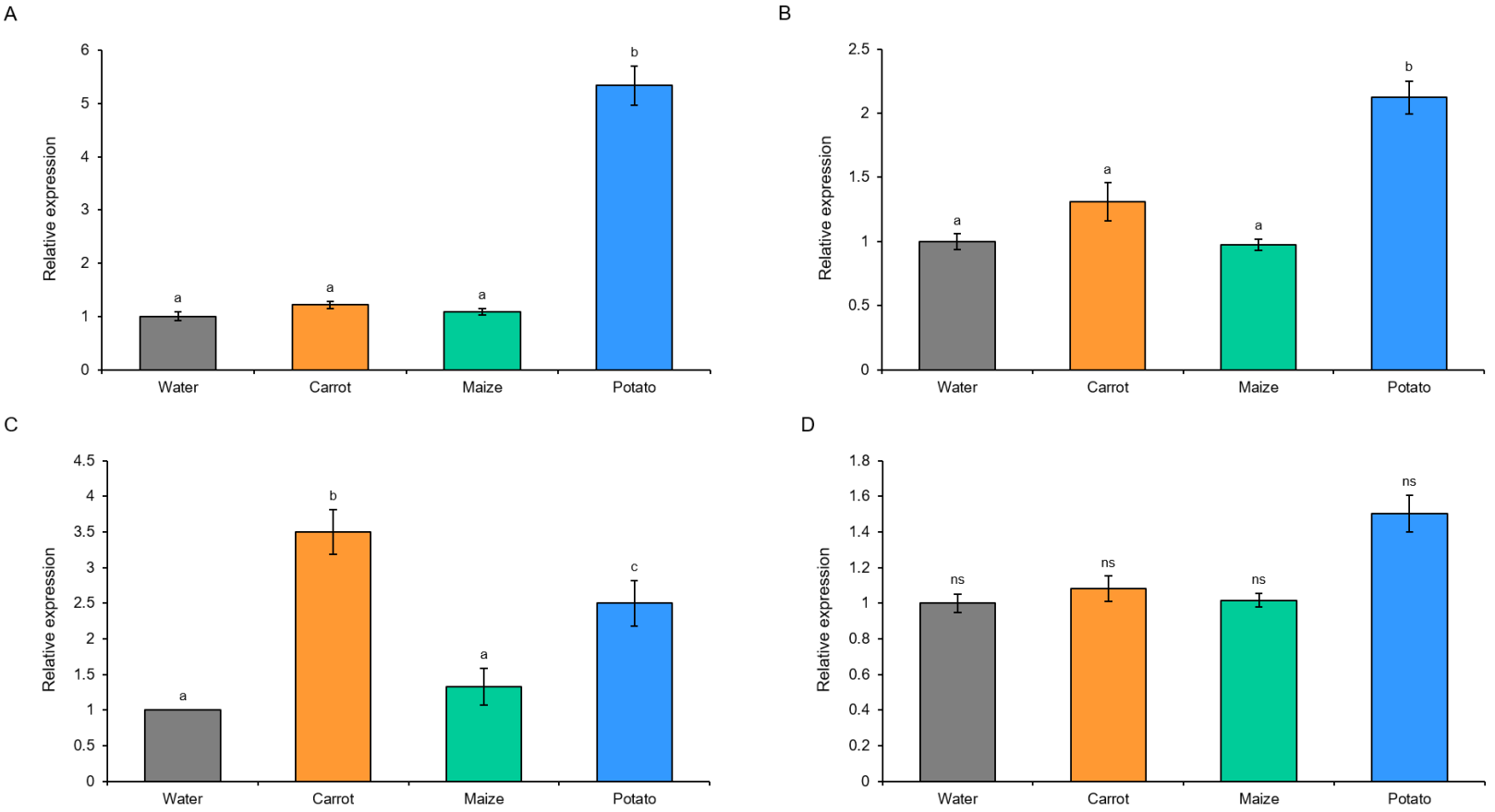


Figure 4.9- Differential gene expression of previously identified and cloned cellulase genes in *G. pallida.*

RNA-Seq was performed for *G. pallida* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate. Relative gene expression of **A) GPLIN_000313600 B) GPLIN_001111200, C) GPLIN_000755200** and **D) GPLIN00036400** was calculated using counts per million (CPM) values derived from the RNA-Seq data. CPM represents the number of sequence reads mapped to a specific gene normalised to the total number of reads in the dataset. Mean expression of 4 (water) or 5 (root exudates) biological repeats. Error bars are +/- SEM. Letters represent homogeneous subsets. (One-way ANOVA, Tukey's post hoc test).

4.4.3.3 Enrichment analysis of differentially expressed genes in *G. pallida* in response to varying root exudates

Gene Ontology and Enrichment analysis was used to highlight some common biological processes, cellular components and molecular functions that are transcriptionally affected by root exudate exposure. This analysis specifically focussed on the genes that were up-regulated. Several molecular function and cellular component GO terms were significantly under-represented among the up-regulated genes in *G. pallida* when exposed to potato root exudate (Figure 4.10). These under-represented molecular functions included metabolic processes, whilst the under-represented cellular components encompassed various GO terms associated with organelles (Figure 4.10). No other root exudate exposure induced significant enrichment of GO terms within these categories in *G. pallida* and there were no significantly enriched GO terms relating to biological processes response to any of the root exudates.

As the focus of this study was initially directed towards cell wall-degrading enzymes, an investigation was undertaken to ascertain whether genes encoding these, and other effectors had been up-regulated in response to root exudates. While specific GO terms directly relating to effector proteins are lacking, this omission can impede their identification during gene enrichment analysis. Nevertheless, meticulous manual searches identified a notable abundance of genes with annotations indicative of potential effectors that were significantly up-regulated in *G. pallida* J2s when exposed to potato root exudates. Among the 775 up-regulated genes in *G. pallida* in response to potato root exudate, 65 genes encoding probable effectors were identified. Among others, these included genes encoding SPRY domain containing proteins, predicted secretory proteins, glutathione synthetase effectors, and proteins localised to the gland cells, collectively representing 8.4 % of the overall up-regulated gene pool in response to this root exudate. Notably, 16 (24.6 %) of these genes were considered to be cell wall-degrading enzymes such as pectate lyases, beta-1,4-endoglucanases, cell wall-associated hydrolases and glycosyl hydrolase family proteins.

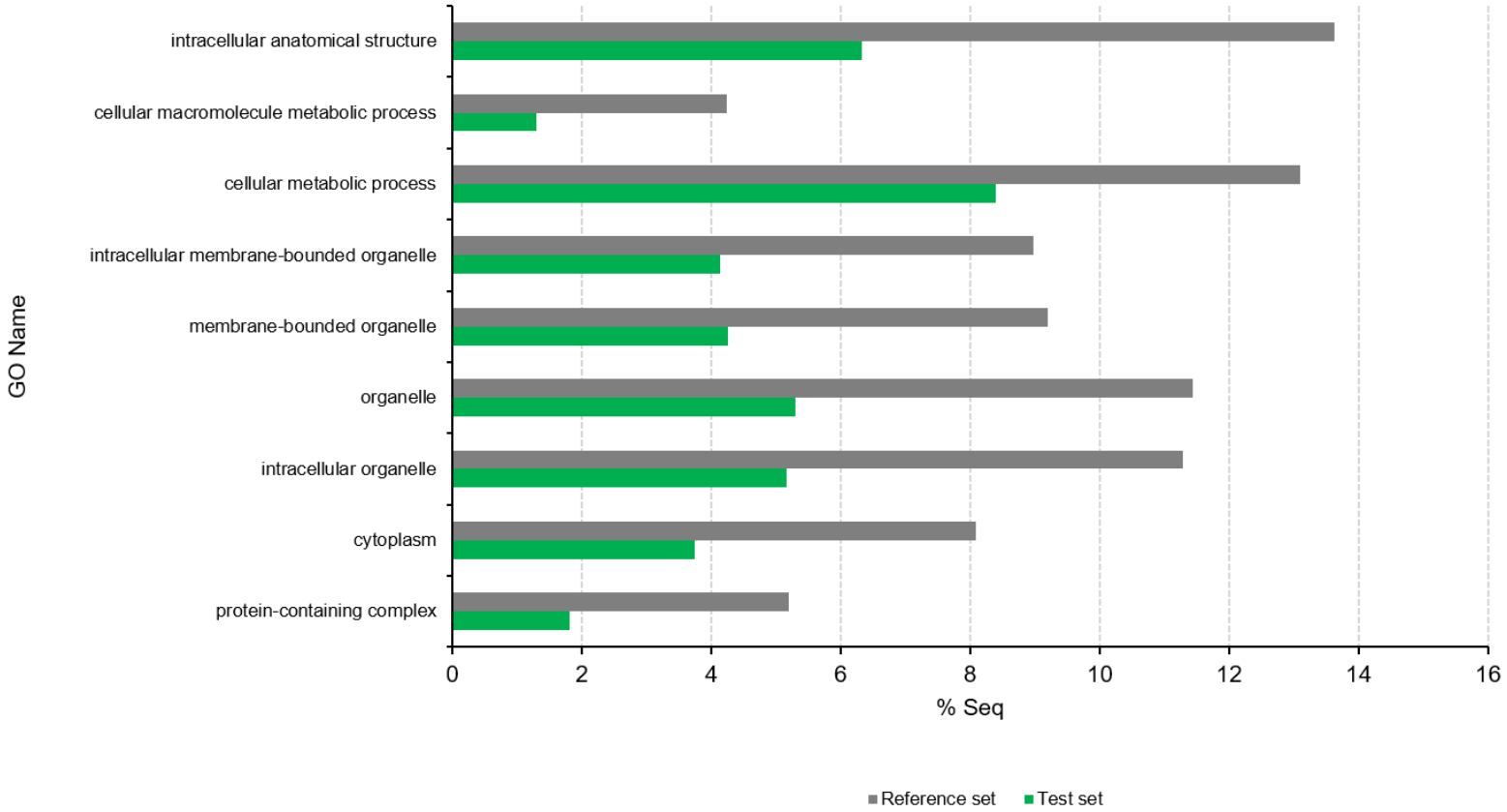


Figure 4.10- Gene ontology (GO) identifiers in genes up-regulated in *G. pallida* in response to potato root exudate exposure.

Over and under-represented gene ontology (GO) IDs for molecular functions and cellular components, reduced to most specific (P ≤ 0.01), retrieved using Blast2GO, of up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5). Reference set represents the percentage of GO IDs within the genome of *G. pallida.* Test set represents the percentage of GO IDs within the genes up-regulated (FDR ≤ 0.05, FC ≥ 1.5) in response to potato root exudate exposure.

4.4.4 Sequencing of RNA from *M. incognita* following root exudate exposure

RNA-Seq was also used to understand the transcriptional responses of *M. incognita* to root exudate detection. Total RNA was extracted from 20,000 J2s which had been exposed to either sterile tap water or cabbage, carrot, maize, potato or tomato root exudate for four hours. Seven biological replicates for each condition were generated and the quality of each RNA sample was checked using the Agilent 2100 bioanalyser (Figure 4.11).

Five of the best quality RNA samples for each condition were chosen to be sequenced. However, for maize and tomato root exudate exposure groups, only four replicates were included as the RNA quality was too low (RIN<6) in the remaining three (Table 4.10).

RNA was sequenced using the NextSeq2000 platform which generated FASTQ files. Each sample yielded over 18 million reads, with the majority yielding >25 million, and over 2,000 million bases, with the majority yielding >3,000 million (Table 4.11). The quality of reads within these files was assessed using the Preprocessing and FastQC Quality Check tools on OmicsBox v.2.2.4. All samples were deemed to have produced good quality reads and all were subsequently included in further analysis (Table 4.11).

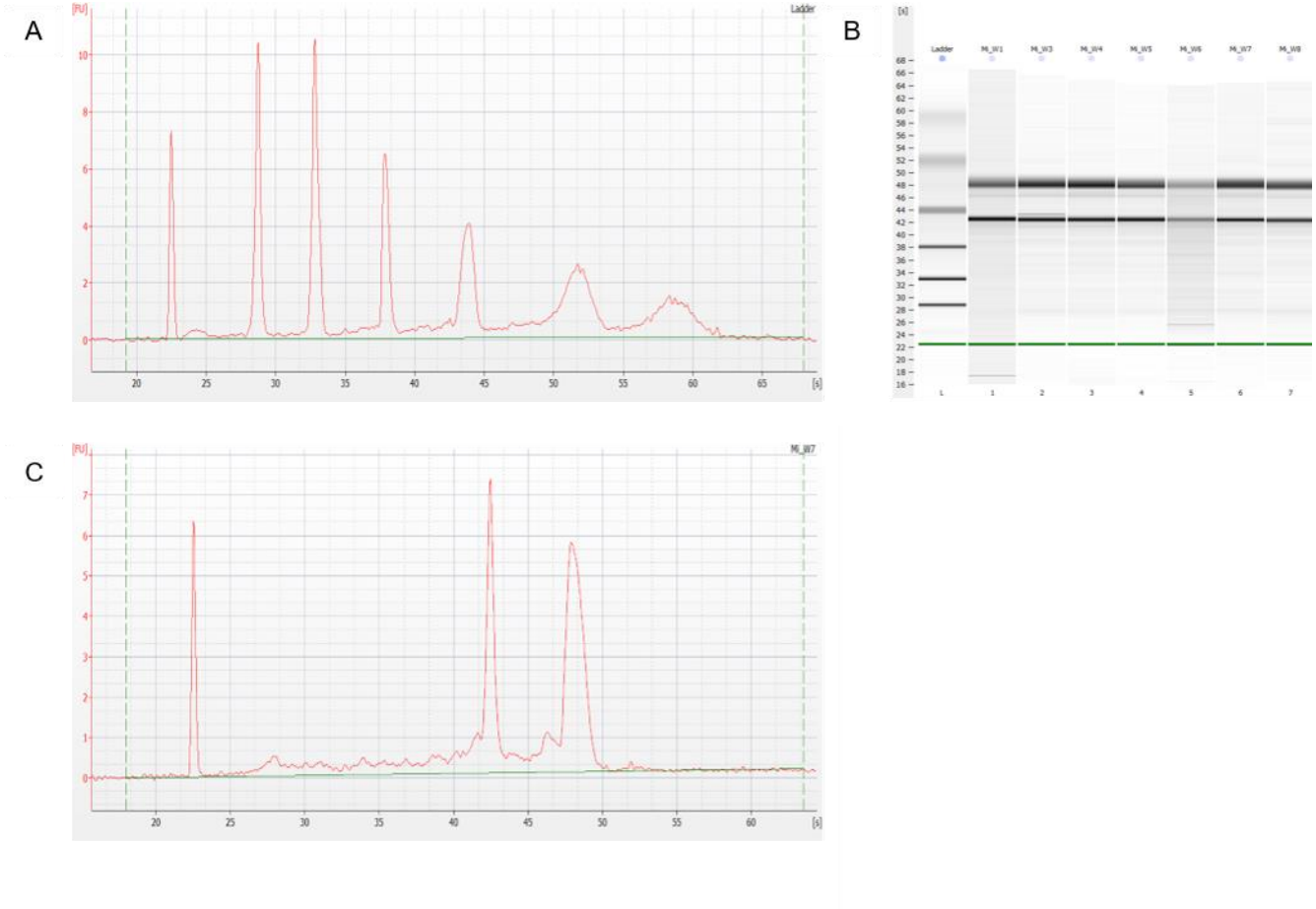


Figure 4.11- *M. incognita* RNA quality.

A) Electropherogram of RNA ladder. Comparison for RNA integrity for *M. incognita* RNA samples.
B) Gel electrophoresis of a variety of *M. incognita* RNA samples. RNA shows good integrity compared to the RNA ladder. Clear, sharp bands represent 28S and 18S rRNA.
C) Electropherogram of representative *M. incognita* RNA sample. Integrity was compared to the RNA ladder.

Nematode species	Condition	Total number of samples sequenced by RNA-Seq (n)	Total number of samples utilised in RNA-Seq analysis (n)
M. incognita	Water exposure (control)	5	5
M. incognita	Cabbage root exudate exposure	5	5
M. incognita	Carrot root exudate exposure	5	5
M. incognita	Maize root exudate exposure	4	4
M. incognita	Potato root exudate exposure	5	5
M. incognita	Tomato root exudate exposure	4	4

Table 4.10- Total number of *M. incognita* samples used for RNA-Seq.

Five samples from each treatment group were sequenced except for maize and tomato root exudate exposure groups where four samples were sequenced. All samples sequenced produced high quality reads and so all were utilised in the subsequent RNA-Seq analysis.

Treatment	Replicate number	Total number of reads	Yield (Million bases)	Uniquely mapped reads (%)
Water control	1	27,842,794	6,244,483	49.94
	2	26,206,612	6,142,445	52.35
	3	29,602,019	6,093,129	45.258
	4	31,843,508	7,368,148	51.354
	5	24,079,868	5,604,128	51.682
Tomato root exudate	1	28,365,144	6,700,001	52.362
	2	26,759,227	6,476,938	53.628
	3	28,648,823	7,816,196	60.03
	4	28,608,961	4,608,882	35.881
Maize root exudate	1	18,722,708	4,270,365	51.248
	2	28,505,546	6,540,946	51.903
	3	22,734,964	5,207,314	51.275
	4	26,790,519	6,238,303	52.77
Carrot root exudate	1	28,466,277	6,769,071	52.429
	2	25,755,091	6,544,173	56.472
	3	26,242,289	6,424,644	53.993
	4	30,466,226	7,426,792	53.99
	5	25,966,109	5,956,058	50.803

Table 4.11- Sequencing and alignment of RNA from *M. incognita* exposed to root exudates.

M. incognita J2s were exposed to either sterile tap water, or cabbage, carrot, maize, potato or tomato root exudate and RNA-Seq was performed using the NextSeq2000™ platform. Reads in these files were aligned to the *M. incognita* genome (Blanc-Mathieu *et al*., 2017) using Omics Box v2.2.4.

Cabbage root exudate	1	25,826,608	6,202,412	53.121
	2	27,049,777	6,759,119	55.5
	3	24,876,896	4,716,948	42.051
	4	25,028,385	5,628,075	49.604
	5	25,598,348	4,866,670	42.28
Potato root exudate	1	23,221,441	2,848,830	27.092
	2	27,909,750	3,738,553	29.653
	3	26,074,461	3,007,632	25.485
	4	28,892,929	3,966,660	30.521
	5	25,685,103	3,340,301	28.754

Table 4.11 continued- Sequencing and alignment of RNA from *M. incognita* exposed to root exudates.

M. incognita J2s were exposed to either sterile tap water, or cabbage, carrot, maize, potato or tomato root exudate and RNA-Seq was performed using the NextSeq2000™ platform. Reads were aligned to the *M. incognita* genome using Omics Box v2.2.4.

4.4.4.1 Differential gene expression in *M. incognita* J2s in response to varying root exudates

Differential gene expression was observed in *M. incognita* J2s in response to all root exudate exposures compared to controls (Figure 4.12). Genes that had a significant (FDR ≤ 0.05) fold change (FC) of ≥ 1.5 or ≤ - 1.5 were considered up- or down-regulated, respectively. Genes that showed the highest fold changes (FC) (FDR ≤ 0.05) in response to root exudate exposure vary between treatments and these are listed in Tables 4.12-4.21.

Potato root exudate had the strongest effect on transcription in *M. incognita* J2s, causing by far the greatest number of differentially expressed genes compared to the control group (Table 4.22). This was followed by maize root exudate exposure, then carrot and then tomato. Exposure to cabbage root exudate had the weakest influence on the *M. incognita* J2s as it stimulated the smallest number of differentially expressed genes compared to the control group (Table 4.22).

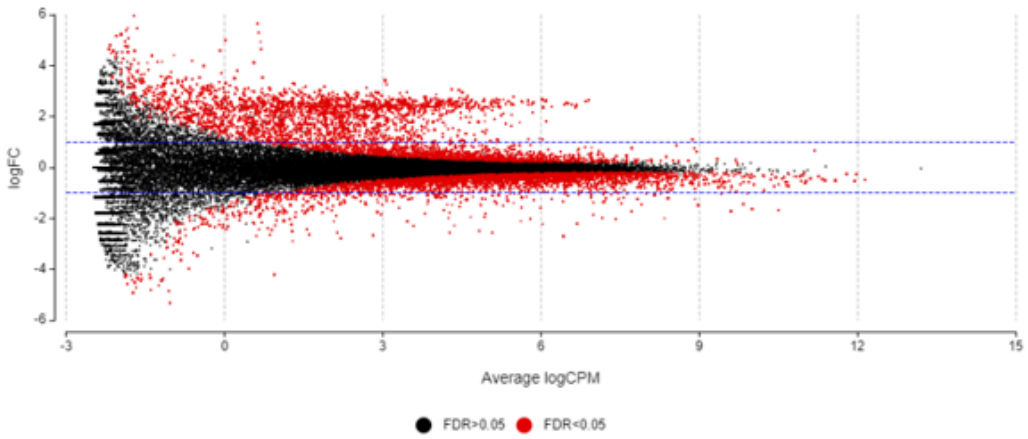
Exposure to potato root exudate resulted in significant differential expression of 3,777 genes in *M. incognita* (Table 4.22). Among these, 2,589 were up-regulated, whilst 1,188 were down-regulated compared to the control group. A total of 823 genes were differentially expressed in response to maize root exudate exposure (Table 4.22), with 599 being up-regulated and 224 down-regulated. Carrot root exudate exposure led to the differential expression of 435 genes (Table 4.22), out of which, 178 were up-regulated and 257 were down-regulated. Similarly, exposure to tomato root exudate triggered the differential expression of 104 genes (Table 4.22), with 72 being up-regulated and 32 down-regulated. Finally, cabbage root exudate, resulted in the upregulation of 32 out of 51 differentially expressed genes, whilst the remaining 19 were down-regulated. The number of differentially expressed genes did not correlate with cellulose concentration in the root exudates (Figure 4.2).

In addition to comparing gene expression in *M. incognita* J2s exposed to root exudates and those exposed to sterile tap water, differential gene expression between root exudate exposures was also compared (Table 4.22). Unsurprisingly, most notable differences were seen between groups of nematodes exposed to potato root exudate compared those exposed to the other root exudates, with the largest difference being between pools of nematodes exposed to potato, and pools exposed to maize root exudates (Table 4.22). Interestingly, there were more differences between potato and any other root exudate than between potato and the water control.

This work initially centred on the evaluation of differential gene expression of cellulase encoding genes in *G. pallida* and *M. incognita.* It is therefore noteworthy that genes

responsible for encoding cell wall-degrading enzymes, and other effectors rank among the top 20 up-regulated genes exhibiting the most substantial fold changes for each respective root exudate treatment (Tables 4.12, 4.14, 4.18, 4.20). These include genes encoding astacins, putative avirulence proteins, pectate lyases, and putative secretory effector proteins. In contrast, genes that encode pectate lyases are also featured within the top 20 down-regulated genes in response to cabbage and maize root exudates (Tables 4.15 and 4.21). Specifically, pectate lyase 1, Minc3s00094g04359, is included in both of these lists.

A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FDR<0.05

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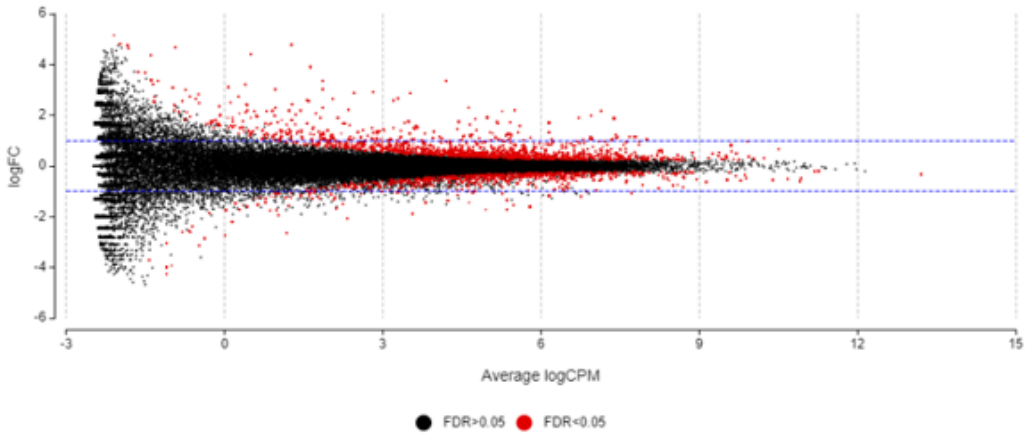
logFC
Average logCPM
FDR>0.05
FDR<0.05

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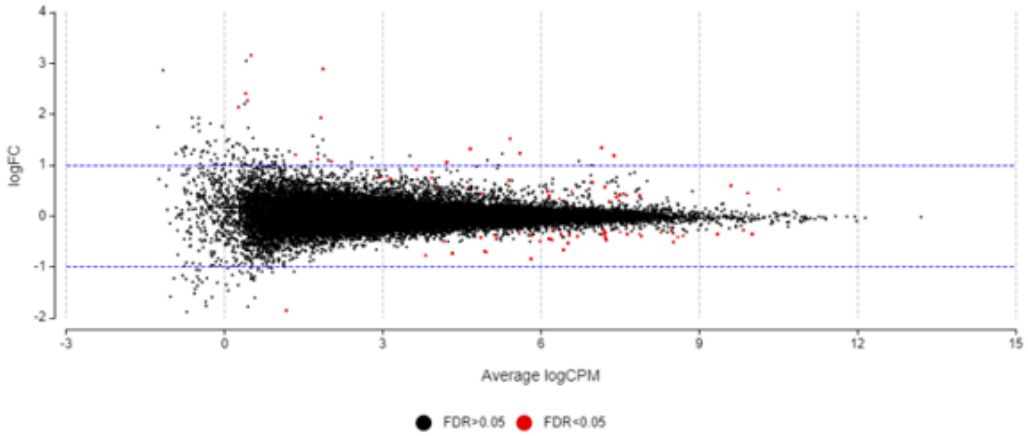
logFC
Average logCPM
FDR>0.05
FDR<0.05

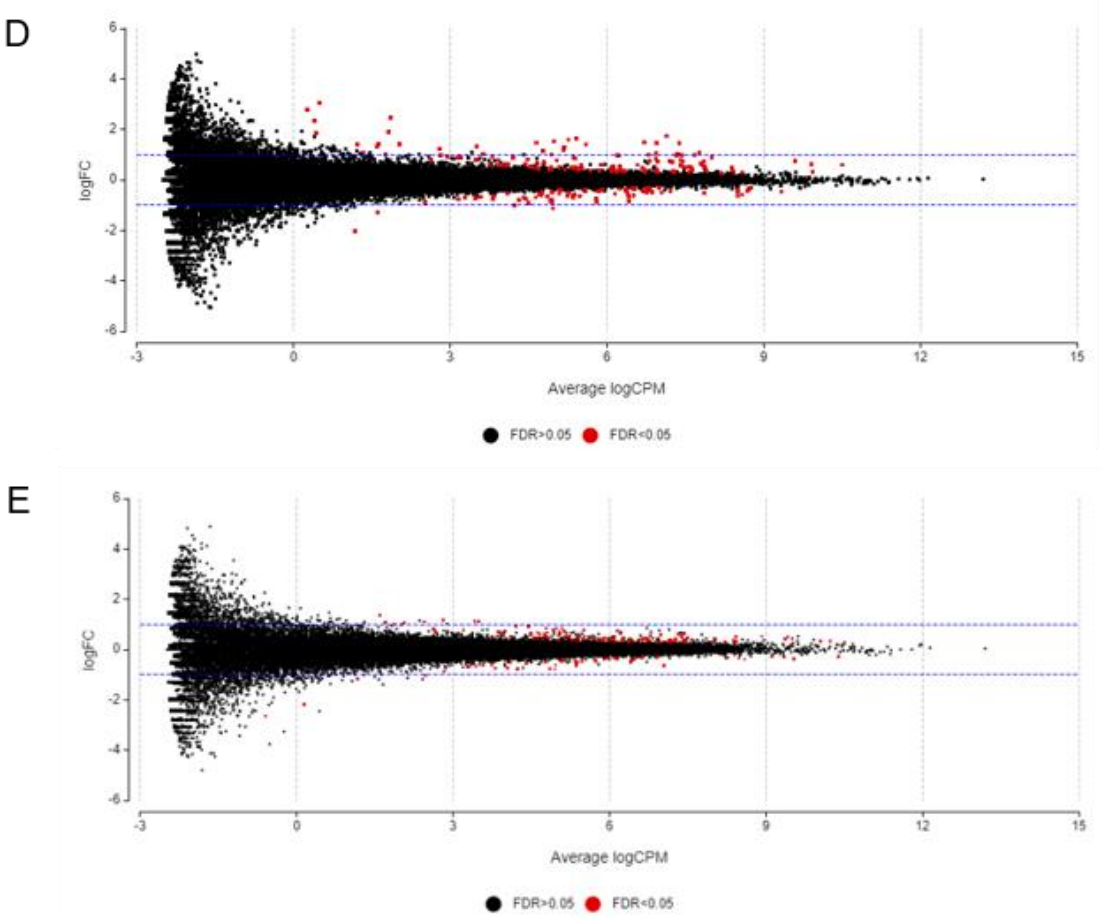

Figure 4.12- Differentially expressed genes in *M. incognita* in response to varying root exudates.

A) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *M. incognita* J2s exposed to **potato root exudate** and *M. incognita* J2s exposed to the water control.

B) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *M. incognita* J2s exposed to **maize root exudate** and *M. incognita* J2s exposed to the water control.

C) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *M. incognita* J2s exposed to **carrot root exudate** and *M. incognita* J2s exposed to the water control. Significantly up-regulated genes are coloured red and significantly down-regulated genes are coloured blue.

D) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *M. incognita* J2s exposed to **tomato root exudate** and *M. incognita* J2s exposed to the water control. Significantly up-regulated genes are coloured red and significantly down-regulated genes are coloured blue.

E) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *M. incognita* J2s exposed to **cabbage root exudate** and *M. incognita* J2s exposed to the water control. Significantly differentially regulated genes are coloured red.

Those that are above 0 are up-regulated and those that are below 0 are down-regulated. Dotted blue lines indicate the implemented fold change (FC) cut offs.

Gene ID	Fold change (FC)	Description
Minc3s07314g40978	62.15	Galectin domain-containing protein
Minc3s00102g04600	50.52	Elongation of very long chain fatty acids protein
Minc3s00919g18888	44.80	Unnamed protein product
Minc3s06303g39641	44.64	MFP2b family protein
Minc3s00291g09541	41.98	Galectin domain-containing protein
Minc3s01931g27277	39.23	Pectate lyase 1
Minc3s00268g09017	37.48	Apyrase apy-1
Minc3s01157g21252	37.37	Unnamed protein product
Minc3s01881g26934	35.92	Unnamed protein product
Minc3s01571g24807	33.74	MSP domain protein
Minc3s09880g43612	32.04	Unnamed protein product
Minc3s02296g29314	31.02	Integrase catalytic domain-containing protein
Minc3s02104g28308	30.64	Elongation of very long chain fatty acids protein
Minc3s00280g09293	27.76	Unnamed protein product
Minc3s00583g14627	27.23	Putative esophageal gland cell secretory protein 10
Minc3s01465g24054	26.82	Protein CBR-ARX-4
Minc3s00620g15204	25.07	Pectate lyase 1
Minc3s03801g34854	25.06	Unnamed protein product
Minc3s01464g24044	24.06	Elongation of very long chain fatty acids protein
Minc3s01150g21203	23.81	Unnamed protein product

Table 4.12- Up-regulated genes in *M. incognita* exposed to potato root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *M. incognita* when exposed to potato root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s00836g17933	-39.92	Unnamed protein product
Minc3s00527g13759	-30.30	Unnamed protein product
Minc3s00262g08890	-28.95	Collagen triple helix repeat protein
Minc3s00072g03560	-27.88	Unnamed protein product
Minc3s00738g16705	-26.13	Histone acetyltransferase
Minc3s00410g11840	-24.78	Protein CBG19672
Minc3s02699g31244	-23.74	HMG box
Minc3s00636g15428	-22.91	Unnamed protein
Minc3s00078g03797	-22.07	Unnamed protein product
Minc3s11545g44967	-22.06	Unnamed protein product
Minc3s02848g31797	-21.49	Zinc finger BED domain-containing protein 1-like protein
Minc3s00824g17789	-20.85	Nuclease HARBI1-like protein
Minc3s01722g25860	-20.84	Unnamed protein product
Minc3s00009g00577	-20.75	PAP_central domain-containing protein
Minc3s02883g31952	-20.24	Zinc finger protein
Minc3s01350g23020	-20.22	CRE-AQP-4 protein
Minc3s02422g29942	-19.77	Unnamed protein product
Minc3s00821g17752	-19.43	Unnamed protein product
Minc3s00010g00669	-18.51	Unnamed protein product
Minc3s05019g37444	-18.46	Unnamed protein product

Table 4.13- Down-regulated genes in *M. incognita* exposed to potato root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *M. incognita* when exposed to potato root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s00944g19157	35.40	Unnamed protein product
Minc3s00056g02916	27.89	MAP-1 protein
Minc3s00627g15290	27.50	Pept_C1 domain-containing protein
Minc3s01465g24054	26.93	Protein CBR-ARX-4
Minc3s02101g28283	25.69	Unnamed protein product
Minc3s11702g45087	24.85	Unnamed protein product
Minc3s01155g21238	21.11	Unnamed protein product
Minc3s02099g28270	20.51	Cytochrome oxidase subunit 1
Minc3s02713g31295	14.95	Serine carboxypeptidase domain-containing protein
Minc3s02006g27721	12.98	Zinc finger, C3HC4 type
Minc3s00168g06604	12.81	Unnamed protein product
Minc3s01280g22423	10.40	Peptidase_M16_C domain-containing protein
Minc3s05958g39089	10.23	Peptidase family m13 domain-containing protein
Minc3s00870g18290	10.23	Endonuclease/Exonuclease/phosphatase family domain-containing protein
Minc3s00367g11103	10.14	Serpentine type 7TM GPCR chemoreceptor srt domain-containing protein
Minc3s00069g03422	9.30	Unnamed protein product
Minc3s00076g03740	9.23	Unnamed protein product
Minc3s01226g21884	8.62	Galactosyl transferase GMA12/MNN10 family domain-containing protein
Minc3s07290g40952	8.48	Expansin-like protein
Minc3s00619g15191	8.12	Unnamed protein product

Table 4.14- Up-regulated genes in *M. incognita* exposed to maize root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *M. incognita* when exposed to maize root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s01686g25593	-19.15	Unnamed protein product
Minc3s05164g37712	-15.90	Protein F14F9.5
Minc3s05050g37507	-15.23	Guanylate cyclase
Minc3s00032g01935	-13.17	Unnamed protein product
Minc3s00002g00077	-8.85	Galectin domain-containing protein
Minc3s01501g24304	-8.26	MYND-type domain-containing protein
Minc3s00112g04917	-7.22	Unnamed protein product
Minc3s11742g45117	-6.69	Unnamed protein product
Minc3s00094g04362	-6.26	Unnamed protein product
Minc3s02992g32341	-6.11	Unnamed protein product
Minc3s01984g27589	-6.09	Ras family domain-containing protein
Minc3s01549g24636	-5.85	L-type lectin-like domain-containing protein
Minc3s01293g22547	-5.24	Unnamed protein product
Minc3s04287g35939	-4.64	G_PROTEIN_RECEP_F1_2 domain-containing protein
Minc3s01569g24791	-4.21	Pept_C1 domain-containing protein
Minc3s00075g03700	-3.86	Pept_C1 domain-containing protein
Minc3s00094g04359	-3.68	Pectate lyase 1
Minc3s00431g12198	-3.61	G_PROTEIN_RECEP_F1_2 domain-containing protein
Minc3s04459g36327	-3.37	Zinc finger, RING-type domain and Zinc finger, RING/FYVE/PHD-type domain-containing protein
Minc3s07985g41772	-3.37	Unnamed protein product

Table 4.15- Down-regulated genes in *M. incognita* exposed to maize root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *M. incognita* when exposed to maize root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s00028g01761	13.49	Ground-like domain-containing protein
Minc3s02832g31725	9.62	Unnamed protein product
Minc3s00032g01958	5.45	Unnamed protein product
Minc3s01321g22761	5.44	Unnamed protein product
Minc3s04287g35939	4.56	G_PROTEIN_RECEP_F1_2 domain-containing protein
Minc3s00002g00077	4.31	Galectin domain-containing protein
Minc3s00221g07909	4.10	Unnamed protein product
Minc3s04240g35823	3.76	Unnamed protein product
Minc3s00575g14494	2.73	Unnamed protein product
Minc3s00343g10630	2.66	Dual oxidase
Minc3s03010g32419	2.60	Major sperm protein
Minc3s04131g35557	2.59	MCM2/3/5 family protein
Minc3s00109g04827	2.56	Unnamed protein product
Minc3s00769g17106	2.52	Unnamed protein product
Minc3s00301g09735	2.48	Trans-thyretin-related family domain family member
Minc3s01685g25586	2.47	CGG triplet repeat-binding protein 1
Minc3s05388g38148	2.45	Unnamed protein product
Minc3s04619g36675	2.41	Dual specificity phosphatase, catalytic domain-containing protein
Minc3s00024g01543	2.40	Unnamed protein product
Minc3s01396g23460	2.37	Unnamed protein product

Table 4.16- Up-regulated genes in *M. incognita* exposed to carrot root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *M. incognita* when exposed to carrot root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s01226g21884	-3.13	Galactosyl transferase GMA12/MNN10 family domain-containing protein
Minc3s00102g04600	-2.79	Elongation of very long chain fatty acids protein
Minc3s06236g39536	-2.59	Putative avirulence protein precursor
Minc3s00002g00163	-2.51	E3 ubiquitin-protein ligase UBR5
Minc3s05915g39029	-2.42	Unnamed protein product
Minc3s01327g22806	-2.38	PDZ domain-containing protein
Minc3s02563g30650	-2.35	CCR4-Not complex component
Minc3s03337g33554	-2.33	Oxidoreductase, short chain dehydrogenase/reductase family protein
Minc3s01890g26993	-2.30	Progestin and adipoq receptor family member 3
Minc3s04601g36631	-2.29	Unnamed protein product
Minc3s00750g16857	-2.27	Unnamed protein product
Minc3s01452g23928	-2.26	Unnamed protein product
Minc3s00431g12206	-2.24	Unnamed protein product
Minc3s00366g11088	-2.23	Tyrosinase_Cu-bd domain-containing protein
Minc3s05485g38318	-2.23	Unnamed protein product
Minc3s09884g43617	-2.23	Unnamed protein product
Minc3s00060g03111	-2.20	Protein CBG18268
Minc3s04299g35979	-2.20	Unnamed protein product
Minc3s00434g12271	-2.18	PID domain-containing protein
Minc3s00017g01119	-2.17	Integrator complex subunit 2 domain-containing protein

Table 4.17- Down-regulated genes in *M. incognita* exposed to carrot root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *M. incognita* when exposed to carrot root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s01155g21238	8.29	Unnamed protein product
Minc3s02491g30303	6.85	Zinc finger protein
Minc3s00619g15191	5.50	Unnamed protein product
Minc3s00076g03740	5.08	Unnamed protein product
Minc3s00490g13201	3.75	Zinc finger protein
Minc3s01481g24163	3.60	Unnamed protein product
Minc3s00671g15902	3.34	L-lactate dehydrogenase
Minc3s00875g18361	3.13	Unnamed protein product
Minc3s01740g25976	3.01	Unnamed protein product
Minc3s00110g04846	2.88	Glycogen recognition site of AMP-activated protein kinase domain-containing protein
Minc3s00011g00712	2.79	Aquaporin or aquaglyceroporin related
Minc3s03118g32848	2.78	Unnamed protein product
Minc3s04466g36352	2.76	Aquaporin or aquaglyceroporin related
Minc3s02003g27699	2.73	L-lactate dehydrogenase
Minc3s00013g00831	2.67	PAP central domain-containing protein
Minc3s02713g31295	2.66	Serine carboxypeptidase domain-containing protein
Minc3s00743g16773	2.64	Unnamed protein product
Minc3s06784g40310	2.63	Unnamed protein product
Minc3s00138g05793	2.51	Glycogen recognition site of AMP-activated protein kinase domain-containing protein
Minc3s06236g39536	2.50	Putative avirulence protein precursor

Table 4.18- Up-regulated genes in *M. incognita* exposed to tomato root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *M. incognita* when exposed to tomato root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s00094g04362	-4.11	Unnamed protein product
Minc3s00030g01815	-2.45	Unnamed protein product
Minc3s00558g14270	-2.19	Unnamed protein product
Minc3s01283g22455	-2.01	Pept_C1 domain-containing protein
Minc3s00237g08286	-1.89	Pept_C1 domain-containing protein
Minc3s00925g18947	-1.88	Pept_C1 domain-containing protein
Minc3s07705g41429	-1.85	DPBB_1 domain-containing protein
Minc3s00741g16747	-1.83	Unnamed protein product
Minc3s00638g15461	-1.80	Carboxylic ester hydrolase
Minc3s04001g35256	-1.80	Unnamed protein product
Minc3s01290g22523	-1.74	Cellulase domain-containing protein
Minc3s06058g39255	-1.72	Tyrosinase_Cu-bd domain-containing protein
Minc3s00237g08287	-1.70	Pept_C1 domain-containing protein
Minc3s03709g34577	-1.69	Cellulase domain-containing protein
Minc3s01111g20823	-1.67	Pectate lyase 3
Minc3s01283g22454	-1.66	Pept_C1 domain-containing protein
Minc3s07601g41307	-1.66	Unnamed protein product
Minc3s06238g39541	-1.65	Invertebrate-type lysozyme 3
Minc3s00925g18941	-1.61	Pept_C1 domain-containing protein
Minc3s12088g45350	-1.61	Unnamed protein product

Table 4.19- Down-regulated genes in *M. incognita* exposed to tomato root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *M. incognita* when exposed to tomato root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s06236g39536	2.58	Putative avirulence protein precursor
Minc3s00750g16857	2.25	Unnamed protein product
Minc3s04299g35979	2.19	Unnamed protein product
Minc3s00579g14555	2.15	Unnamed protein product
Minc3s02024g27834	2.15	Peptidase family m13 domain-containing protein
Minc3s02480g30258	2.12	EGF-like domain-containing protein
Minc3s00694g16191	2.09	Unnamed protein product
Minc3s00485g13126	2.02	Serine carboxypeptidase domain-containing protein
Minc3s00012g00796	1.98	G_PROTEIN_RECEP_F1_2 domain-containing protein
Minc3s05958g39089	1.89	Peptidase family m13 domain-containing protein
Minc3s00212g07668	1.88	Unnamed protein product
Minc3s00944g19159	1.81	Unnamed protein product
Minc3s03733g34638	1.80	Guanylate cyclase
Minc3s00009g00641	1.75	Astacin (Peptidase family m12a) domain-containing protein
Minc3s00160g06399	1.73	Unnamed protein product
Minc3s00337g10473	1.71	MFS transporter, SP family
Minc3s00056g02893	1.71	Repetitive domains
Minc3s00297g09645	1.70	Serine carboxypeptidase
Minc3s07544g41248	1.68	Candidate secreted effector Minc08014
Minc3s02593g30804	1.66	Putative avirulence protein precursor

Table 4.20- Up-regulated genes in *M. incognita* exposed to cabbage root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *M. incognita* when exposed to cabbage root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
Minc3s00691g16143	-6.28	Unnamed protein product
Minc3s04287g35939	-4.58	G_PROTEIN_RECEP_F1_2 domain-containing protein
Minc3s00094g04362	-2.23	Unnamed protein product
Minc3s00126g05418	-2.03	Cytochrome oxidase assembly factor 4
Minc3s00925g18947	-1.72	Pept_C1 domain-containing protein
Minc3s01283g22455	-1.71	Pept_C1 domain-containing protein
Minc3s01624g25179	-1.69	Gamma-glutamyltranspeptidase domain-containing protein
Minc3s00044g02449	-1.68	VAP1 protein
Minc3s01111g20823	-1.68	Pectate lyase 3
Minc3s04396g36198	-1.67	Cytochrome oxidase assembly factor 4
Minc3s08265g42065	-1.61	Unnamed protein product
Minc3s00301g09730	-1.60	Dumpy: shorter than wild-type
Minc3s00252g08613	-1.59	Guanylate cyclase
Minc3s12088g45350	-1.57	Unnamed protein product
Minc3s00775g17181	-1.55	VAP1 protein
Minc3s00094g04359	-1.54	Pectate lyase 1
Minc3s00558g14270	-1.53	Unnamed protein product
Minc3s00007g00489	-1.53	Otopetrin domain containing protein
Minc3s00991g19648	-1.51	Unnamed protein product

Table 4.21- Down-regulated genes in *M. incognita* exposed to cabbage root exudate.

Down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *M. incognita* when exposed to cabbage root exudate compared to when exposed to a water. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

	Water (control)	Carrot root exudate	Cabbage root exudate	Maize root exudate	Potato root exudate	Tomato root exudate
Water (control)						
Carrot root exudate	435					
Cabbage root exudate	51	245				
Maize root exudate	823	1,199	819			
Potato root exudate	3,776	4,260	4,456	5,112		
Tomato root exudate	104	364	33	352	4,127	

Table 4.22- Total number of differentially expressed genes in *M. incognita* J2s in response to varying root exudates.

M. incognita J2s were exposed to either sterile tap water, or cabbage, carrot, maize, potato or tomato root exudate and RNA-Seq was performed using the NextSeq 2000™ platform. Differential gene expression analysis revealed numbers of genes that were significantly differentially expressed between treatment groups compared to the water control, as well as each of the treatment groups compared to each other (FDR ≤ 0.05, FC ≥ 1.5 or ≤ -1.5).

4.4.4.2 Differential gene expression is host-specific in *M. incognita*

Exposure to different root exudates caused varying levels of differential gene expression in *M. incognita* J2s. The differential expression was largely host-specific and, although there is some overlap, the majority of the genes that showed the highest fold changes (FDR ≤ 0.05) in response to root exudate exposure vary between root exudate treatments (Tables 4.12-4.21).

No genes were commonly up- or down-regulated in response to all five root exudate treatments (Figure 4.13). The general trend was that only a small number of genes were differentially expressed in more than one root exudate treatment and the majority of differences in expression seen compared to the water control were unique to each individual root exudate treatment. To emphasise this, the cell wall-degrading enzymes and effectors mentioned previously (Section 4.4.4.1), which featured among the top 20 up-regulated genes in *M. incognita* in response to various root exudates, exhibited exclusivity across the various root exudate treatments, with each treatment's list of top 20 up-regulated genes encompassing a unique set of these genes (Tables 4.12, 4.14, 4.18, 4.20).

Upon exposure to potato root exudate, *M. incognita* differentially expressed a total of 3,776 genes (Table 4.22). Of these, only 101 were commonly differentially regulated in response to another root exudate, meaning 97.3 % of the genes displaying differential regulation in response to potato root exudate were unique to this particular treatment. While other root exudate exposures displayed greater overlap in differentially expressed genes, there were still considerable proportions of differentially regulated genes that were unique to each of the treatments.

Two genes displayed common up-regulation in response to exposure to root exudates from four different host plants (cabbage, maize, potato and tomato) (Figure 4.13A). One of these genes is annotated as encoding a peptidase family m13 domain-containing protein, while the other is described as an "unnamed protein product". Notably, the peptidase family m13 domain-containing protein ranked among the top 20 most highly up-regulated genes in *M. incognita* in response to both cabbage and maize root exudate (Tables 4.14 and 4.20). On the other hand, the unnamed protein exhibited the second highest fold change among all of the up-regulated genes in response to cabbage root exudate (Table 4.20). Interestingly, this gene also featured among the top 20 down-regulated genes in response to carrot root exudate (Table 4.17).

A further nine genes, with a variety of functions, were among the entire complement of up-regulated genes in response to three or more root exudate treatments (Figure 4.13A). These included a G-protein coupled receptor (GPCR), a zinc finger protein, a sulfatase

domain-containing protein, a putative avirulence protein precursor and an astacin (peptidase family m12A) domain-containing protein. The remaining four were annotated as “unnamed protein products”. The putative avirulence protein precursor was among the top 20 up-regulated genes in *M. incognita* response to tomato root exudate (Table 4.18) and all, but two of the unnamed proteins, were among the top 20 up-regulated genes in *M. incognita* response to cabbage root exudate (Table 4.20).

To confirm the host-specific expression patterns of the previously investigated cellulase genes, *Mi-eng-1* and *Mi-eng-4,* a more comprehensive analysis was undertaken during the RNA-Seq analysis. Initially, qRT-PCR had assessed the expression of these genes following the exposure of *M. incognita* J2s to potato and maize root exudates (Figure 4.5). Building on these preliminary findings, the RNA-Seq data revealed significant differences in expression of *Mi-eng-1* in nematodes exposed to maize root exudates, compared to those exposed to cabbage, carrot or potato root exudates (Figure 4.14A). While there are significant differences between root exudate treatments, the exposure to any of cabbage, carrot, maize, potato or tomato root exudate does not cause significant differential expression of *Mi-eng-1* when compared to the control (Figure 4.14A). Interestingly, the previous qRT-PCR analysis had demonstrated a significant up-regulation of *Mi-eng-4* in response to potato and maize root exudates, relative to the control, as well as a significant difference in expression between root exudate treatments (Figure 4.5B). However, the RNA-Seq results contradict these findings, indicating a down-regulation of this gene in response to both maize and potato root exudates compared to the control, and revealing no significant difference in expression between these two root exudate treatments (Figure 4.14B). *Mi-eng-4* did however exhibit host-specific gene expression, with significant differences observed between nematodes exposed to potato root exudate and those exposed to cabbage, carrot or tomato root exudate (Figure 4.14B). *Mi-eng-2* was also previously identified as a gene of interest in Chapter 3, however attempts to clone it were unsuccessful. The expression of this gene was therefore subjected to more in-depth examination in the RNA-Seq analysis, but it was not significantly up-regulated in response to any root exudate treatments; in fact, this gene showed a significant downregulation in nematodes exposed to maize and potato root exudates when compared to those exposed to the water control (Figure 4.14C).

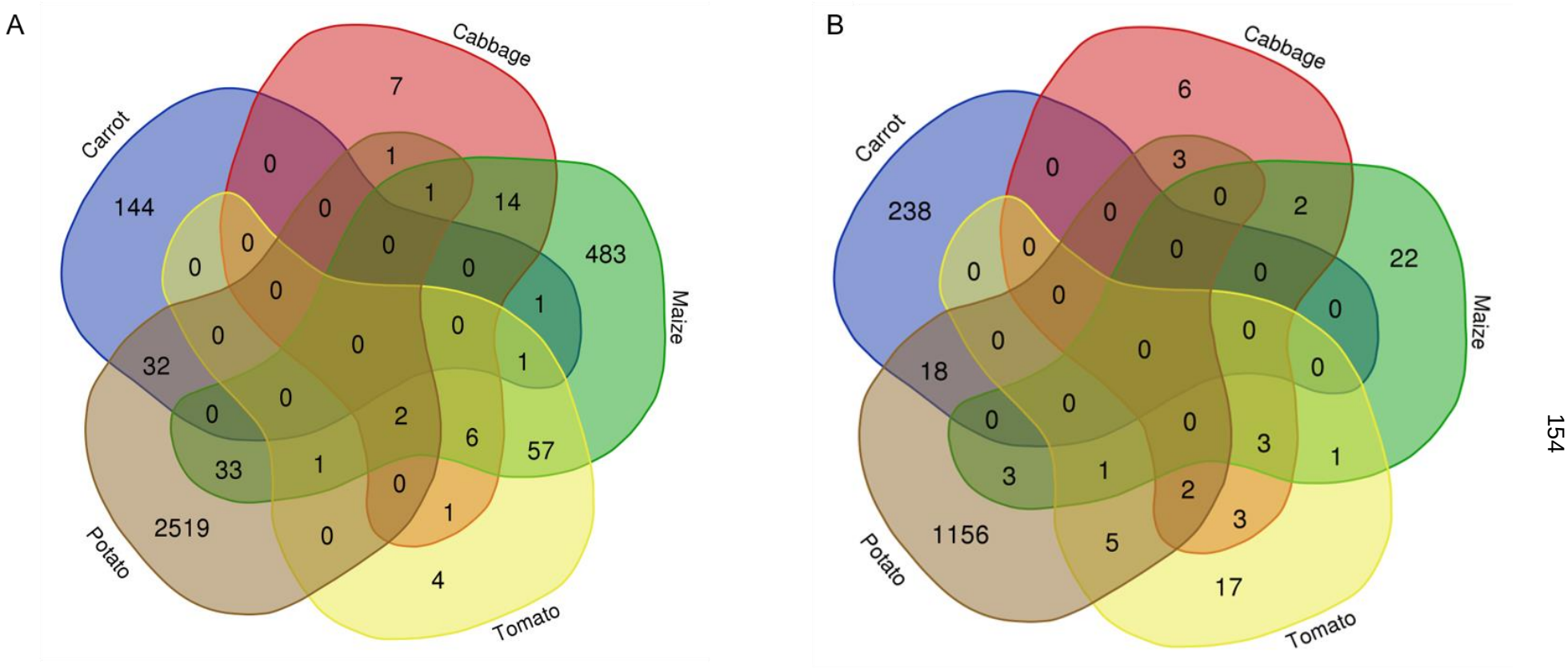

Figure 4.13- Comparisons of genes up- and down-regulated in *M. incognita* J2s in response to root exudates from cabbage, carrot, maize, potato and tomato plants.

RNA-Seq was performed for *M. incognita* J2s after exposure to either sterile tap water, or cabbage, carrot, maize, potato or tomato root exudate. Differential gene expression analysis revealed the different numbers of up- and down-regulated genes compared to the control. Venn diagrams display numbers of **A) up-regulated** (FDR ≤ 0.05, FC ≥ 1.5) and **B) down-regulated** (FDR ≤ 0.05, FC ≤ -1.5) genes in each root exudate treatment and indicate common up- or down-regulated genes across root exudate treatment groups compared to the control.

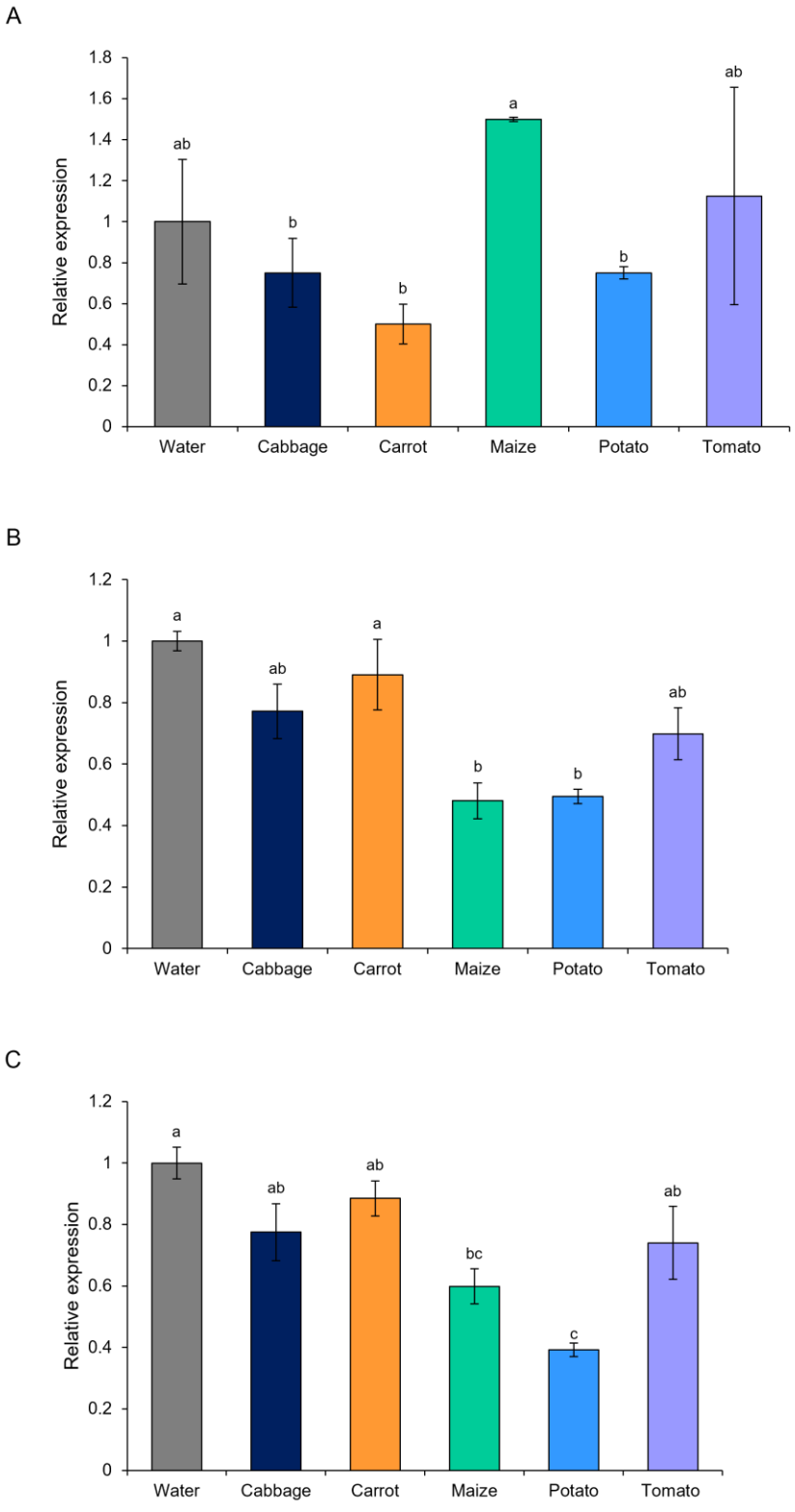

Figure 4.14- Differential gene expression of previously identified and cloned cellulase genes in *M. incognita.*

RNA-Seq was performed for *M. incognita* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate. Relative gene expression of **A) *Mi-eng-1*** **B) *Mi-eng-2*** and **C) *Mi-eng-4*** was calculated using counts per million (CPM) values derived from the RNA-Seq data. CPM represents the number of sequence reads mapped to a specific gene normalised to the total number of reads in the dataset. Mean expression of 5 (or 4(tomato and maize)) biological repeats. Error bars are +/- SEM. Letters represent homogeneous subsets. (One-way ANOVA, Tukey's post hoc test).

4.4.4.3 Enrichment analysis of differentially expressed genes in *M. incognita* in response to varying root exudates

Gene Ontology and Enrichment analysis was used to highlight some common biological processes, molecular functions and cellular components that are affected transcriptionally by root exudate exposure. This analysis specifically focussed on the genes that were up-regulated. Notably, significant enrichment of a number of GO terms in all three categories were observed among the genes that exhibited increased expression in response to maize root exudate (Figure 4.15). Interesting GO terms included those relating to GPCR signalling pathways in the biological processes category (Figure 4.15). Significantly enriched GO terms in all three categories were also observed among the genes up-regulated in response to potato root exudate (Figures 4.16 and 4.17). Conversely, there were no significantly enriched GO terms in the cellular component category among the up-regulated genes in *M. incognita* in response to tomato root exudate. There were however significantly enriched GO terms among the up-regulated genes in the biological processes and molecular functions categories, all of which were found to be over-represented in comparison to the genomic baseline (Figure 4.18). In contrast, none of the categories exhibited significant enrichment of GO terms among the up-regulated genes in *M. incognita* when exposed to either cabbage or carrot root exudate.

There was little overlap in the significantly under- and over-represented GO terms among the up-regulated genes in *M. incognita* in response to tomato, potato and maize root exudate treatments (Figure 4.19). All of the over-represented GO terms represented by the up-regulated genes in *M. incognita* in response to potato root exudate exposure were unique (Figure 4.19A). Six common over-represented GO terms (water transport, cysteine biosynthetic process from serine, water channel activity, cysteine metabolic process, (S)-2-hydroxy-acid oxidase activity, 2-hydroxyglutarate dehydrogenase activity) were seen in the significantly over-expressed GO terms represented by the up-regulated genes in response to both maize and tomato root exudates. Seven GO terms, including: DNA polymerase complex, protein transport, DNA-directed DNA polymerase activity, ribonucleoprotein complex, nuclear protein-containing complex, intracellular organelle lumen, and DNA biosynthetic process, were also commonly under-represented in the genes up-regulated in response to both maize and potato root exudates.

It is noteworthy that GO terms associated with zinc-related stress responses exhibit significant over-representation specifically among the genes that are up-regulated in response to potato root exudate (Figure 4.16). Importantly, these GO terms did not exhibit significant enrichment in response to any of the other root exudate treatments. In addition, apyrases are known to be involved in the stress responses of

C. elegans (Uccelletti *et al*., 2008) and among the most highly up-regulated genes in *M. incognita* upon exposure to potato root exudate, is an apyrase encoding gene (Table 4.13). Genes associated with the stress response, such as this, appear to exhibit specific activation in response to potato root exudate.

As with *G. pallida,* the initial focus of this study was directed towards cell wall-degrading enzymes but these, and other effector encoding genes are unlikely to be highlighted in the gene enrichment analysis, as there are no specific GO terms directly associated with effector proteins. However, manual searches revealed numerous genes up-regulated in *M. incognita* in response to the various root exudates that were annotated as potential effectors. These included a variety of proteins including cell wall-degrading enzymes, predicted secretory proteins, and proteins localised to the gland cells. Specifically, upon exposure to cabbage root exudate, 8 of the 32 up-regulated genes were identified as potential effectors. Similarly, 7 likely effectors were recognised among the 178 up-regulated genes in response to carrot root exudate, with 3 of these (42.9 %) considered cell wall-degrading enzymes. In contrast, maize root exudate triggered the up-regulation of 599 genes in *M. incognita,* with 29 of them identified as probable effectors. 11 (37.9 %) of these were probable cell wall-degrading enzymes. Furthermore, tomato root exudate induced the up-regulation of 72 genes, of which 2 were deemed probable effectors. Lastly, potato root exudate exposure resulted in the up-regulation of 2,589 genes, among which, 45 were recognised as potential effectors, with 17 (37.8 %) being categorised as cell wall-degrading enzymes.

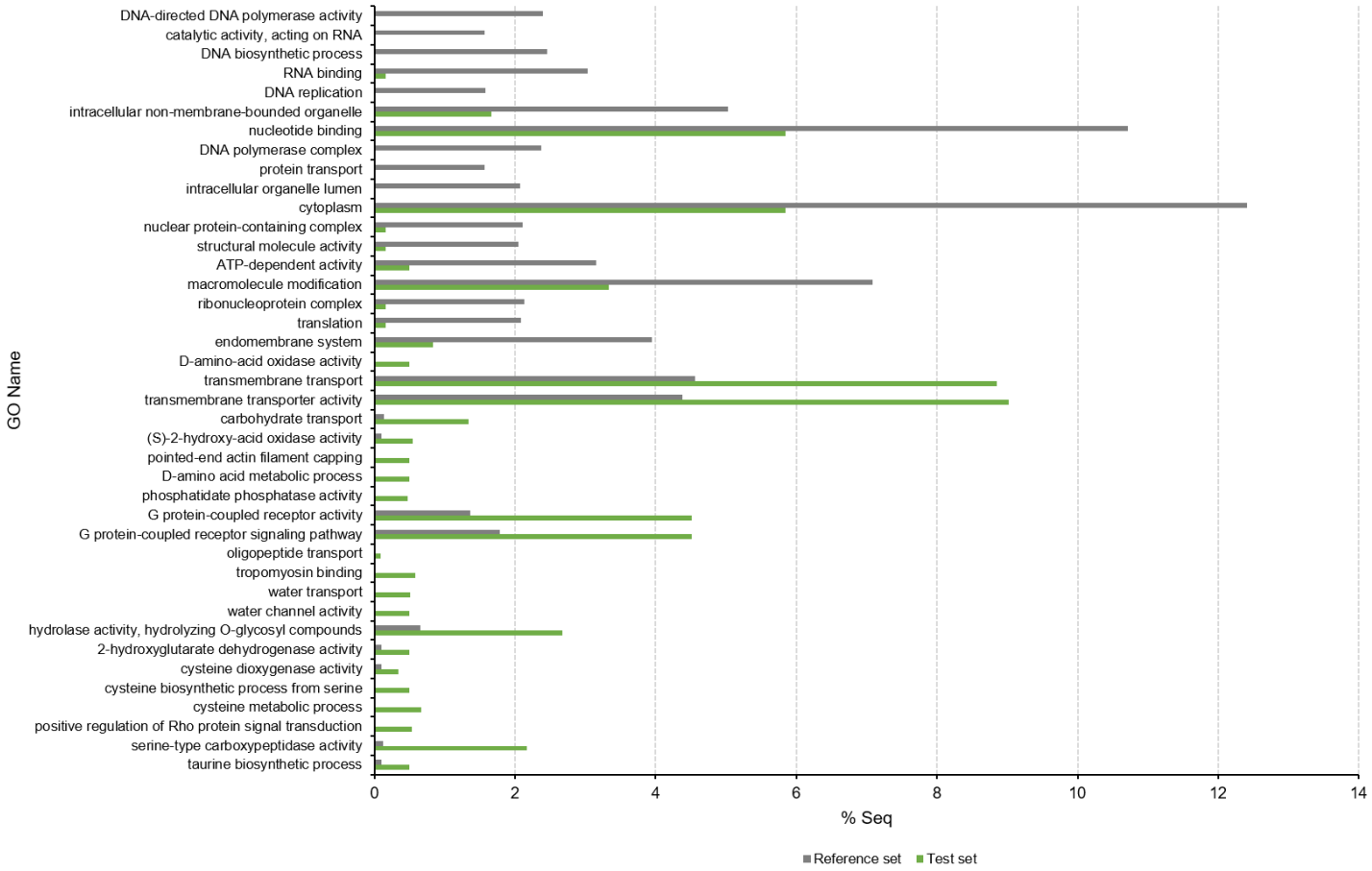

Figure 4.15- Gene ontology (GO) identifiers in genes up-regulated in *M. incognita* in response to maize root exudate exposure.

Over and under-represented gene ontology (GO) IDs for biological processes, molecular functions and cellular components, reduced to most specific (P ≤ 0.01), retrieved using Blast2GO, of up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5). Reference set represents the percentage of GO IDs within the genome of *M. incognita.* Test set represents the percentage of GO IDs within the genes up-regulated (FDR ≤ 0.05, FC ≥ 1.5) in response to maize root exudate exposure.

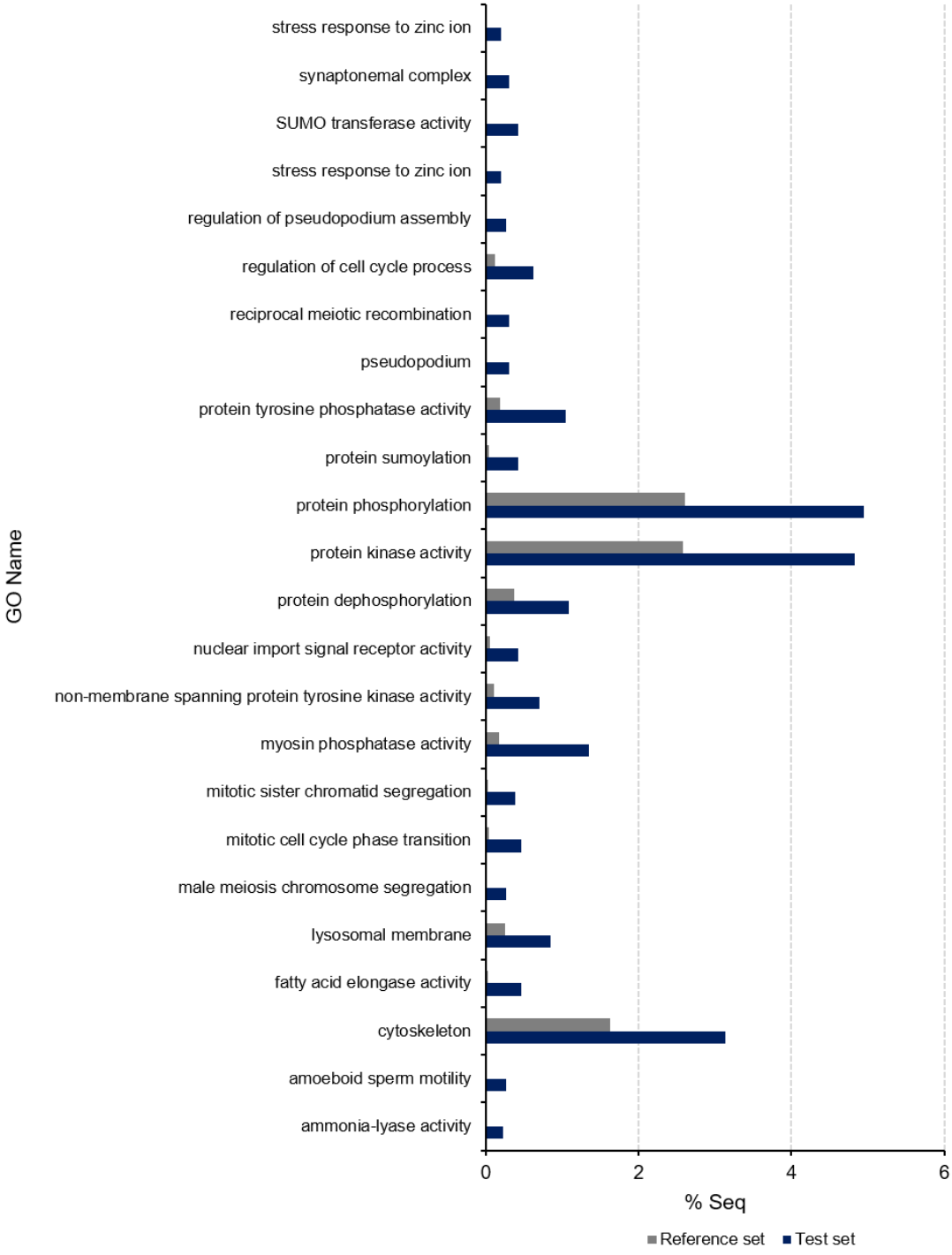

Figure 4.16- Over-represented gene ontology (GO) identifiers in genes up-regulated in *M. incognita* in response to potato root exudate exposure.

Over-represented gene ontology (GO) IDs for biological processes, molecular functions and cellular components, reduced to most specific ($P \leq 0.0001$), retrieved using Blast2GO, of up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5). Reference set represents the percentage of GO IDs within the genome of *M. incognita.* Test set represents the percentage of GO IDs within the genes up-regulated (FDR ≤ 0.05, FC ≥ 1.5) in response to potato root exudate exposure.

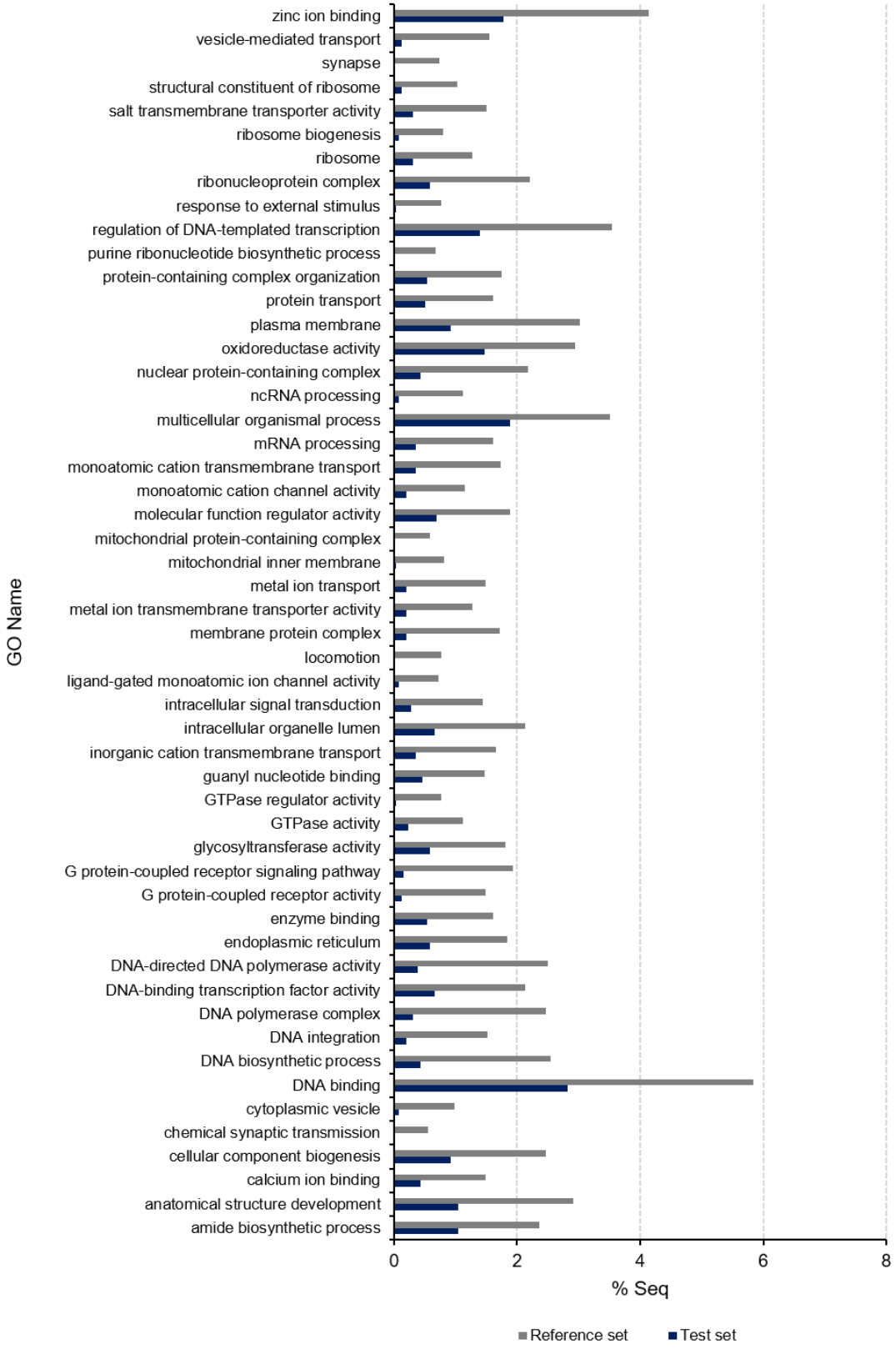

Figure 4.17- Under-represented gene ontology (GO) identifiers in genes up-regulated in *M. incognita* in response to potato root exudate exposure.

Under-represented gene ontology (GO) IDs for biological processes, molecular functions and cellular components, reduced to most specific (P ≤ 0.0001), retrieved using Blast2GO, of up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5). Reference set represents the percentage of GO IDs within the genome of *M. incognita.* Test set represents the percentage of GO IDs within the genes up-regulated (FDR ≤ 0.05, FC ≥ 1.5) in response to potato root exudate exposure.

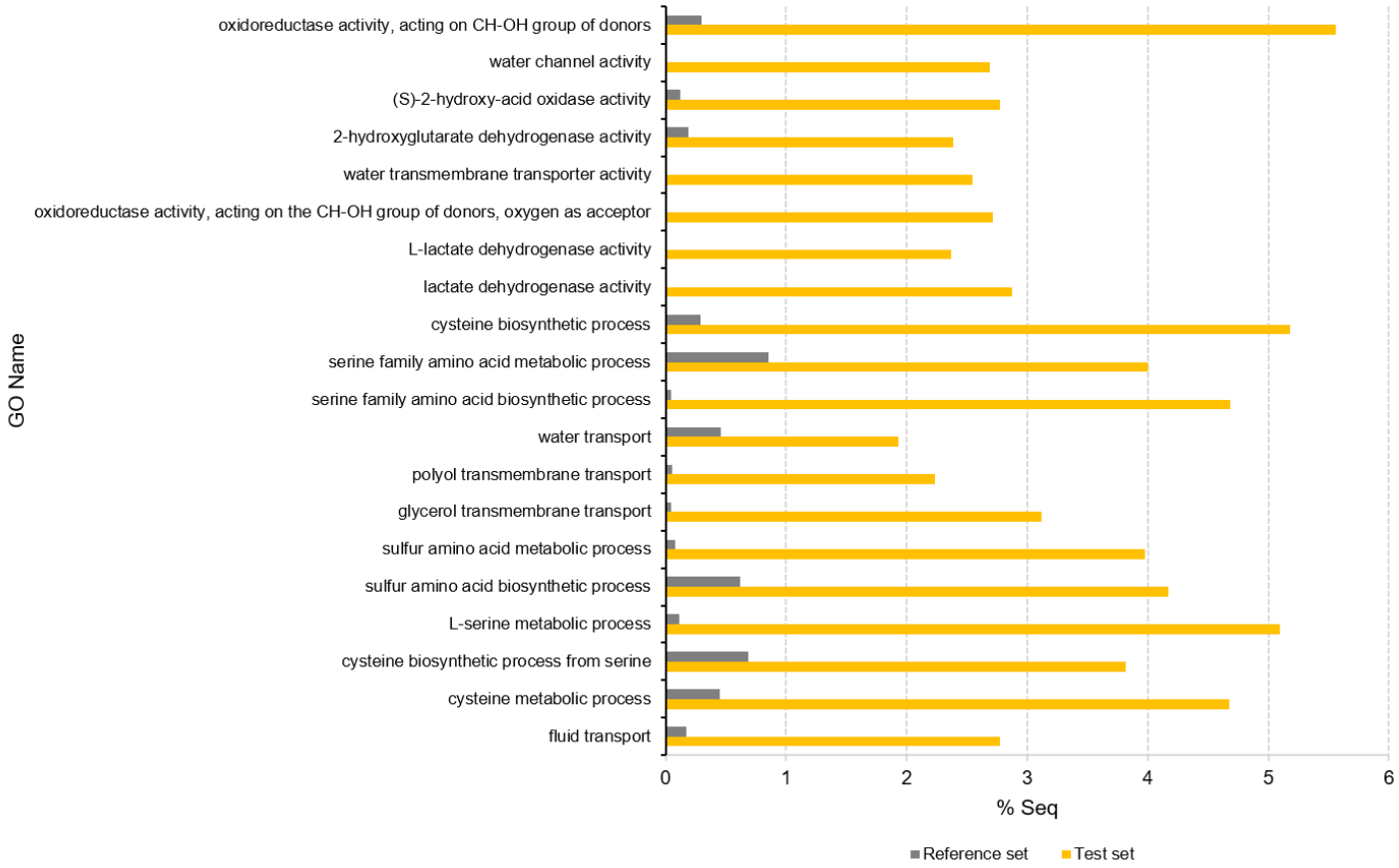

Figure 4.18- Gene ontology (GO) identifiers in genes up-regulated in *M. incognita* in response to tomato root exudate exposure.

Under-represented gene ontology (GO) IDs for biological processes and molecular functions, retrieved using Blast2GO, of up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5). Reference set represents the percentage of GO IDs within the genome of *M. incognita.* Test set represents the percentage of GO IDs within the genes up-regulated (FDR ≤ 0.05, FC ≥ 1.5) in response to tomato root exudate exposure.

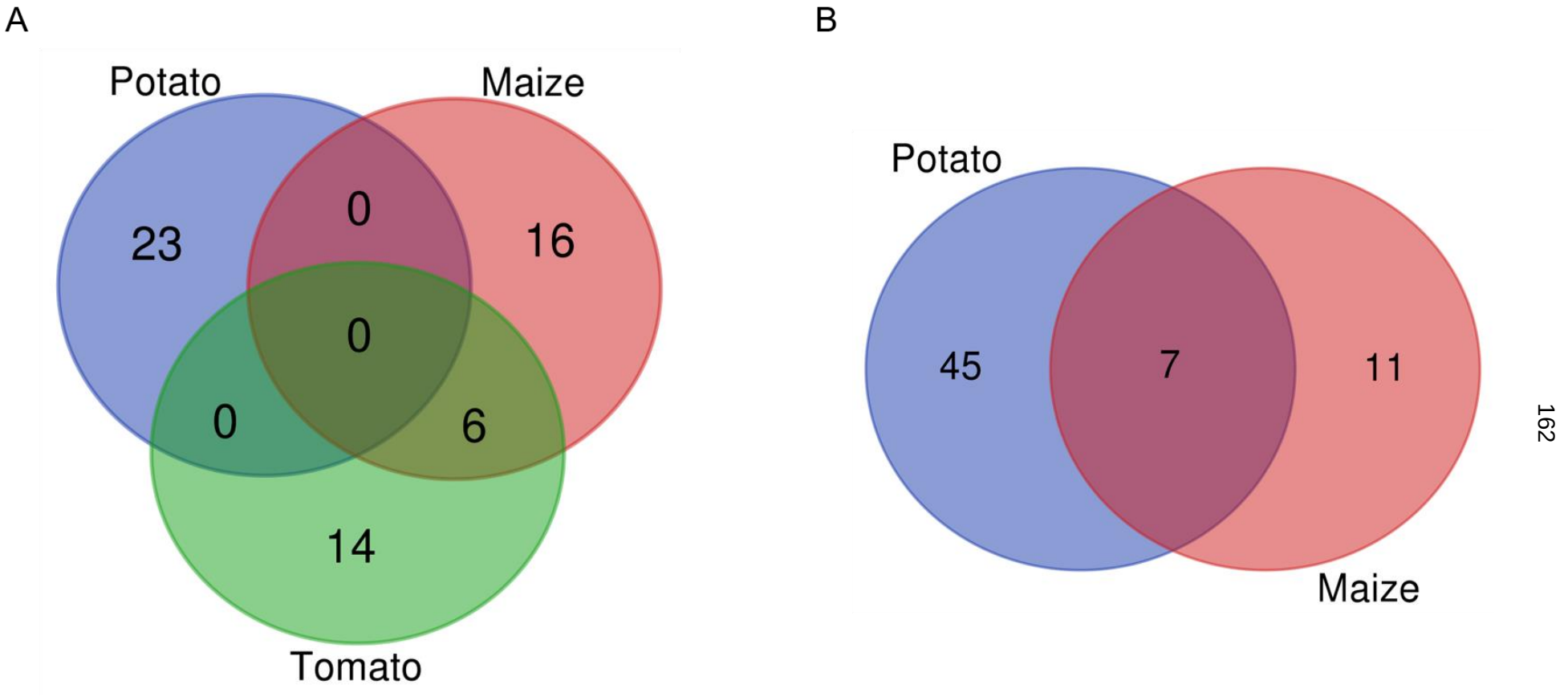

Figure 4.19- Comparisons of over- and under-represented GO terms represented by up-regulated genes in *M. incognita* J2s in response to root exudates.

RNA-Seq was performed for *M. incognita* J2s after exposure to either sterile tap water, or cabbage, carrot, maize, potato or tomato root exudate. Gene Ontology and Enrichment analysis revealed over-and under-represented GO terms represented by the up-regulated genes in each root exudate treatment (Fisher's exact test (FDR ≤ 0.05)). Venn diagrams display numbers of **A) over-represented** (FDR ≤ 0.05) and **B) under-represented** (FDR ≤ 0.05) GO terms in each root exudate treatment compared to the control. No GO terms were significantly enriched in response to cabbage or carrot root exudates.

4.4.5 Differential gene expression in *R. similis* in response to varying root exudates

RNA-Seq data for *R. similis,* exposed to either sterile tap water, coffee, maize or potato root exudate were available, but had not been previously analysed. Given the partial overlap in the previous assessment of root exudates with *G. pallida* and *M. incognita,* this dataset was briefly analysed as before to provide further insights into the transcriptional responses of plant parasitic nematodes to host-derived cues. Analysing transcription data from *R. similis* offers insights into whether or not the differences observed in the responses of *G. pallida* and *M. incognita* are stem from differences in host range, attributed to *R. similis'* broad host range, or if they are evolutionarily determined, influenced by the phylogenetic proximity of *R. similis* and *G. pallida.*

Differential gene expression analysis confirmed that this nematode also responds to host detection with transcriptional alterations (Figure 4.20). Many genes in *R. similis* exhibited significant up- or down-regulation (FC ≥ 1.5 or ≤ -1.5) in response to each root exudate treatment compared to the control (Figure 4.20). In line with the findings in *G. pallida* and *M. incognita,* potato root exudates induced the most pronounced transcriptional response in *R. similis* (Figure 4.21). A total of 781 genes showed differential expression upon exposure to this root exudate, with 366 genes being up-regulated, and 415 genes being down-regulated (Figure 4.21). The 20 genes with the most substantial fold changes are detailed in Tables 4.23 and 4.24. Noteworthy up-regulated genes include the RBP-1 protein (G1952.t1) (Table 4.23) which is an orthologue of a known effector in *G. pallida* (Carpentier *et al*., 2012). In contrast, maize root exudate induced differential expression of 126 genes in *R. similis* (Figure 4.21), with 80 genes being up-regulated and 46 genes down-regulated. The genes displaying the largest fold changes in response to this root exudate are provided in Tables 4.25 and 4.26. Of note is a pectate lyase gene (G10324.t1) within the list of the top 20 down-regulated genes in response to this exudate (Table 4.26). Interestingly, pectate lyase genes was also observed within the top 20 down-regulated genes in *M. incognita* when exposed to maize and cabbage root exudates. Furthermore, exposure to coffee root exudate resulted in the differential expression of a total of 202 genes in *R. similis* (Figure 4.21). 101 of these genes were up-regulated and the remaining 101 were down-regulated. The top 20 genes with the most prominent fold changes can be found in Tables 4.27 and 4.28.

As observed in *G. pallida* and *M. incognita,* the transcriptional changes in *R. similis* in response to various host root exudates appeared to be host-specific. Each of the potato, maize and coffee root exudates induced a distinct transcriptional response in this nematode, with minimal overlap in the differentially expressed genes among treatment

groups (Figure 4.20). Notable similarities are seen in the responses of all three nematode species to root exudate exposure. Specifically, the up-regulation of genes encoding GPCRs is common.

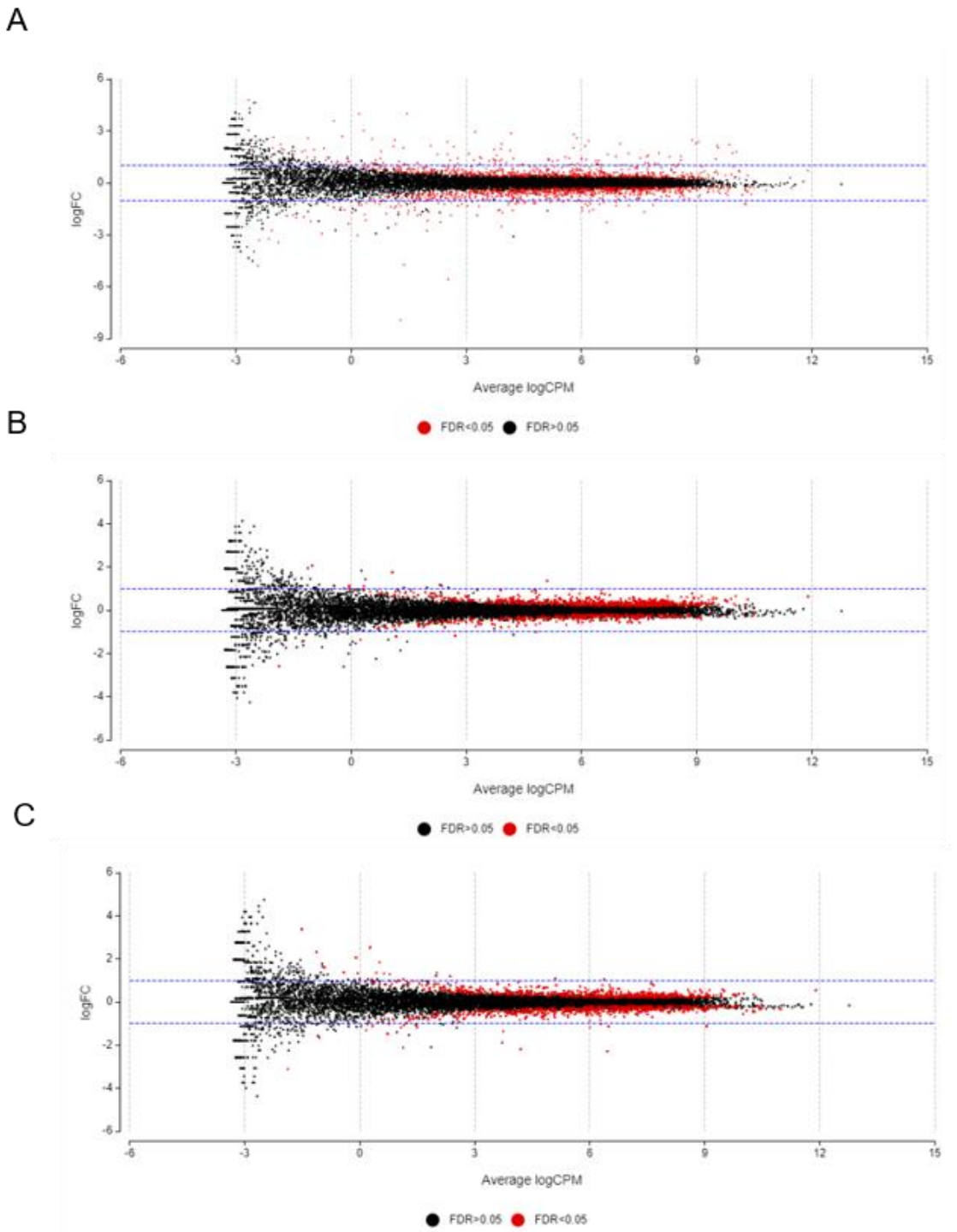

Figure 4.20- Differentially expressed genes in *R. similis* in response to varying root exudates.

A) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *R. similis* exposed to **potato root exudate** and *R. similis* exposed to the water control.

B) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *R. similis* exposed to **maize root exudate** and *R. similis* exposed to the water control.

C) Log fold change (logFC) and average expression (Log counts per million (LogCPM)) of genes between *R. similis* exposed to **coffee root exudate** and *R. similis* exposed to the water control. Significantly differentially regulated genes are coloured red.

Those that are above 0 are up-regulated and those that are below 0 are down-regulated. Dotted blue lines indicate the implemented fold change (FC) cut offs.

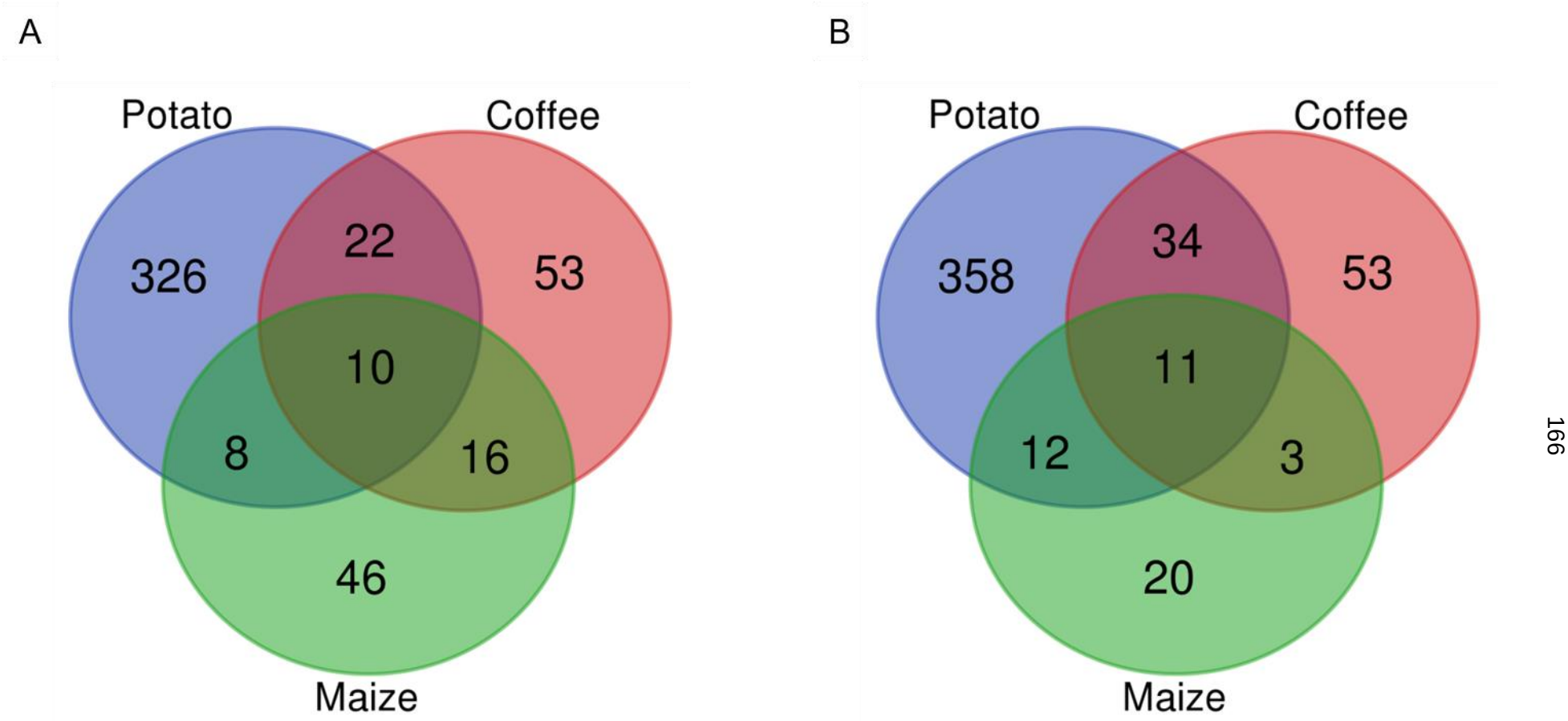

Figure 4.21- Comparisons of genes up- and down-regulated in *R. similis* in response to root exudates from potato, maize and coffee plants.

RNA-Seq data for *R. similis* after exposure to either sterile tap water, or potato, maize or coffee root exudate was obtained, and differential gene expression analysis revealed the different numbers of up- and down-regulated genes compared to the control. Venn diagrams display numbers of **A) up-regulated** (FDR ≤ 0.05, FC ≥ 1.5) and **B) down-regulated** (FDR ≤ 0.05, FC ≤ -1.5) genes in each root exudate treatment and indicate common up- or down-regulated genes across root exudate treatment groups compared to the control.

Gene ID	Fold change (FC)	Description
G8824.t1	27.47	Unnamed protein
G11804.t1	24.72	Low-density lipoprotein receptor domain class A domain-containing protein
G11572.t1	24.58	Unnamed protein
G9091.t1	19.69	Papain family cysteine protease
G1679.t1	16.47	Leishmanolysin domain-containing protein
G11793.t1	16.46	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein
G10418.t1	16.01	Unnamed protein
G8422.t1	15.89	Transmembrane amino acid transporter protein
G14653.t1	15.82	Unnamed protein
G4104.t1	11.88	Acyl-protein thioesterase 1
G4798.t1	8.21	Unnamed protein
G4105.t1	8.05	Acyl-protein thioesterase 1
G9178.t1	7.66	Unnamed protein
G4453.t1	7.23	Unnamed protein
G7903.t1	6.86	HAD hydrolase, family IA, variant 3
G9831.t1	6.46	Unnamed protein
G14851.t1	6.20	Unnamed protein
G5097.t1	6.05	Unnamed protein
G7548.t1	5.92	Unnamed protein
G1952.t1	5.77	RBP-1 protein

Table 4.23- Up-regulated genes in *R. similis* exposed to potato root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *R. similis* when exposed to potato root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
G752.t1	-244.22	PAN domain-containing protein
G8331.t1	-48.03	Ground region domain containing protein
G14006.t4	-27.61	Unnamed protein
G6605.t1	-26.57	Unnamed protein product
G574.t1	-22.81	CRAL/TRIO domain-containing protein
G1085.t1	-20.29	Putative V-type proton atpase 116 kda subunit a
G3486.t1	-15.54	Bacterial transferase hexapeptide (six repeats) domain-containing protein
G8333.t1	-10.95	Hypothetical protein Mgra_00005074
G856.t1	-10.03	Microtubule associated protein (MAP65/ASE1 family) domain-containing protein
G7309.t1	-9.42	Protein roadkill
G6011.t1	-8.29	Unnamed protein
G9476.t1	-7.63	Cytochrome p450 domain-containing protein
G4654.t1	-6.83	Unnamed protein
G1618.t1	-6.79	Kunitz/Bovine pancreatic trypsin inhibitor domain-containing protein
G12455.t1	-6.72	ZP domain-containing protein
G2338.t1	-5.76	Carboxylic ester hydrolase
G11056.t1	-5.24	C6 domain-containing protein
G9051.t1	-5.12	Unnamed protein product
G8334.t1	-4.85	Ground-like domain-containing protein
G15346.t1	-4.80	Pept_C1 domain-containing protein

Table 4.24- Down-regulated genes in *R. similis* exposed to potato root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *R. similis* when exposed to potato root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
g10714.t1	16.3116	DH domain-containing protein
g8802.t1	6.729979	KH domain-containing protein
g13018.t1	4.157768	Receptor protein serine/threonine kinase
g7548.t1	3.832817	Na(+)/H(+) exchange regulatory cofactor NHE-RF2
g7875.t1	3.376413	zinc finger, c4 type (two domains) domain-containing protein
g4725.t1	2.771233	Na(+)/H(+) exchange regulatory cofactor NHE-RF1
g9997.t1	2.722611	hypothetical protein DdX_02324
g14027.t1	2.562731	P-type Cu(+) transporter
g7270.t1	2.194109	ATP synthase assembly factor FMC1 domain containing protein
g10822.t2	2.178862	Protein C29E4.12
g1299.t1	2.161233	rasGEF domain-containing protein
g15068.t2	2.152904	Sodium/calcium exchanger 2
g7691.t1	2.006196	coiled-coil domain-containing protein MTMR15
g10743.t1	1.963237	Fanconi-associated nuclease
g7085.t1	1.937452	MFS domain-containing protein
g15648.t1	1.936405	integrator complex subunit 2 domain-containing protein
g4937.t1	1.923333	Iron sulphur-containing domain, CDGSH-type, subfamily and Iron sulphur-containing domain, CDGSH-type-containing protein
g896.t2	1.916708	60S ribosomal protein L30
g865.t1	1.890435	beta-galactoside-binding lectin
g7033.t1	1.889506	Unnamed protein

Table 4.25- Up-regulated genes in *R. similis* exposed to maize root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *R. similis* when exposed to maize root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
G7044.t1	-17.97	Unnamed protein
G13490.t1	-5.99	Unnamed protein
G856.t1	-4.59	Carboxylic ester hydrolase
G14865.t2	-2.66	Unnamed protein product
G11139.t1	-2.58	Unnamed protein
G5391.t1	-2.31	Unnamed protein
G7023.t1	-2.29	Unnamed protein
G789.t1	-2.27	Unnamed protein
G15619.t1	-2.12	Unnamed protein
G3530.t1	-2.01	Unnamed protein
G13694.t1	-2.00	Unnamed protein
G5969.t1	-1.92	Esophageal gland-localized secretory protein 12
G4834.t1	-1.82	Hypothetical protein GPALN_003117
G356.t1	-1.82	Hypothetical protein GPALN_010142
G13460.t1	-1.80	Unnamed protein
G7975.t1	-1.77	Unnamed protein
G6598.t1	-1.74	Zinc finger, c4 type (two domains) domain-containing protein
G12080.t1	-1.73	Putative glutathione S-transferase 5
G10324.t1	-1.72	Pectate lyase 1
G1959.t1	-1.71	Unnamed protein

Table 4.26- Down-regulated genes in *R. similis* exposed to maize root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *R. similis* when exposed to maize root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
G11804.t1	26.04	Low-density lipoprotein receptor domain class A domain-containing protein
G9091.t1	21.25	Papain family cysteine protease
G11793.t1	17.88	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein
G825.t1	17.75	Unnamed protein
G11766.t2	17.74	Unnamed protein
G7353.t1	10.32	Collagen triple helix repeat protein
G6834.t2	9.03	Diacylglycerol kinase catalytic domain-containing protein
G14141.t1	5.77	Unnamed protein
G7548.t1	4.99	Unnamed protein
G9707.t1	4.16	Protein Skeletor, isoforms D/E
G5675.t1	3.89	Calcium binding EGF domain protein
G8879.t1	3.55	Unnamed protein
G6014.t1	3.12	Serpentine type 7TM GPCR chemoreceptor srh domain-containing protein
G4811.t1	3.02	Unnamed protein product
G7668.t1	2.58	BPTI/Kunitz inhibitor domain-containing protein
G14566.t1	2.56	Calcium-dependent protein kinase 1
G6253.t1	2.55	Surface-associated antigen 2
G7180.t1	2.50	Unnamed protein
G601.t1	2.49	Unnamed protein
G2039.t1	2.42	Glycosyl hydrolases family 31 domain-containing protein

Table 4.27- Up-regulated genes in *R. similis* exposed to coffee root exudate.

Top 20 up-regulated genes (FDR ≤ 0.05, FC ≥ 1.5) by fold change in *R. similis* when exposed to coffee root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

Gene ID	Fold change (FC)	Description
G5457.t1	-20.30	Unnamed protein
G7310.t1	-8.61	Speckle-type poz protein
G856.t1	-6.01	Carboxylic ester hydrolase
G15596.t1	-4.90	3-phosphoinositide dependent protein kinase-1
G8141.t1	-4.75	Hypothetical protein Mgra_00009956
G6077.t1	-4.59	Unnamed protein
G7638.t1	-4.35	Serpentine type 7TM GPCR chemoreceptor srx domain-containing protein
G299.t1	-3.69	Unnamed protein
G1661.t1	-3.15	Unnamed protein product
G271.t1	-2.79	Unnamed protein product
G1678.t1	-2.58	SHSP domain-containing protein
G6011.t1	-2.47	Unnamed protein
G14155.t1	-2.27	Hypothetical protein AAVH_12829
G12123.t1	-2.26	Unnamed protein
G12315.t1	-2.26	Unnamed protein
G5380.t1	-2.20	Haloacid dehalogenase-like hydrolase domain-containing protein
G1016.t1	-2.18	Cytochrome p450 domain-containing protein
G7014.t1	-2.16	Unnamed protein product
G14214.t1	-2.12	Serpentine type 7TM GPCR receptor class ab chemoreceptor domain-containing protein
G10958.t1	-2.08	Unnamed protein

Table 4.28- Down-regulated genes in *R. similis* exposed to coffee root exudate.

Top 20 down-regulated genes (FDR ≤ 0.05, FC ≤ -1.5) by fold change in *R. similis* when exposed to coffee root exudate compared to when exposed to a water control. Descriptions of genes are derived from Blast2GO descriptions. Differential expression analysis was performed using the Pairwise Differential Gene Expression Analysis tool on Omics Box v.2.2.4 which compares normalised gene counts for each gene and tests for statistical significance using a Fisher's exact test.

4.4.6 Selection of genes of interest for further analysis in *G. pallida* and *M. incognita*

Although there were a considerable number of differentially regulated genes in *G. pallida* and *M. incognita* J2s following exposure to different root exudate treatments, only a limited subset were chosen for further analysis (Tables 4.29-4.30). The selection criteria for these genes were based on their assumed function, fold change magnitude, common up-regulation, and the potential significance of further investigation. Whilst genes exhibiting down-regulation in response to root exudate treatments were of interest, they were not considered for further analysis at this time.

Gene	Description	Exudate inducing up-regulation	Reason for choosing
GPLIN_001463700	Cell wall-associated hydrolase	Carrot, maize and potato	Highly up-regulated in response to potato (FC 10.8). Cell wall-degrading enzyme- likely to be involved in host penetration.
GPLIN_001116500	Cell wall-associated hydrolase	Maize and potato	Biggest FC in response to potato (FC 348.81).
GPLIN_000255800	Unnamed protein	Maize and potato	Biggest FC in Maize (FC 60.31). Unnamed- could be novel gene involved in parasitism.
GPLIN_000153100	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein	Potato	Could be involved in cell signalling after root exudate detection.
GPLIN_001116400	Unnamed protein product	Carrot, maize and potato	Up-regulated in response to all three treatments. Highly up-regulated in response to potato (FC 243). Unnamed- could be novel gene involved in parasitism.
GPLIN_000936300	Glutathione synthetase	Potato	Could be interesting to investigate potential role as effector.

Table 4.29- Genes of interest for further investigation in *G. pallida.*

RNA-Seq was performed on total RNA extracted from *G. pallida* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate for four hours. Differential gene expression analysis was carried out on Omics Box v.2.2.4 which compared normalised gene counts for each gene (in each condition) and tests for statistical significance using a Fisher's exact test. Up-regulated genes listed here were chosen for further investigation and the reasons for choosing each are stated.

GPLIN_000969900	Secretory protein 4D06	Potato	4D06 is a known effector family in *Heterodera* species.
GPLIN_001634900	Zinc finger protein	Potato	Could be a transcription factor regulating genes involved in parasitism.
GPLIN_000969800	Putative effector protein	Potato	Is a putative effector so it would be interesting to see how it could be involved in parasitism.
GPLIN_001203600	NHR-34 protein	Potato	Many NHRs are implicated in regulating transcription in response to a chemical stimulus and it would be interesting to investigate if this plays a role in the response to root exudate components.

Table 4.29 continued- Genes of interest for further investigation in *G. pallida*.

RNA-Seq was performed on total RNA extracted from *G. pallida* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate for four hours. Differential gene expression analysis was carried out on Omics Box v.2.2.4 which compared normalised gene counts for each gene (in each condition) and tests for statistical significance using a Fisher's exact test. Up-regulated genes listed here were chosen for further investigation and the reasons for choosing each are stated.

Gene	Description	Exudate inducing up-regulation	Reason for choosing
Minc3s05958g39089	Peptidase family m13 domain-containing protein	Cabbage, maize, potato, tomato	One of only two genes up-regulated in response to four root exudates. Potential effector - likely to be involved in host penetration.
Minc3s00750g16857	Unnamed protein product	Cabbage, maize, potato, tomato	Second of two genes up-regulated in response to four root exudates. Unnamed- could be novel gene involved in parasitism.
Minc3s04148g35606	Serpentine type 7TM GPCR chemoreceptor srd domain-containing protein	Maize and potato	Could be involved in cell signalling after root exudate detection.
Minc3s07290g40952	Expansin-like protein	Maize	Expansins are known effectors in potato cyst nematodes so this gene is likely to have a similar function in *M. incognita*.
Minc3s00362g11016	Pectate lyase 3	Maize	Could play a role in host penetration as pectate lyases are cell wall-degrading enzymes.
Minc3s06236g39536	Putative avirulence protein precursor	Cabbage, maize and tomato	Avirulence proteins are secreted from gland cells and have been shown to function in planta as ligand-mimicking effectors.

Table 4.30- Genes of interest for further investigation in *M. incognita.*

RNA-Seq was performed on total RNA extracted from *M. incognita* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate for four hours. Differential gene expression analysis was carried out on Omics Box v.2.2.4 which compared normalised gene counts for each gene (in each condition) and tests for statistical significance using a Fisher's exact test. Up-regulated genes listed here were chosen for further investigation and the reasons for choosing each are stated.

Minc3s00218g07814	Tyrosinase and metridin shk toxin domain containing protein	Maize and potato	Could function as an effector as it is secreted.
Minc3s02639g30985	Beta-1,4-endoglucanase	Maize	Beta-1-4-endoglucanase – cell wall-degrading enzyme likely to be involved in host penetration. Over-represented GO term in maize.
Minc3s00009g00641	Astacin (Peptidase family m12a) domain-containing protein	Cabbage, maize and tomato	Astacins are thought to be involved in host-tissue penetration.
Minc3s05895g38985	Putative esophageal gland cell secretory protein 40	Carrot	Could function as an effector as it is thought to be secreted from the gland cells.
Minc3s00015g01000	Candidate secreted effector Minc00331	Carrot	Would be interesting to confirm role as effector.
Minc3s01441g23854	Zinc finger, C2H2 type family protein	Maize and potato	Could be a transcription factor regulating genes involved in parasitism.

Table 4.30 continued- Genes of interest for further investigation in *M. incognita.*

RNA-Seq was performed on total RNA extracted from *M. incognita* J2s after exposure to either sterile tap water, or carrot, maize or potato root exudate for four hours. Differential gene expression analysis was carried out on Omics Box v.2.2.4 which compared normalised gene counts for each gene (in each condition) and tests for statistical significance using a Fisher's exact test. Up-regulated genes listed here were chosen for further investigation and the reasons for choosing each are stated.

4.5 Discussion

4.5.1 Behavioural modifications

Plant parasitic nematodes must sense and respond to root exudates in order to successfully infect and establish within their hosts. An important infection behaviour is stylet thrusting that allows the nematode to physically damage host cells in order to gain entry into the root and form a feeding site.

Previous studies have shown that root exudates from *A. thaliana* stimulate a stylet thrusting response in *M. incognita* (Teillet *et al*., 2013) and the results presented here build on these findings to show that root exudates from other plant species induce the same response in this nematode. In addition, it was found that exposure to root exudate also induces a stylet thrusting response in *G. pallida.* In plant parasitic nematodes, stylet thrusting is thought to be regulated by the neurotransmitter serotonin (Crisford *et al*., 2020). The active compounds in root exudates, of which the precise nature and identity remain unidentified, could potentially be activating serotonin signalling pathways, leading to the changes in behaviour observed.

Given the importance of stylet thrusting in host infection it was notable that the intensity of this behaviour seemed to be affected by root exudate identity in *G. pallida,* with significant variations in stylet thrusting activity being found among different root exudate exposures in this species. Exposure to root exudates from potato and cabbage plants induced a significantly stronger response in nematodes compared to exposure to maize root exudate. These results may be attributed to *G. pallida's* narrow host range and its preference for potato as a host (Moens *et al*., 2018). Potato is known to secrete a range of compounds into the soil, some of which distinguish them from other host plants in terms of root exudate composition. Notably, certain compounds, like solanoeclepin, have been shown to induce hatching in potato cyst nematodes (Shimizu *et al*., 2023). These compounds may also have the potential to induce stylet thrusting in J2s and the isolation and subsequent examination of these compounds could shed light on their impact on stylet thrusting behaviours.

Conversely, although exposure to root exudate significantly increased stylet thrusting activity in *M. incognita* compared to the control, no significant differences were seen in the stylet thrusting response between root exudate exposures. A similar behavioural response to varying root exudates could be due to the broad host range of *M. incognita* and could contribute to its ability to infect multiple different host species.

4.5.2 Differential expression of cellulase genes by qRT-PCR

Following the confirmation of a change in a behaviour that enhances the success of root invasion in both *G. pallida* and *M. incognita* upon exposure to root exudates from varying plants, transcriptional changes that facilitate root invasion were explored.

It has previously been established that both *G. pallida* (Kooliyottil *et al*., 2019, Kud *et al*., 2021) and *M. incognita* (Teillet *et al*., 2013) show transcriptional plasticity in response to host plants. However, there is currently no available data on the specific transcriptional changes in effectors following host detection.

Cell wall-degrading enzymes, such as cellulases, catalyse the breakdown of cell wall components of host cells and their secretion is vital for host infection as they allow the nematodes to successfully enter and migrate through host tissue. *P. coffeae* has previously shown host-dependent gene expression of cell wall-degrading enzymes, which play a vital role in facilitating host entry (Bell *et al*., 2019). It was therefore determined whether or not *G. pallida* and *M. incognita* also differentially express effectors such as these. qRT-PCR analysis confirmed that some selected genes encoding cellulases have differential expression in response to varying root exudates in both *G. pallida* and *M. incognita.* The transcriptional responses seen in these nematodes were shown to be host-specific, with different root exudate exposures conferring unique gene expression profiles of these genes. This mirrors the findings of Bell *et al*. (2019).

Unlike in *P. coffeae,* the expression of putative cellulase genes in *M. incognita* did not correlate with the concentration of their substrate. This was surprising as *P. coffeae* and *M. incognita* share a relatively broad host range (Silva and Inomoto, 2002). The differences in observed gene expression could, however, reflect the species' specific adaptations to their different ecological niches. *P. coffeae* and *M. incognita* have different lifestyles and have therefore evolved different strategies of host infection and nutrient acquisition. The regulation of cellulase genes in *M. incognita* might be influenced by factors other than substrate concentration, for example host-specific factors, or other environmental cues.

The qRT-PCR analysis of putative cellulase gene expression in *G. pallida* revealed that the expression of GPLIN_000313600 was significantly higher in response to potato root exudates compared to both exposure to and maize or carrot root exudates and the water control. In contrast, expression of the other tested cellulase gene in *G. pallida* (GPLIN_000536400) was not responsive to root exudate treatment. Similar to the stylet thrusting response, the transcriptional changes observed for GPLIN_000313600 and GPLIN_001111200 are probably also attributed to *G. pallida's* preference for potato as a host (Moens *et al*., 2018). It is logical for this nematode to exhibit a stronger response

towards a more suitable host, as this increases its likelihood of survival. On the other hand, results in Chapter 3 revealed that GPLIN_000536400 is most highly expressed in adult males. This suggests that the enzyme encoded by this gene might play a role later in the life-cycle, potentially aiding the exit of males from the root. Consequently, the absence of significant expression differences in response to root exudates for this gene could be attributed to it not being as heavily involved in the infection process as GPLIN_000313600, or other putative effectors. It would be interesting in the future to test if exposure to root exudate or root extract also induces expression of some cell wall-degrading enzyme genes in male nematodes, or if those genes are solely developmentally regulated.

4.5.3 Comprehensive gene expression analysis

The initial findings revealing host-specific differential regulation of cellulase encoding genes in *G. pallida* and *M. incognita* prompted the expansion of the gene expression analysis. Through RNA-Seq, a comprehensive dataset of genes differentially expressed in response to varying root exudates was generated, providing valuable insights into the molecular strategies employed by these nematodes to infect their hosts.

Exposure to potato root exudate had a very strong and distinctive effect on gene expression in *G. pallida,* while the exposure of this nematode to carrot and maize root exudates had comparably weaker effects. In contrast, exposure to cabbage, carrot, maize, potato and tomato root exudates were all found to have distinctive effects on gene expression in *M. incognita.* Venn diagrams emphasised the limited overlap among genes exhibiting significant up- or down-regulation in response to these root exudate treatments, confirming the uniqueness and specificity of the differential gene expression associated with each respective root exudate. These findings strengthen the previous conclusions drawn from the qRT-PCR analysis that gene expression changes in response to root exudate exposure in *M. incognita* and *G. pallida* is host-specific.

4.5.3.1 *G. pallida* responds only to its preferred host

There were notable differences in numbers of genes that are differentially expressed in *G. pallida* J2 nematodes when exposed to root exudate from their preferred host, potato, compared to when exposed to other root exudates. This suggests that this nematode is able to distinguish hosts from non-hosts according to root exudate composition which varies between plant species (Barber and Martin, 1976, Bertin *et al*., 2003). Only four and 12 genes were differentially expressed in response to carrot and maize root exudates, respectively, compared to over 1,400 in response to potato. The minimal reaction to non-hosts suggests that the transcriptional responses are host-specific. These could

confer an evolutionary advantage for these nematodes, as expending energy and resources in attempts to infect non-hosts could be detrimental to their survival.

Interestingly, within the substantial number of genes that were differentially regulated in *G. pallida* in response to potato root exudate, gene enrichment analysis highlighted no significantly over-represented GO terms. This highlights the fact that many plant parasitic nematode-specific genes that are involved in the parasitic interaction are not annotated, have no standard protein domains and/or encode novel proteins of unknown function. Manual searches for genes likely to encode cell wall-degrading enzymes and other effectors identified 65 genes likely to belong to this category among the genes up-regulated in response to potato root exudate, however, this number is likely to be under-estimated due to the large number of genes that remain unannotated. This highlights the need for the characterisation of effector proteins in plant parasitic nematodes.

4.5.3.2 *M. incognita* responds to a variety of hosts

Differences in the numbers of differentially expressed genes were also seen when comparing *M. incognita* exposed to various root exudates with the control group. The intensity of the transcriptional response is somewhat comparable between cabbage, carrot, maize and tomato root exudate treatments, but the complement of genes was unique to each one. Overall, there was very little overlap in the genes that were up- or down-regulated in response to each root exudate. It was hypothesised that, like *G. pallida, M. incognita* might also react most strongly to root exudates from its most favourable host, tomato (Sikora and Fernández, 2005). However, this was not the case and tomato root exudate had a relatively weak effect, causing the differential expression of only 104 genes. In contrast, the differential expression in response to potato root exudate exceeded 3,000 genes, whilst maize and carrot root exudates caused differential expression of 823 and 435 genes, respectively. It is, however, worth mentioning that all of the *M. incognita* J2s used in this experiment were obtained from tomato roots. Despite being washed in tap water for at least 24 hours before exposure to root exudate, it is plausible that transcriptional responses to the culture plant persisted, thereby minimising the effects of the re-exposure to tomato root exudate. If nematodes had been cultured on an independent plant, the results may have been different.

Similar to the case in *G. pallida,* a subset of genes that exhibited up-regulation in response to root exudate exposure appeared to encode likely effectors, including cell wall-degrading enzymes. Due to the absence of dedicated GO terms, gene enrichment analysis did not highlight these. Instead, manual searches were employed to identify these specific genes. Again, the resultant figures are prone to under-estimation.

In addition to this challenge, many genes in *G. pallida* and *M. incognita* remain unannotated, thus rendering their functions unknown. This lack of comprehensive annotation made it difficult to gain a clear understanding of the genuine molecular responses elicited by root exudate exposure. While the anticipated up-regulation of genes encoding cell wall-degrading enzymes and other effectors was substantiated by this work, the inability to discern the functional roles of many of the other genes, prevented a broader comprehension of the nematode responses. This further highlights the need for the functional characterisation of a broader array of genes within these nematode species to provide meaningful annotations.

4.5.4 Comparing responses to those of *R. similis*

RNA-Seq data obtained from the burrowing nematode, *R. similis* exposed to coffee, maize and potato root exudates was also analysed as a further comparison. As a migratory endoparasite, this nematode shares the same lifestyle as *P. coffeae*. Similar to root-knot nematodes, *R. similis* exhibits a broad host range which extends to more than 365 different species of plant (Moens and Perry, 2009). However, in terms of phylogeny, it is most closely related to cyst nematodes. These characteristics position *R. similis* as a valuable comparator for interpreting the underlying factors contributing to the observed differences between *G. pallida* and *M. incognita.*

Data analysis revealed that like *M. incognita,* this nematode also showed a varied response to each root exudate treatment with potato having the strongest influence on transcription. This suggests that *R. similis* also displays host-specific gene expression. For all three of these nematode species, the impact of potato root exudate on gene expression was the greatest. This was a surprising result for *M. incognita* and *R. similis* because potato is not the preferred host of either of these species (Sikora and Fernández, 2005, Sarah, 1996).

The comparable response of *R. similis* to *M. incognita* suggests that the mode of parasitism does not significantly influence the transcriptional responses of plant-parasitic nematodes to host signals. Furthermore, the differences observed between the responses of *R. similis* and *G. pallida* imply that transcriptional responses are not solely dictated by evolutionary factors. These findings indicate that differences in host range likely play a pivotal role in driving the observed variances in transcriptional responses seen between *G. pallida* and *M. incognita.*

4.5.5 Up-regulation of genes involved in nematode stress responses upon exposure to potato root exudate

A number of the genes that were up-regulated in response to potato root exudate in *M. incognita* were found to be associated with stress responses in these nematodes. In addition, genes encoding nuclear hormone receptors (NHRs) were also found to be significantly up-regulated in these nematodes and in *G. pallida* and *R. similis* exclusively in response to potato root exudate. NHRs are transcription factors that are often implicated in the stress and detoxification response in *C. elegans* (Guerrero *et al*., 2021, Goh *et al*., 2018, Hu *et al*., 2018, Hu, 2007, Jones *et al*., 2013b). It is therefore feasible that they also play an important role in coordinating the stress and detoxification responses across multiple species of plant parasitic nematode.

Interestingly, these apparent stress responses were not observed when nematodes of all three species were exposed to the other types of root exudate. This suggests that potato root exudates may contain unique compounds, capable of inducing stress in plant parasitic nematodes. This is highlighted by the fact that genes up-regulated in *G. pallida* in response to potato root exudate have been found to fall into two main categories: effectors, and those associated with a stress response (Kud *et al*., 2021).

In the root systems of potatoes, secondary metabolites encompass a diverse range of compounds, including glycoalkaloids, calystegine alkaloids, protease inhibitors, lectins, phenolic compounds, and chlorophyll (Friedman, 2006). Notably, nematodes have demonstrated sensitivity to steroidal glycoalkaloids derived from potato roots. While other plants in the *Solanaceae* family may contain glycoalkaloids as well, the specific types and concentrations found in potatoes can differ significantly. Previous research has shown that glycoalkaloids have an influence on plant parasitic nematodes. For example, the detection of potato glycoalkaloids has been specifically linked with hatching induction in *G. rostochiensis* (Ochola *et al*., 2020, Shimizu *et al*., 2020). Conversely, they have been shown to play a role in host-plant resistance. Potato glycoalkaloids such as with solasodine and solamargine have been found to trigger reduced rates of hatching, infection and reproduction in *G. pallida* (Sivasankara Pillai and Dandurand, 2021). The observed stress responses in plant-parasitic nematodes could therefore plausibly stem from their detection of glycoalkaloids present in the potato root exudates to which they were exposed. Alternatively, the up-regulation of genes involved in stress responses observed in *M. incognita, G. pallida* and *R. similis* may be attributed to the nematodes' detection of potato-specific protease inhibitors. Protease inhibitors are known to be involved in the plant's defence mechanism and have been effectively employed in genetically modified plants to enhance their resistance against pests. For example, protease inhibitors from the potato protease inhibitor families, Pin1 and PKPI, that were

expressed in *Nicotiana benthamiana* led to reduced infection of the bacterial pathogen *Pseudomonas syringae* and provided protection against two fungal pathogens (Turrà *et al*., 2020). Similarly, the co-expression of potato type I and type II proteinase inhibitors in cotton plants provided protection against the insect herbivore *Helicoverpa armigera* (Dunse *et al*., 2010). These protease inhibitors may also be present in potato root exudate and could also exert influence on plant parasitic nematodes, potentially triggering the up-regulation of genes involved in the stress response. Future work could focus on identifying the specific compounds responsible for inducing these responses. This could potentially lead to the development of effective control mechanisms.

4.5.6 Cell wall-degrading enzymes

Among the genes up-regulated in both *G. pallida* and *M. incognita* in response to the various root exudate treatments were a considerable number of genes encoding cell wall-degrading enzymes and other effectors. In *M. incognita,* while there was some overlap among these genes, the majority displayed up-regulation exclusively in response to a specific host. The variations in cell wall composition among different plant species, discussed in Chapter 3, could be reflected in the composition of root exudate, potentially explaining the distinct patterns of up- and/or down-regulation observed in genes encoding these cell wall-degrading enzymes in response to root exudates from different plants.

In *G. pallida,* the gene exhibiting the most substantial up-regulation in response to potato root exudate is annotated as a cell wall-associated hydrolase. Notably, this particular gene also ranks among the top 20 up-regulated genes with the most substantial fold changes in *G. pallida* J2s exposed to maize root exudate. In addition, another cell wall-associated hydrolase consistently features within the top 20 up-regulated genes in response to potato, maize and carrot root exudates in this species. The consistent up-regulation of these genes suggests the vital involvement of cell wall-degrading enzymes in *G. pallida's* early infection process. In *M. incognita,* a substantial proportion of the up-regulated effector genes identified in response to various root exudate treatments are annotated as cell wall-degrading enzymes. This observation implies that J2s of this species also rely heavily on this specific class of effector to initiate infection of the root.

Despite the presence of a beta-1,4-endoglucanase precursor gene among the top 20 down-regulated genes in *G. pallida* following exposure to potato root exudate, an examination of RNA-Seq data available on WormBase Parasite has confirmed that this particular gene exhibits its highest expression in adult males, with a minimal expression

observed in J2s. This observation implies that this gene is likely to be employed by male nematodes during their exit from the root, which occurs later in their life-cycle and could provide an explanation as to why it is down-regulated in J2s preparing for infection. Expression profiles of other genes encoding cell wall-modifying proteins in *G. pallida* confirm that they are most highly expressed during the adult male phase of the life-cycle (Thorpe *et al*., 2014). This provides evidence for the notion that a subset of the cell.

wall-degrading enzymes possessed by *G. pallida* are utilised during this life stage.

Furthermore, an array of pectate lyase genes exhibited down-regulation in *M. incognita* in response to exposure to cabbage and maize root exudate, and similar trends were observed in *R. similis.* This may arise from natural variations of pectin concentrations in the cell walls of these particular plant hosts (Voragen *et al*., 2009). For example, if the nematode has detected a specific host plant with a low concentration of pectin, such as grasses or more woody tissue (Voragen *et al*., 2009), it may adapt to down-regulate pectate lyase genes that are unnecessary for penetrating the host's cell walls. Alternatively, these genes may initially be down-regulated to avoid triggering host plant defence responses until the nematode is established within the root tissues. In *M. graminicola,* the activity of a pectate lyase, *Mg-Pel1,* triggered host immunity in rice plants (Chen *et al*., 2021). Similarly, the activity of a pectate lyase in the fungal pathogen, *Verticillium dahliae,* triggered the host defence responses of tobacco and cotton plants (Yang *et al*., 2018). The pectate lyase activity of *M. incognita* could therefore also feasibly trigger the host plant defence responses, providing additional support for this suggestion. There are numerous plausible explanations for this observation, however further research is necessary to distinguish which is correct.

4.5.7 qRT-PCR vs RNA-Seq

Although the results generally suggest host-specific differential regulation of cellulase genes, a discrepancy emerged between the qRT-PCR and RNA-Seq findings in the case of *Mi-eng-4* in *M. incognita*, specifically in the context of comparing expression levels between root exudate treatment with the control. According to the qRT-PCR analysis, *Mi-eng-4* showed significant up-regulation in response to exposure to both potato and maize root exudates. However, contradictory results emerged from the RNA-Seq analysis, indicating a significant down-regulation of *Mi-eng-4* expression in nematodes exposed to potato and maize root exudates when compared to those exposed to the control. Discordant results may have arisen from variations in the separate nematode pools analysed in each method, reflecting their biological diversity.

4.5.8 Root exudates as a proxy for soil environment conditions

Although using root exudates in laboratory experiments, such as those described in this chapter, can provide valuable insights into host-nematode interactions, it is important to recognise that they may not fully represent the complex soil environment where nematodes naturally respond to their hosts. Factors such as soil type, microbial interactions and root architecture can influence the concentration and composition of root exudates in the soil (Canarini *et al*., 2019), which is not replicated in a laboratory environment. Root exudates generated in the laboratory are also often more concentrated than those found in the soil. This oversimplification of the soil environment may lead to an incomplete understanding of nematode responses.

To overcome these limitations, future research should consider incorporating more realistic soil conditions. Extracting nematodes directly from host roots or from soil samples around different plant species, for example, would provide a more accurate representation of nematode responses in their natural habitats. Alternatively, some research uses root exudates collected directly from the soil, which reflects soil conditions more closely (Phillips *et al*., 2008).

4.5.9 Conclusions

The results confirm the ability of *G. pallida* and *M. incognita* to respond in a host-specific manner to the detection of root exudates from varying hosts and non-hosts. *G. pallida* exhibits a strong transcriptional response almost exclusively to its preferred host. In contrast, *M. incognita* consistently demonstrates a comparable and robust response to numerous potential hosts (excluding potato), whilst differentially expressing distinct sets of genes for each one. This, combined with *R. similis* transcriptome data, and known effects on *P. coffeae,* suggests that the ability of plant parasitic nematodes to tailor gene expression to host root exudates is dependent on host range.

Studying gene expression in plant parasitic nematodes is important as it provides information about plant parasitic nematode biology including their development, reproduction and interactions with their host plants. Identifying the genes that are activated or repressed in response to specific environmental cues, such as root exudates described in this chapter, can provide valuable insights into the molecular basis of nematode parasitism which could ultimately lead to more effective management of plant parasitic nematodes which in turn could help to protect global food security in the future.

4.6 Summary

- Root exudate exposure induces stylet thrusting in *G. pallida* and *M. incognita*. In the case of *G. pallida,* the magnitude of the response is host-specific.

- Root exudate exposure induces the differential expression of a cellulase gene in *G. pallida* and two cellulase genes in *M. incognita,* in a host-specific manner, as confirmed by qRT-PCR.

- RNA-Seq analysis confirmed that *G. pallida, M. incognita* and *R. similis* show transcriptional plasticity in response to root exudates from varying plants and suggests that the transcriptional responses are host-specific.

- *G. pallida* shows a strong transcriptional response to its preferred host, potato, but the response towards non-host plants is almost negligible.

- *M. incognita* and *R. similis* display transcriptional responses to a range of potential host root exudates, but potato induces the strongest response.

- Differences in host range are likely to be responsible for variances in transcriptional responses to host root exudates.

Chapter 5
Functional characterisation of genes of interest identified in the RNA-Seq analysis

5.1 Introduction

Many of the genes identified in Chapter 4 as being differentially regulated in *G. pallida* and *M. incognita* in response to different root exudates are hypothesised to encode effectors. Nematode effectors play an important role in parasitism and are usually secreted into the host, through the stylet, from the dorsal and sub-ventral pharyngeal glands (Vieira and Gleason, 2019, Hussey *et al*., 2002), or from other organs such as the amphids (Rehman *et al*., 2016, Semblat *et al*., 2001).

It is not yet understood exactly how sensing of root exudate components leads to the altered gene expression in the pharyngeal gland cells that has been observed in *G. pallida* and *M. incognita*. As previously mentioned, serotonin signalling is involved in nematode behaviour, and it is hypothesised that differential gene expression is also most likely attributed to neurological pathways. Stimuli are probably perceived by sensory neurones in the amphids and phasmids, and signalling pathways are activated to induce the changes observed. G-protein coupled receptors might be involved in subsequent signal transduction as, compared to ionotropic receptors, the binding of neurotransmitters to G-protein coupled receptors, mediates slower and longer lasting effects, such as changes to transcription (Koelle, 2018). As this type of receptor responds to external stimuli to generate intracellular responses, it is likely that they are involved in the responses to the detection of root exudates observed in Chapter 4.

The pharyngeal glands are highly specialised secretory cells where secretory proteins are synthesised. These proteins are subsequently stored within secretory granules which are derived from the Golgi apparatus. Upon secretion, exocytosis enables the release of these proteins from the granules, facilitating their transit through the oesophagus and ultimately to the stylet (Hussey *et al*., 2002). A substantial proportion of genes with spatial expression in the dorsal and sub-ventral pharyngeal glands, and that encode a signal peptide, are likely to encode effectors.

Nematode effectors are known to cause changes in plant cell morphology, physiology, gene expression and function (Bird, 1996, Gheysen and Fenoll, 2002, Przybylska and Spychalski, 2021, Sultana *et al*., 2022, Vilela *et al*., 2023) to aid in the parasitic processes of plant parasitic nematodes. During infection, some effectors such as cell wall-degrading enzymes, facilitate host penetration and migration through the root tissue (Rai *et al*., 2015b). When cell wall components are degraded, oligosaccharides

are released as pathogen-associated molecular patterns (PAMPs) which triggers plant immunity (Wan *et al*., 2021). The majority of effectors that have been characterised in plant parasitic nematodes have been linked to the suppression of host defences and this allows nematodes to evade detection during infection (Ali *et al*., 2015, Goverse and Smant, 2014, Quentin *et al*., 2013). Once inside the root, different effectors are involved in nutrient acquisition and, in sedentary parasitic nematodes, including *G. pallida* and *M. incognita,* they enable the formation of permanent feeding sites (Lilley*et al*., 2018, Verma *et al*., 2018). The success and survival of these sedentary plant parasitic nematodes relies heavily on their ability to migrate through host root tissue and establish a feeding site.

The possession of genes encoding effectors that aid in feeding site formation is unique to sedentary plant parasitic nematodes within the nematode phylum. Other phytopathogens also secrete effectors which are specific to their own needs in order to successfully infect their hosts. For example, fungi are known to construct hydrophobic spaces within the plant root through the secretion of hydrophobins (Tanaka and Kahmann, 2021, Soanes *et al*., 2002). This allows them to successfully penetrate the root because their mycelia attach primarily to hydrophobic surfaces, whereas the inside of the root is hydrophilic (Zhang *et al*., 2022). Knock-down of genes encoding hydrophobins in the Fusarium head blight fungus, *Fusarium graminearum,* causes reduced virulence and pathogenicity (Quarantin *et al*., 2019), suggesting an important role for these effectors in fungal infection.

Gene knock-down is a useful tool to investigate gene function. In plant parasitic nematodes, it was first achieved by RNAi in the cyst nematodes *G. pallida* and *H. glycines* (Urwin *et al*., 2002). Since then, RNAi has been utilised to knock-down the expression and determine the function of effectors in root-lesion, root-knot and cyst nematodes (Bakhetia *et al*., 2007, Banakar *et al*., 2020, Hada *et al*., 2020, Kumar *et al*., 2022, Kumar *et al*., 2017, Mani *et al*., 2020, Somvanshi *et al*., 2020, Tian *et al*., 2019, Joseph *et al*., 2012). Knock-down of effector genes typically reduces parasitic success in multiple nematode species, emphasising the essential role played by effectors in the process of parasitism.

This type of functional analysis would prove particularly valuable for genes that remain uncharacterised and are currently designated as unnamed proteins or denoted by the abbreviation “N/A”. The knock-down of such genes by RNAi paired with infection assays to investigate the nematode’s infection ability, could uncover novel functions. If these methods were applied to the genes that were identified as being up-regulated in response to root exudates in *G. pallida* and *M. incognita* in Chapter 4, our understanding of the molecular host-parasite interactions of these plant parasitic nematodes could be

enhanced. As not all of the genes that showed significant up-regulation in response to root exudates encode effectors, the outcomes of these experiments would also allow for the identification of other genes that might be involved in the parasitic process. Furthermore, studying the genes that are up-regulated in response to a limited number or only one root exudate exposure could provide valuable insights into host-specific responses.

Further to RNAi, in situ hybridisation is a useful tool in determining gene function. *This method* allows visualisation of spatial gene expression in a variety of different organisms. The spatial expression patterns of genes provide valuable insights into their potential roles, cellular context and involvement in biological processes. For example, in *C. elegans,* the *hlh-1* gene is almost exclusively expressed in the muscle cells which correlates with its function in muscle development and function (Guan *et al*., 2020). Similarly, the *dpy-2* and *dpy-10* genes are predominantly expressed in the hypodermis of *C. elegans* which relates to their function in nematode body morphogenesis (Levy *et al*., 1993). In plant parasitic nematodes, effector-encoding genes demonstrate preferential expression in the pharyngeal glands (Davis *et al*., 2008, Hussey, 1989, Hussey *et al*., 2002, Mitchum *et al*., 2013, Vieira and Gleason, 2019), correlating with their subsequent secretion into the host. As the spatial expression of genes encoding effectors relates to their function, if genes that are not well characterised or still have an unknown function are found to be expressed in the pharyngeal glands of *G. pallida* or *M. incognita*, it strongly suggests that they are secreted from the nematode and share similar functions with known effectors, thereby facilitating host infection.

This chapter endeavours to functionally characterise a subset of genes identified in Chapter 4 as being significantly up-regulated in response to root exudate detection. Understanding the potential functions of these genes will provide an improved understanding of the molecular infection mechanisms employed by plant parasitic nematodes.

5.2 Aims

- To determine the spatial expression of a subset of genes that are differentially expressed in response to varying root exudates in *G. pallida* and *M. incognita* using *in situ* hybridisation.

- To use RNAi to knock-down the expression of these genes.

- To investigate the functions of these genes using infection assays and assess whether or not they are involved in parasitism.

5.3 Materials and Methods

5.3.1 *In situ* hybridisation of genes of interest in *G. pallida* and *M. incognita*

The RNA-Seq analysis carried out in Chapter 4 identified a large number of differentially expressed genes in *G. pallida* and *M. incognita* exposed to different root exudates. A subset from each species was chosen for further characterisation on the basis of assumed function and relative expression levels. The initial list of selected genes from Chapter 4 was narrowed down based on expression level, quality of annotation and gene model. The first step in the functional characterisation of the selected genes was to determine their spatial expression using *in situ* hybridisation.

Firstly, single-stranded digoxigenin-labelled antisense DNA probes were generated for genes of interest in *G. pallida* an *M. incognita* (Section 2.2.7.1). Control, sense probes were generated in the same way. Primers used in these reactions are detailed in Tables 5.1 and 5.2.

Nematodes were fixed, permeabilised, hybridised and stained as previously described in Section 2.2.7.2.

5.3.2 RNA interference (RNAi) for genes of interest in *G. pallida* and *M. incognita*

After *in situ* hybridisation had confirmed the spatial expression of a subset of the genes of interest, RNAi was used to knock-down their expression in order to investigate their potential roles in parasitism.

Dicer-substrate small interfering RNAs (DsiRNAs) were designed for *G. pallida* RNAi candidates: unnamed protein (GPLIN_001116400), effector homologue 4D06 (GPLIN_000969900), putative effector protein (GPLIN_000969800) and the GPCR (GPLIN_000153100) using the design tool supplied by Integrated DNA Technologies available at: https://eu.idtdna.com/pages/products/functional-genomics/dsirnas-and-trifecta-rnai-kits (Table 5.3). DsiRNAs were also designed in the same way for *M. incognita* RNAi candidates: secretory protein 40 (Minc3s05895g38985), putative avirulence protein (Minc3s06236g39536), astacin (Minc3s00009g00641), expansin-like protein (Minc3s07290g40952) and the GPCR (Minc3s04148g35606) (Table 5.4). To ensure that there were no off-target effects, each potential sequence was blasted against the *G. pallida* or *M. incognita* genome using the blast tool available on WormBase Parasite. DsiRNAs that aligned to non-target genes containing regions where

more than 16 bases of their coding sequence aligned with the first 19 bases of the DsiRNA were not selected.

Triplicate pools of approximately 15,000 *G. pallida* J2s or 20,000 *M. incognita* J2s were incubated overnight, rotating at room temperature with octopamine (100 mM) and either DsiRNA (10 µg/ml) targeted to the gene of interest or a negative control DsiRNA (10 µg/ml) that had no specific targets in these species. Nematodes were then washed extensively with sterile tap water to remove DsiRNA that had not been taken up and re-suspended in sterile tap water. Approximately 1,250 nematodes were removed from each treatment for use in infection assays and the remaining nematodes were pelleted and flash-frozen in liquid nitrogen for RNA extraction.

5.3.3 Analysis of the success of gene knock-down by RNAi

5.3.3.1 RNA extraction and cDNA synthesis

To determine whether or not the RNAi had successfully knocked-down the genes of interest, gene expression was analysed by qRT-PCR. Total RNA was extracted from the frozen nematodes using an E.Z.N.A® Plant RNA Kit (Omega Bio-Tek) (including additional DNase steps) (Section 2.2.1). First strand cDNA was then synthesised from 200 ng RNA using iScript™ cDNA synthesis kit (Bio-Rad) (Section 2.2.2).

5.3.3.2 qRT-PCR

Primer pairs were designed for each RNAi candidate gene in both *M. incognita* and *G. pallida* (Tables 5.5 and 5.6). These were each found to have amplification efficiencies of between 95 and 105 percent.

To determine whether or not the knock-down of gene expression by RNAi had been successful, qRT-PCR was performed using a CFX Connect™ Real-Time System (Bio-Rad) (Section 2.2.6). Relative gene expression was calculated using the means of three technical repeats for each of three biological repeats for each RNAi candidate gene using the Livak ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) following the best practice described by Taylor *et al*. (2019). Reference genes for *G. pallida* were Elongation factor 1A (*Gp-EIF1*) and Initiation factor 1 (*Gp-EF1-A*). Reference genes for *M. incognita* were Actin 2 (*Mi-Act-2*) and PTP (*Mi-PTP*).

Expression profiles of the genes of interest were compared between control nematodes and those treated with DsiRNA (Section 2.2.6). Data were analysed using One-way ANOVA and post-hoc Tukey's post-hoc test in SPSS (SPSS v.25; IBM Corporation Armonk, New York, USA).

Gene name	Description	Antisense primer sequence	Antisense primer sequence	Expected product length (bp)	Annealing temperature (°C)
GPLIN_000153100	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein	TCAACGCAAAAGCACTCTCC	CTGATTCTGCTCGGGGTGTA	151	59
GPLIN_001116400	Unnamed protein product	ACGTATTCACCGCAGCAATG	AACCCTTGTTCTGTGTTGCC	194	59
GPLIN_000969900	Effector homologue 4D06	GGTGAGTTGTGCGTCGATTT	TACATTGGCACTTTCTCGGC	163	59
GPLIN_001634900	Zinc finger protein	CCACGACACAATGCCAAACT	TTTGCAACAAACATGTCGGC	161	59
GPLIN_000969800	Putative effector protein	GGTATGTTCCGTGGCAAGTG	ATGAAGATGTGTGCGCGTAC	161	59
GPLIN_001203600	Glutathione synthetase	TGCAGCAACTCCAACTTGTC	GTGCACAATGTTCCCGAGTT	161	59

Table 5.1- Primer sequences used for *in situ* hybridisation probes in *G. pallida.*

Gene name	Description	Antisense primer sequence	Sense primer sequence	Expected product length (bp)	Annealing temperature (°C)
Minc3s05958g39089	Peptidase family m13 domain-containing protein	ACCGTCTATTTGCACCTTCG	GGTAACCCTTGGGCATGAAT	169	58
Minc3s00750g16857	Unnamed protein product	CCATTCCTTCATAATTGGGCGT	TTACTCCAGAAGCCAAGCGT	155	59
Minc3s04148g35606	Serpentine type 7TM GPCR chemoreceptor srd domain-containing protein	TCGAGACAACAATCCAGGCA	ACTCTATGTAGCCGGGTTGC	152	59
Minc3s07290g40952	Expansin-like protein	TTGCTCTTCCAACAACACCG	CAAGCATGTAGTGAGGAATGACA	182	59
Minc3s00362g11016	Pectate lyase 3	TTGAACAACCTTTCCGCCAG	AGTATAAGGCGGCAGATGGA	167	59
Minc3s06236g39536	Putative avirulence protein precursor	ATCTTCTGGCTTGGGTGACA	CGACCAATGCTGTGAGGAAG	154	59
Minc3s05895g38985	Putative oesophageal gland cell secretory protein 40	TCCTCTTCACCTTCCGCTTT	GGCCGAAGATGAAGAGAAGG	165	59

Table 5.2- Primer sequences used for *in situ* hybridisation probes in *M. incognita.*

Minc3s02639g30985	Beta-1,4-endoglucanase	AGACACCTGGTTCGATTGGA	CCCTATGGACGACTTTCAGTT	190	58
Minc3s00009g00641	Astacin (Peptidase family m12a) domain-containing protein	GCAGCACATTCTTATCCCCG	TTGGCATTTACAAGCACCCC	190	59

Table 5.2 continued- Primer sequences used for *in situ* hybridisation probes in *M. incognita.*

Gene ID	Description	Sense DsiRNA sequence	Antisense DsiRNA sequence
GPLIN_001116400	Unnamed protein product	GCGAUACCGUGAGAUCAGCAUUGCT	AGCAAUGCUGAUCUCACGGUAUCGCAG
GPLIN_000969900	Effector homologue 4D06	AAGCGAGAAUUGUGGACGAAAUCGA	UCGAUUUCGUCCACAAUUCUCGCUUCC
GPLIN_000153100	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein	GUCUACAAAGCAUCAACAGAUAAGT	ACUUAUCUGUUGAUGCUUUGUAGACCA
GPLIN_000969800	Putative effector protein	GAAUGGAACAAAUUGGCCAAAGATT	AAUCUUUGGCCAAUUUGUUCCAUUCAG

Table 5.3- Dicer-substrate small interfering RNAs designed for genes of interest in *G. pallida.*

Gene ID	Description	Sense DsiRNA sequence	Antisense DsiRNA sequence
Minc3s05895g38985	Putative oesophageal gland cell secretory protein 40	CGAUAAGUGUGAAUUACAUUUUCAA	UUGAAAAUGUAAUUCACACUUAUCGCA
Minc3s06236g39536	Putative avirulence protein precursor	AAGAUGUCCCUGAAGCUACAAAACC	GGUUUUGUAGCUUCAGGGACAUCUUCA
Minc3s00009g00641	Astacin (Peptidase family m12a) domain-containing protein	GAUUGAAGCUUUUAUCAGAAACUAA	UUAGUUUCUGAUAAAAGCUUCAAUCCA
Minc3s07290g40952	Expansin-like protein	GUGCAAAAAUUGAAUACAAAGGCAA	UUGCCUUUGUAUUCAAUUUUUGCACAU
Minc3s04148g35606	Serpentine type 7TM GPCR chemoreceptor srd domain-containing protein	GAUAAUUCAGUCCCUUUUACCUCTT	AAGAGGUAAAAGGGACUGAAUUAUCAU

Table 5.4- Dicer-substrate small interfering RNAs designed for genes of interest in *M. incognita.*

Gene ID	Description	Forward primer sequence	Reverse primer sequence
GPLIN_001116400	Unnamed protein product	CCTTATGTCTTGGGCTGCAC	TAATGGCCTCTGGATTCCCC
GPLIN_000969900	Effector homologue 4D06	TACATTGGCACTTTCTCGGC	GGTGAGTTGTGCGTCGATTT
GPLIN_000153100	Serpentine type 7TM GPCR chemoreceptor srsx domain-containing protein	CTGATTCTGCTCGGGGTGTA	TCAACGCAAAAGCACTCTCC
GPLIN_000969800	Putative effector protein	ATGAAGATGTGTGCGCGTAC	GGTATGTTCCGTGGCAAGTG
EIF1	Initiation factor- reference gene	GCCTAAAGATGCCTTCGAGC	GGTGATAGTCTTCCTCCCCG
EF1-A	Elongation factor- reference gene	AATGACCCGGCAAAGGAGAG	GTAGCCGGCTGAGATCTGTC

Table 5.5- qRT-PCR primer sequences for RNAi candidate genes in *G. pallida.*

Gene ID	Description	Forward primer sequence	Reverse primer sequence
Minc3s05895g38985	Putative oesophageal gland cell secretory protein 40	GGCCGAAGATGAAGAGAAGG	TCCTCTTCACCTTCCGCTTT
Minc3s06236g39536	Putative avirulence protein precursor	CGACCAATGCTGTGAGGAAG	ATCTTCTGGCTTGGGTGACA
Minc3s00009g00641	Astacin (Peptidase family m12a) domain-containing protein	TTGGCATTTACAAGCACCCC	GCAGCACATTCTTATCCCCG
Minc3s07290g40952	Expansin-like protein	GGCATGCGGAATTGACACTG	GGCATCCAGGGCATTCATTA
Minc3s04148g35606	Serpentine type 7TM GPCR chemoreceptor srd domain-containing protein	ACTCTATGTAGCCGGGTTGC	TCGAGACAACAATCCAGGCA
Actin 2	Actin- reference gene	GATGGCTACAGCTGCTTCGT	GGACAGTGTTGGCGTAAAGG
PTP	Protein tyrosine phosphatase- reference gene	GGAAAGCATTTTCACCTGCGA	ACGGGTTGCCAACAGATGAA

Table 5.6- qRT-PCR primer sequences for RNAi candidate genes in *M. incognita.*

5.3.4 Infection assays

5.3.4.1 Preparation of plants for infection

Shoots of *in vitro* grown potato plants (Désirée) were transferred to Magenta pots containing multiplication medium (Murashige and Skoog medium including vitamins (4.43 g/L) (Murashige and Skoog, 1962), sucrose (20 g/L), plant agar 5.5 g/L (Duchefa), pH 5.8) and were grown *in vitro* for two weeks in a constant temperature room at 20 °C with 16:8 hr light:dark. After this period, rooted plantlets were transferred to CYG growth pouches where they were grown hydroponically for a further one week in a glasshouse at 20 °C with 50-60 percent humidity and 16 hours of light per day. Two plantlets were inserted into each pouch and the paper inside the pouches was kept moist with tap water.

To determine whether or not Hoagland's solution (Hoagland and Arnon, 1950) was beneficial to plants grown from seed, four adzuki bean (*Vigna angularis*) seeds were applied to each of six pouches which were grown for one week hydroponically in glasshouses at 24 °C with 50-60 percent humidity and 16 hours of light per day. Three of the pouches were supplemented with Hoagland's solution every other day and the remaining three pouches were watered with tap water every other day. The success of the growth of the plants was assessed after one week.

In the *M. incognita* infection assays, adzuki beans were used as the host plant species. This choice was deliberate, given the numerous genes of interest that exhibited up-regulation in response to more than one root exudates, presenting a need to account for the differential reactions.

Once optimal growth conditions had been determined, one adzuki bean seed was applied to each of 60 pouches that were grown for one week in a glasshouse at 24 °C with 50-60 percent humidity and 16 hours of light per day. Similarly, one carrot seed was applied to each of 30 pouches that were grown hydroponically for between two and three weeks in glasshouses at 20 °C with 50-60 percent humidity and 16 hours of light per day. The pouches containing adzuki bean and carrot seeds were watered with tap water during this time.

5.3.4.2 Infecting potato roots with *G. pallida* subjected to RNAi

Separate RNAi treatments were performed to knock-down each gene of interest in *G. pallida* J2 populations. For each treatment group, approximately 15 J2s were applied to five infection points on the roots of potato plants that had been grown in pouches. This procedure was repeated for between seven and 10 plants for each gene of interest. A negative control group was generated by treating J2s with a non-target DsiRNA and these were applied to the roots of seven potato plants grown in pouches in the same

way. Pouches of potato plants were returned to the glasshouse at 20 °C with 50-60 percent humidity and 16 hours of light per day and left to grow for seven days.

5.3.4.3 Infecting adzuki bean roots with *M. incognita* subjected to RNAi

The *M. incognita* genes encoding the putative avirulence protein, astacin, expansin-like protein, and a GPCR were all significantly up-regulated in response to two or more root exudate treatments. RNAi was utilised to knock-down the expression of these genes in separate J2 pools of *M. incognita.* A group of J2s treated with a non-target DsiRNA were used as a negative control. Approximately 15 J2s from each treatment group were applied to five infection points on the roots of adzuki bean plants in pouches. This was repeated for seven or eight plants per treatment group. Pouches were returned to a glasshouse at 24 °C with 50-60 percent humidity and 16 hours of light per day where the plants were grown for a further seven days.

5.3.4.4 Infecting carrot roots with *M. incognita* subjected to RNAi

The *M. incognita* putative oesophageal gland cell secretory protein 40 was up-regulated only in response to carrot root exudate. After knocking down this gene in *M. incognita* J2s by RNAi, infection assays were carried out using pouch-grown carrot plants as described for adzuki bean. Six carrot plants were infected with J2s treated with DsiRNA to knock-down the putative oesophageal gland cell secretory protein 40 gene and six were infected with J2s that served as the negative control. The pouches containing the infected roots were grown for a further seven days in a glasshouse at 20 °C with 50-60 percent humidity and 16 hours of light per day.

5.3.4.5 Acid Fuchsin staining of roots infected with nematodes

In order to visualise infection levels, Acid Fuchsin was used to stain the nematodes in the roots. Roots from infected plants were removed from pouches and soaked in sodium hypochlorite solution (1 % available chlorine) for two minutes at room temperature. They were then washed three times in tap water for two minutes before being transferred to boiling Acid Fuchsin stain (Acros Organics) (35 mg/L in 25 % glacial acetic acid). Roots were then rinsed briefly in tap water and placed in acidified glycerol and incubated overnight at 65 °C.

5.3.4.6 Visualising nematodes in the roots of infected plants

Roots were viewed under an Olympus SZX9 stereo-binocular microscope and the number of nematodes in the roots were recorded. For potato roots infected with *G. pallida,* distinctions were made between developmental stages of the nematodes and for adzuki bean and carrot roots infected with *M. incognita,* distinctions were made between nematodes causing swelling of the root and those that were not.

5.4 Results

5.4.1 *In situ* hybridisation of genes of interest in *G. pallida* and *M. incognita*

In situ hybridisation was employed to determine the spatial expression of the genes of interest. Here, nematodes are hybridised with gene-specific single-stranded digoxigenin-labelled DNA probes and then labelled with anti-digoxigenin-AP Fab fragments and stained to reveal the exact location in the nematode where the gene is expressed.

5.4.1.1 Generation of single-stranded digoxigenin-labelled antisense DNA probes for *G. pallida*

DNA probe templates were created for each gene of interest using PCR, cDNA prepared from J2 nematodes and the primers detailed in Table 5.1. Templates were successfully generated for the GPCR (GPLIN_000153100), unnamed protein (GPLIN_001116400), effector homologue 4D06 (GPLIN_000969900), zinc finger protein (GPLIN_001634900), putative effector protein (GPLIN_000969800) and glutathione synthetase (GPLIN_000936300) in *G. pallida* (Figure 5.1).

Successfully amplified templates were combined with digoxigenin DNA labelling mix (Roche, Germany) and either an antisense or sense primer (10 µM) to generate either an antisense or sense probe, respectively for each gene (Figure 5.2). The sense probe acted as the negative control. The generation of probes from all templates was successful.

5.4.1.2 Generation of single-stranded digoxigenin-labelled antisense DNA probes for *M. incognita*

PCR successfully generated DNA templates for the following genes: expansin-like protein (Minc3s07290g40952), unnamed protein (Minc3s00750g16857), putative avirulence protein (Minc3s06236g39536), pectate lyase 3 (Minc3s00362g11016), putative oesophageal gland secretory protein 40 (Minc3s05895g38985), GPCR (Minc3s04148g35606), beta-1,4-endoglucanase (Minc3s02639g30985), peptidase family m13 domain-containing protein (Minc3s05958g39089) and astacin (Minc3s00009g00641) (Figure 5.3). Subsequently, these templates were employed to generate single-stranded digoxigenin-labelled sense and antisense DNA probes (Figure 5.4). Efforts to create DNA templates for the candidate secreted effector (Minc3s00015g01000), and tyrosinase and metridin ShK toxin domain containing protein (Minc3s00218g07814) were unsuccessful. Consequently, it was not possible to generate probes for these genes. Probes were also not successfully created from the DNA

templates of the genes encoding the GPCR or the peptidase family m13 domain-containing protein. Overall, single-stranded digoxigenin-labelled sense and antisense DNA probes were successfully generated for seven of the 12 genes of interest in *M. incognita.*

5.4.1.3 Spatial expression of genes of interest in *G. pallida* and *M. incognita*

To determine the spatial expression of the genes of interest identified in the RNA-Seq analysis, *in situ* hybridisation was carried out. If expression was found to be in the pharyngeal gland cells, as described previously (Section 3.4.3), the location of the resultant staining was compared to that of numerous previously documented genes expressed in these cells of *G. pallida* and *M. incognita.* Measurements from 10 published images indicated that the sub-ventral gland cells are situated roughly 180 µm away from the head of a J2.

1.4.1.3.1 *G. pallida*

In situ hybridisation confirmed that both the unnamed protein and the putative effector protein were expressed in the sub-ventral pharyngeal glands of *G. pallida* (Figure 5.5C and Figure 5.5G). The staining for both of these genes is distinctly localised to the pharyngeal gland cells with minimal staining observed elsewhere within the nematode. Although the staining associated with effector homologue 4D06 is spread throughout the nematode, there is clearly some hybridisation in the region of the sub-ventral gland cells (Figure 5.5E).

The spatial expression of the GPCR, the glutathione synthetase and zinc finger protein could not be determined as the *in-situ* hybridisation was not successful for these genes.

1.4.1.3.2 *M. incognita*

In *M. incognita, in situ* hybridisation confirmed expression of the putative avirulence protein in the sub-ventral pharyngeal glands (Figure 5.6C). Staining associated with this gene specifically localises to the sub-ventral glands, with minimal staining detected in other areas of the nematode (Figure 5.6C). Staining associated with the astacin gene was observed exclusively in the head region, near the amphids (Figure 5.6E), suggesting expression of this gene within these specific regions. It is challenging to precisely determine the spatial expression of the expansin-like protein. While there is slight staining within the expected gland cell region, this staining is insufficient to conclusively confirm gene expression in these cells (Figure 5.6G).

The spatial expression of the unnamed protein, pectate lyase 3, beta-1,4-endoglucanase and putative oesophageal gland cell secretory protein 40 could not be determined as the

in situ hybridisation attempts using the digoxigenin-labelled DNA probes generated for these genes were unsuccessful.

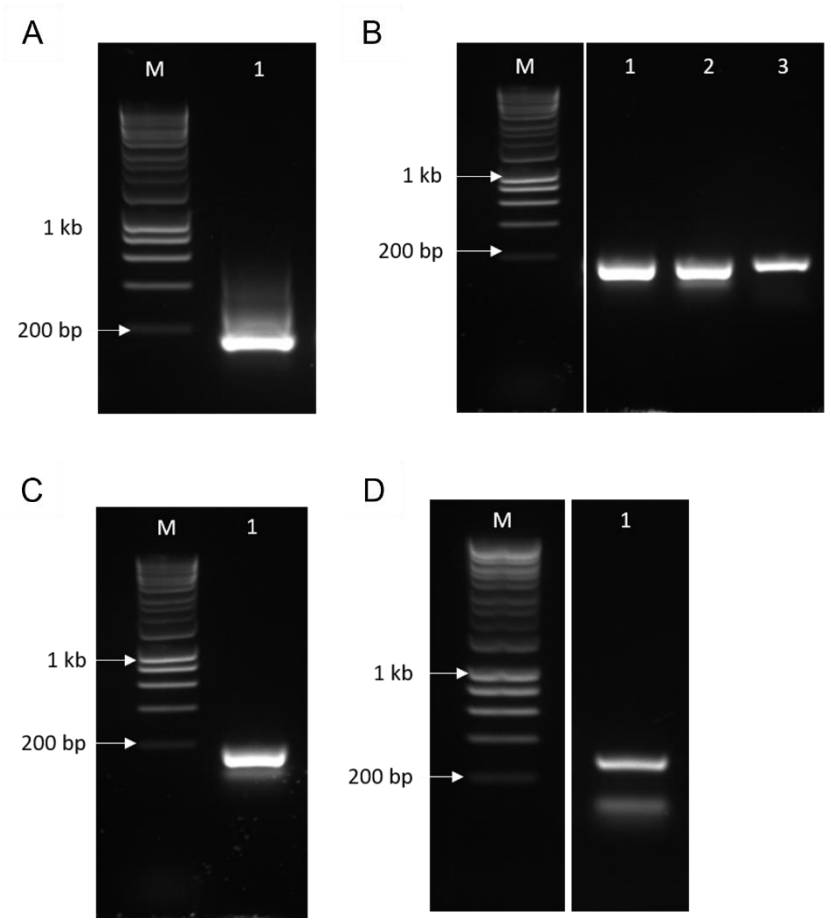

Figure 5.1- Agarose gel electrophoresis of DNA templates for the generation of digoxigenin-labelled antisense DNA probes for *in situ* hybridisation in *G. pallida.*

A) Generation of *in situ* hybridisation template for effector homologue 4D06. Lane 1: PCR product for effector homologue 4D06 (163 bp).

B) Generation of *in situ* hybridisation template for zinc finger protein, unnamed protein and the GPCR. Lane 1: PCR product for zinc finger protein (161 bp). Lane 2: PCR product for unnamed protein (194 bp), Lane 3: PCR product for the GPCR (151 bp).

C) Generation of *in situ* hybridisation template for glutathione synthetase. Lane 1: PCR product for glutathione synthetase (187 bp).

D) Generation of *in situ* hybridisation template for putative effector protein. Lane 1: PCR product for putative effector protein (161 bp).

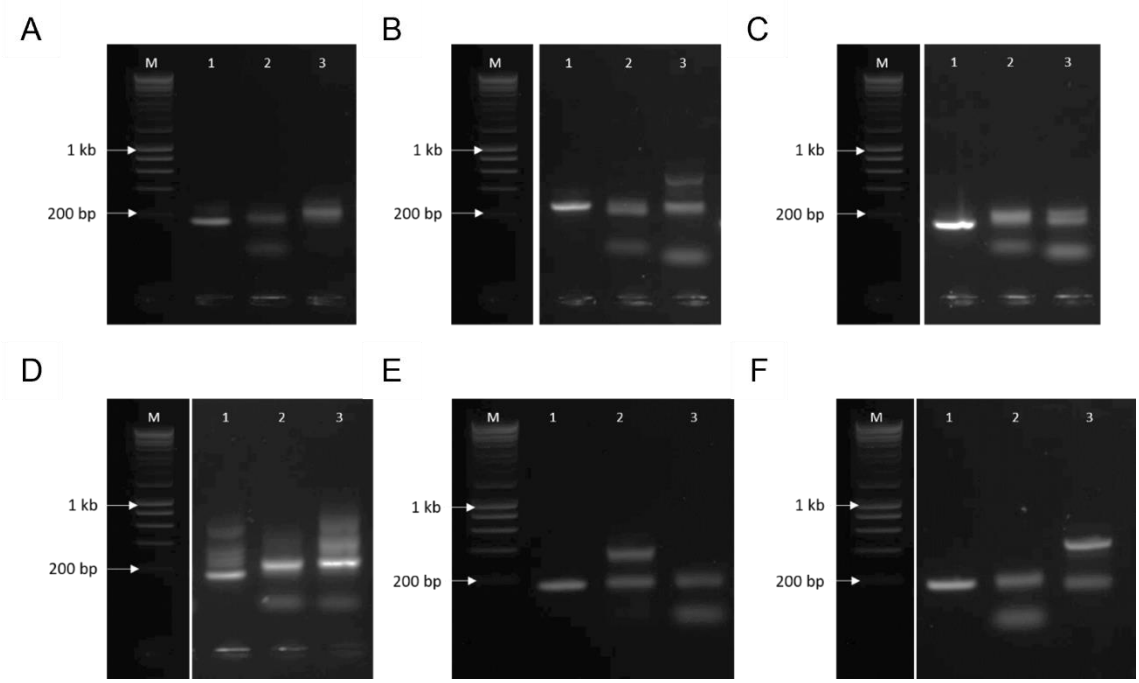

Figure 5.2- Agarose gel electrophoresis of digoxigenin-labelled antisense and sense DNA probes for use in *in situ* hybridisation of genes of interest in *G. pallida.*

A) Generation of sense and anti-sense probes for *in situ* hybridisation of the GPCR. Lane 1: DNA template (151 bp). Lane 2: sense probe. Lane 3: antisense probe.

B) Generation of sense and anti-sense probes for *in situ* hybridisation of the unnamed protein. Lane 1: DNA template (194 bp). Lane 2: sense probe. Lane 3: antisense probe.

C) Generation of sense and anti-sense probes for *in situ* hybridisation of glutathione synthetase. Lane 1: DNA template (187 bp). Lane 2: sense probe. Lane 3: antisense probe.

D) Generation of sense and anti-sense probes for *in situ* hybridisation of effector homologue 4D06. Lane 1: DNA template (163 bp). Lane 2: sense probe. Lane 3: antisense probe.

E) Generation of sense and anti-sense probes for *in situ* hybridisation of putative effector protein. Lane 1: DNA template (161 bp). Lane 2: sense probe. Lane 3: antisense probe.

F) Generation of sense and anti-sense probes for *in situ* hybridisation of zinc finger protein. Lane 1: DNA template (187 bp). Lane 2: sense probe. Lane 3: antisense probe.

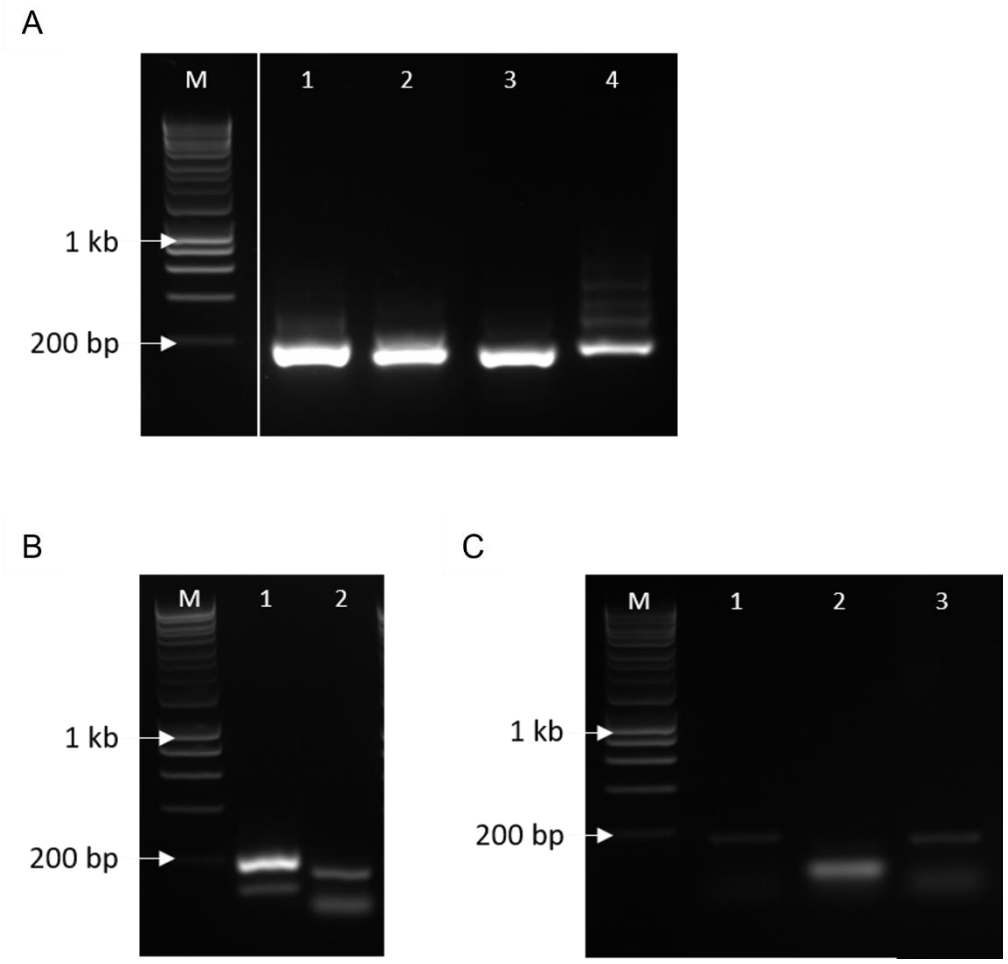

Figure 5.3- Agarose gel electrophoresis of DNA templates for the generation of digoxigenin-labelled antisense DNA probes for *in situ* hybridisation in *M. incognita.*

A) Generation of *in situ* hybridisation template for expansin-like protein, unnamed protein, putative avirulence protein and pectate lyase 3. Lane 1: PCR product for expansin-like protein (182 bp). Lane 2: PCR product for unnamed protein (155 bp). Lane 3: PCR product for putative avirulence protein 154 bp. Lane 4: PCR product for pectate lyase 3 (167 bp).

B) Generation of *in situ* hybridisation template for beta-1,4-endoglucanase and putative oesophageal gland cell secretory protein 40. Lane 1: PCR product for beta-1,4-endoglucanase (190 bp). Lane 2: PCR product for putative oesophageal gland cell secretory protein 40 (165 bp).

C) Generation of *in situ* hybridisation template for peptidase family m13 domain-containing protein, GPCR and astacin. Lane 1: PCR product for peptidase family m13 domain-containing protein (169 bp). Lane 2: PCR product for the GPCR (152). Lane 3: PCR product for astacin (190 bp).

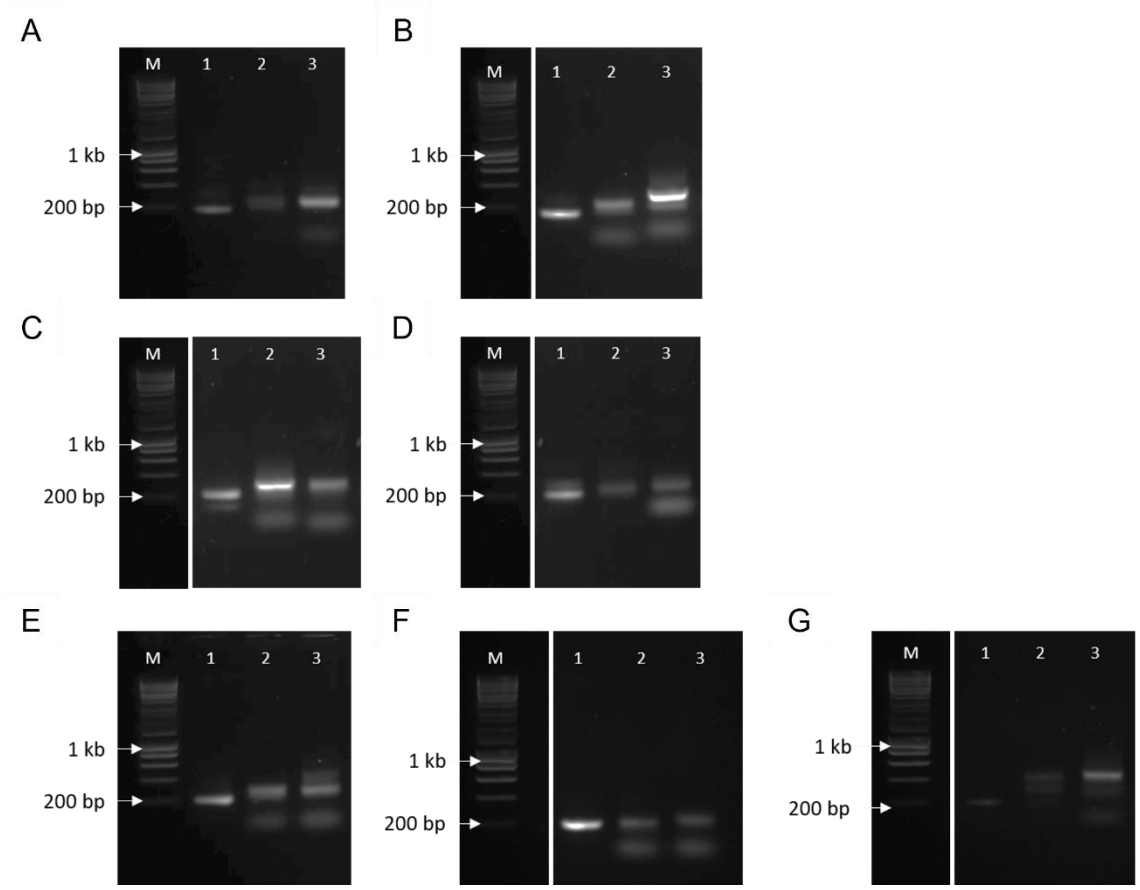

Figure 5.4- Agarose gel electrophoresis of digoxigenin-labelled antisense and sense DNA probes for use in *in situ* hybridisation of genes of interest in *M. incognita.*

A) Generation of sense and anti-sense probes for *in situ* hybridisation of Pectate lyase 3. Lane 1: DNA template (167 bp). Lane 2: sense probe. Lane 3: antisense probe.

B) Generation of sense and anti-sense probes for *in situ* hybridisation of putative avirulence protein. Lane 1: DNA template (154 bp). Lane 2: sense probe. Lane 3: antisense probe.

C) Generation of sense and anti-sense probes for *in situ* hybridisation of beta-1,4-endoglucanase. Lane 1: DNA template (190 bp). Lane 2: sense probe. Lane 3: antisense probe.

D) Generation of sense and anti-sense probes for *in situ* hybridisation of the unnamed protein. Lane 1: DNA template (155 bp). Lane 2: sense probe. Lane 3: antisense probe.

E) Generation of sense and anti-sense probes for *in situ* hybridisation of expansin-like protein. Lane 1: DNA template (182 bp). Lane 2: sense probe. Lane 3: antisense probe.

F) Generation of sense and anti-sense probes for *in situ* hybridisation of putative oesophageal gland cell secretory protein 40. Lane 1: DNA template (165 bp). Lane 2: sense probe. Lane 3: antisense probe.

G) Generation of sense and anti-sense probes for *in situ* hybridisation of astacin. Lane 1: DNA template (190 bp) lane 2 is the sense probe and lane 3 is the antisense probe.

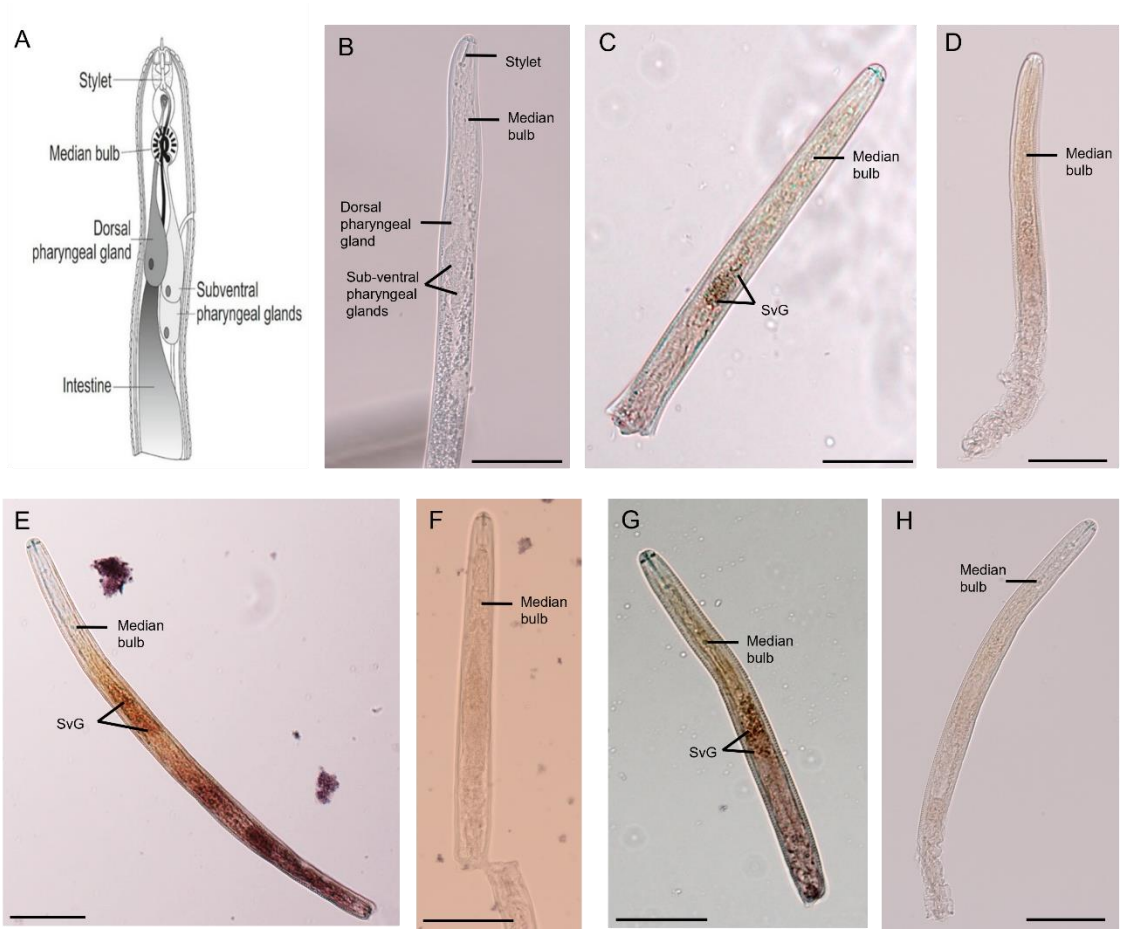

Figure 5.5- Spatial expression of genes of interest in *G. pallida.*

A) Anatomy of a plant parasitic nematode.

B) Microscope image of a J2 with the dorsal and sub-ventral pharyngeal glands highlighted.

C) *In situ* hybridisation of **unnamed protein** using an antisense probe.

D) *In situ* hybridisation of **unnamed protein** using a sense probe.

E) *In situ* hybridisation of **effector homologue 4D06** using an antisense probe.

F) *In situ* hybridisation of **effector homologue 4D06** using a sense probe.

G) *In situ* hybridisation of **putative effector protein** using an antisense probe.

H) *In situ* hybridisation of **putative effector protein** using a sense probe.

SvG denotes sub-ventral pharyngeal glands. Brown/purple staining highlights the spatial expression of the gene of interest. Size markers are 50 µm.

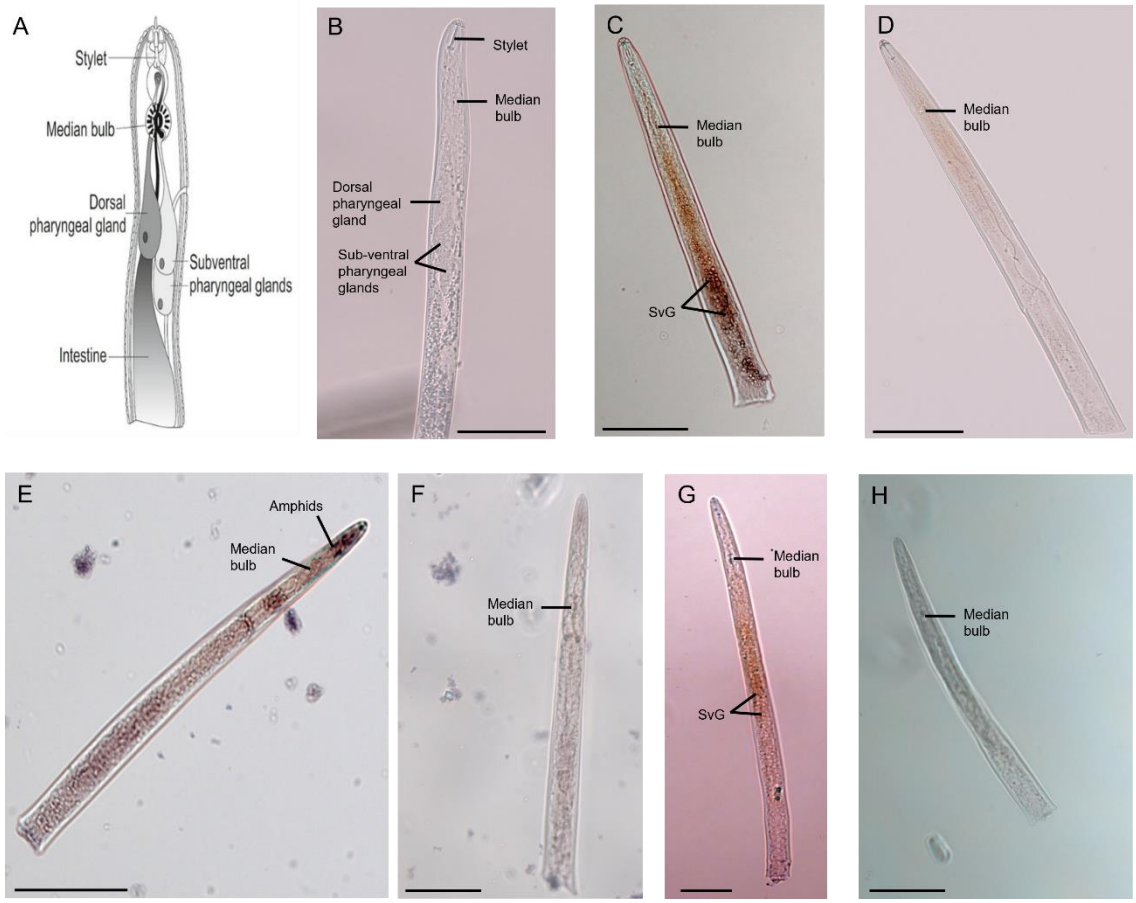

Figure 5.6- Spatial expression of genes of interest in *M. incognita.*

A) Anatomy of a plant parasitic nematode.

B) Microscope image of a J2 with the dorsal and sub-ventral pharyngeal glands highlighted.

C) *In situ* hybridisation of **avirulence protein** using an antisense probe.

D) *In situ* hybridisation of **avirulence protein** using a sense probe.

E) *In situ* hybridisation of **astacin** using an antisense probe.

F) *In situ* hybridisation of **astacin** using a sense probe.

G) *In situ* hybridisation of **expansin-like protein** using an antisense probe.

H) *In situ* hybridisation of **expansin-like protein** using a sense probe.

SvG denotes sub-ventral pharyngeal glands. Brown/purple staining highlights the spatial expression of the gene of interest. Size markers are 50 µm

5.4.2 RNAi successfully knocks-down genes in *G. pallida* and *M. incognita*

Once the spatial expression of the unnamed protein, 4D06 effector homologue and the putative effector protein in *G. pallida,* and the putative avirulence protein, astacin and expansin-like protein in *M. incognita* had been confirmed, RNAi was employed to knock-down the expression of these, and genes of interest that encoded G-protein coupled receptors in *G. pallida* and *M. incognita* (GPLIN_000153100 and Minc3s04148g35606). Although the spatial expression of the putative oesophageal gland cell secretory protein 40 in *M. incognita* could not be successfully determined through *in situ* hybridisation, RNAi experiments targeting this gene were also still conducted, as its localisation to the sub-ventral glands had previously been reported (Niu *et al*., 2016). DsiRNAs were generated for each target gene and J2s were soaked overnight with a gene-specific or negative control DsiRNA and octopamine to stimulate its uptake. To determine whether or not the RNAi was successful in knocking down the expression of the target genes, qRT-PCR was performed to assess relative gene expression.

In *G. pallida,* genes that encode effector homologue 4D06*,* the GPCR and putative effector protein were significantly down-regulated in nematodes treated with DsiRNA that targeted these genes compared to nematodes treated with a negative control DsiRNA (Figure 5.7). Although not significantly down-regulated, the relative expression of the gene encoding the unnamed protein was reduced by approximately 25 percent, on average, compared to the control (Figure 5.7).

Similarly, the genes encoding putative avirulence protein, astacin, expansin-like protein and the GPCR were all significantly down-regulated in *M. incognita* J2s that had been treated with DsiRNA targeting these genes compared to nematodes treated with a negative control DsiRNA (Figure 5.8). The relative expression of the putative oesophageal gland cell secretory protein 40 was not significantly altered by the DsiRNA treatment (Figure 5.9), suggesting this gene is not amenable to RNAi at the J2 stage. As this was the case, no further analysis was performed with these nematodes.

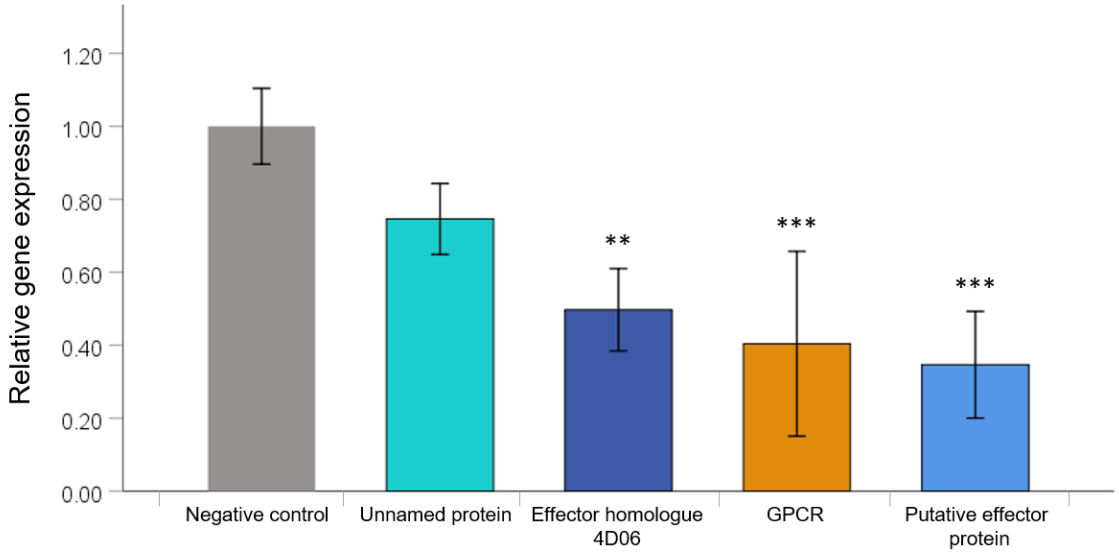

Figure 5.7- RNAi induced knock-down of genes of interest in *G. pallida.*

Genes of interest were knocked down in separate pools of *G. pallida* J2s using RNAi. Subsequent amplification of specific cDNA sequences by qRT-PCR showed that as a result of the RNAi treatment, the relative expression of genes encoding effector homologue 4D06, the GPCR and putative effector protein was significantly reduced compared to the negative control. The relative expression of the unnamed protein encoding gene was reduced by approximately 25 %. Values are means of 3 biological repeats +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** p≤0.01, *** p≤0.001) (One-way ANOVA, Dunnett's post hoc test).

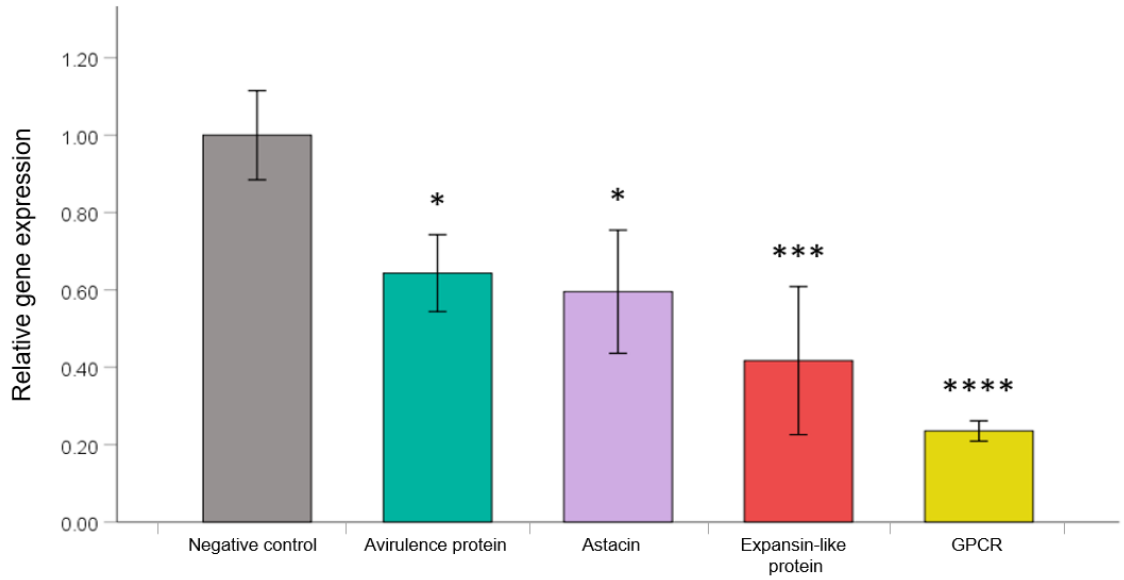

Figure 5.8- RNAi induced knock-down of genes of interest in *M. incognita.*

Genes of interest were knocked down in separate populations of *M. incognita* J2s using RNAi. Subsequent amplification of these specific cDNA sequences by qRT-PCR showed that as a result of the RNAi treatment, the relative expression of genes encoding putative avirulence protein, astacin, expansin-like protein and the GPCR was significantly reduced compared to the negative control. Values are means of 3 biological repeats +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** p≤0.01, *** p≤0.001) (One-way ANOVA, Dunnett's test).

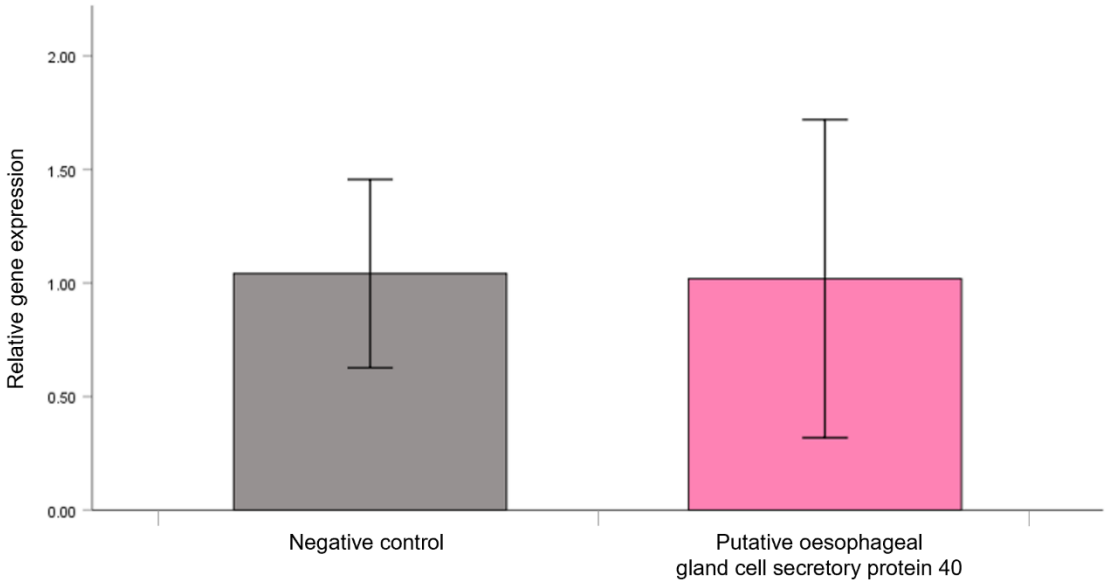

Figure 5.9- Putative oesophageal gland cell secretory protein 40 in *M. incognita* is not amenable to RNAi.

The gene encoding putative oesophageal gland cell secretory protein 40 was targeted for knock-down in *M. incognita* J2s using RNAi. Subsequent amplification of cDNA for this gene by qRT-PCR showed that the RNAi treatment had not been successful in knocking down the expression of this gene. Values are means of 3 biological repeats +/- SEM. No significant difference was seen between the groups (Independent samples T-test).

5.4.3 Hoagland's solution is not beneficial for plants grown from seed, in pouches, for infection assays

The genes that had been knocked-down in *G. pallida* and *M. incognita* were suspected to play a role in early parasitism and in order to determine whether or not this was the case, infection assays were carried out.

M. incognita infection assays were performed using adzuki bean plants that were grown from seed, hydroponically, in pouches. To determine whether or not supplementation with Hoagland's solution would be beneficial to the growth of these plants, four seeds were planted in each of six pouches. Three of these pouches were supplemented with Hoagland's solution, whilst the remaining three were watered with tap water.

The visual assessment of plants grown in pouches and watered with tap water indicated better growth compared to those supplemented with Hoagland's solution (Figure 5.10). Therefore, in subsequent experiments, adzuki beans grown in pouches from seed were exclusively watered with tap water throughout the duration of the experiment. Potato plants grown in pouches from rooted shoot cuttings were also watered exclusively with tap water.

5.4.4 Gene knock-down causes reduced numbers of *G. pallida* in potato roots

To investigate whether or not effector homologue 4D06, the GPCR, putative effector protein and the unnamed protein play a role in parasitising potato plants, potato roots were infected with *G. pallida* J2s subjected to separate RNAi treatments to knock-down the expression of each of these genes. They were grown for seven days in glasshouse conditions. After this period, the roots were stained with Acid Fuchsin which allowed for the visualisation and counting of nematodes within the roots. This was replicated between seven and 10 infected plants per gene of interest and the mean number of nematodes per plant was calculated for each treatment group. Significantly reduced numbers of nematodes were seen in the roots of all the potato plants that had been infected with *G. pallida* J2s treated to knock-down the expression of the genes of interest compared to potato roots that had been infected with *G. pallida* J2s treated with the negative control (Figure 5.11).

Morphological differences were observed between the nematodes seen within the roots (Figure 5.12). It was possible to distinguish nematodes that were at the J2 stage of their life-cycle from those that were at the J3 stage (Figure 5.12). As a result, the numbers of nematodes at each life stage were also counted and compared to the negative control. A significantly lower proportion of nematodes in the roots of infected potato plants had

progressed to the J3 stage in populations of *G. pallida* that had been treated to knock-down the expression of the GPCR, putative effector protein or the unnamed protein compared to the control (Figure 5.13). Conversely, the knock-down of the effector homologue 4D06 in *G. pallida* had no effect on the proportion of J3 stage nematodes in the roots of potato (Figure 5.13).

Figure 5.10- Adzuki bean seeds watered with tap water grow better than those supplemented with Hoagland's solution after one week.

Adzuki beans were grown in pouches supplemented with either Hoagland's solution (top row) or water (bottom row). Seeds watered with tap water grew visibly better than those supplemented with Hoagland's solution.

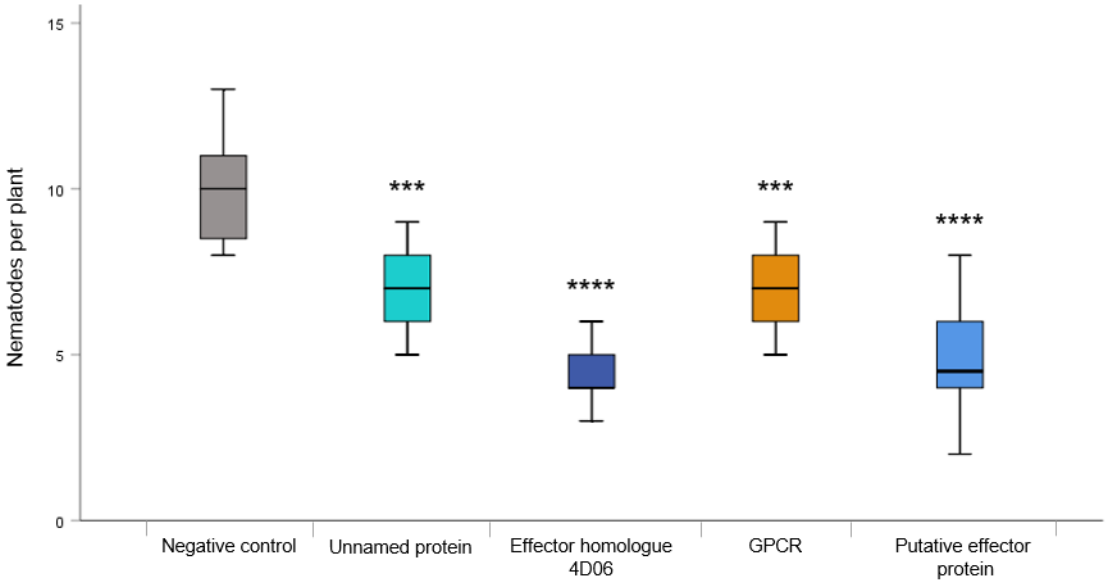

Figure 5.11- Knock-down of genes of interest by RNAi affects number of *G. pallida* in potato roots.

Genes of interest were knocked down in separate pools of *G. pallida* J2s using RNAi. Subsequent infection of potato plantlets revealed that nematodes treated as J2s to knock-down the expression of genes encoding the unnamed protein, effector homologue 4D06, the GPCR and putative effector protein were significantly reduced in number in the roots of potato plants after seven days compared to the control. The dark line in the centre of the boxplot represents the median number of nematodes per plant and the coloured box illustrates the interquartile range of the data set. The ends of the whiskers show the minimum and maximum number of recorded nematodes per plant. For effector homologue 4D06, the median line can be seen just above the bottom of the box indicating the interquartile range. Values are means (negative control n=7, effector homologue 4D06 n=9, unnamed protein, GPCR and putative effector protein n=10) +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** p≤0.01, *** p≤0.001) (One-way ANOVA, Dunnett's post hoc test).

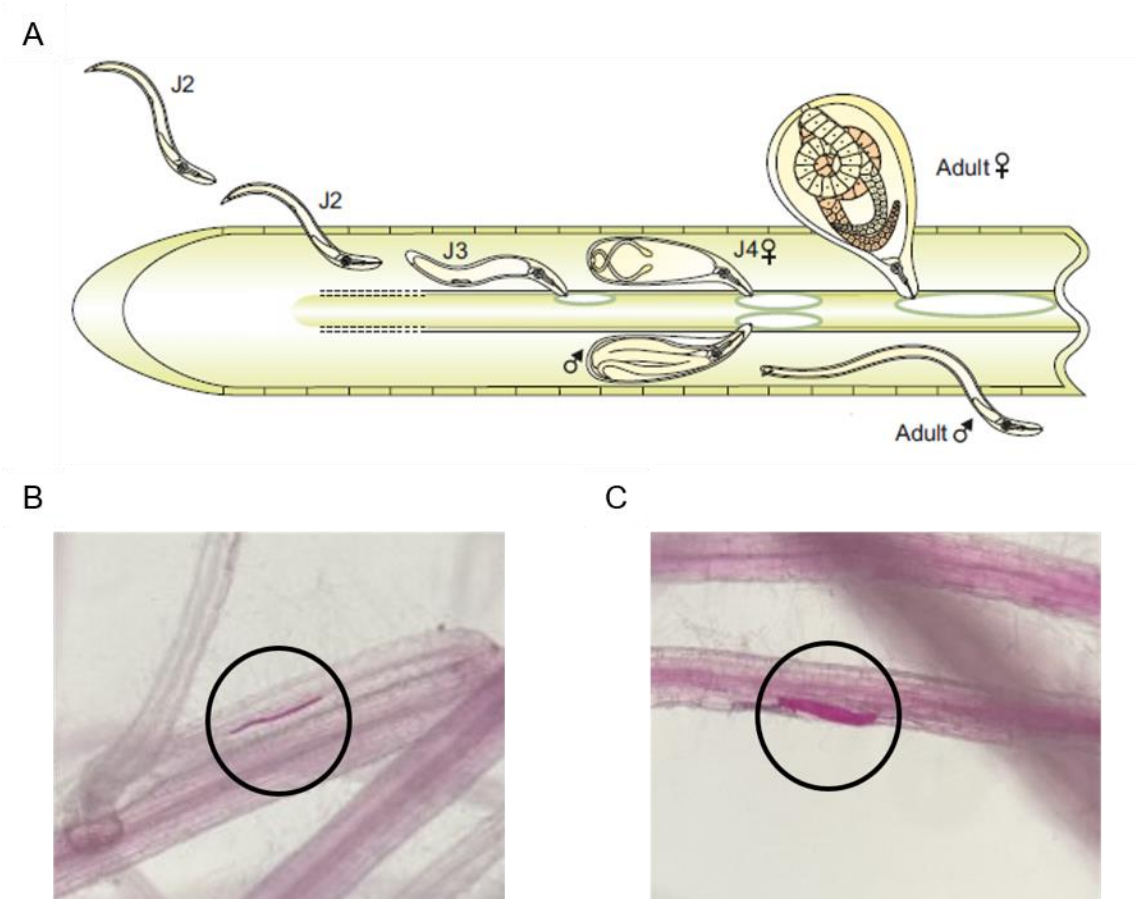

Figure 5.12- Morphological differences of *G. pallida* in the roots of potato plants.

A Cyst nematode life-cycle. *G. pallida* J2s migrate through the root to the vascular cylinder where they set up their permanent feeding sites. Here, they moult into J3 and J4 stages before becoming adults.

B) J2 stage *G. pallida* in the root of potato. Nematode matching the typical size and shape of a parasitic-stage J2 *G. pallida* in the root of potato plant.

C) J3 stage *G. pallida* in the root of potato. Nematode in the root of a potato plant matching the typical size and shape of a J3 *G. pallida* in the root of potato plant.

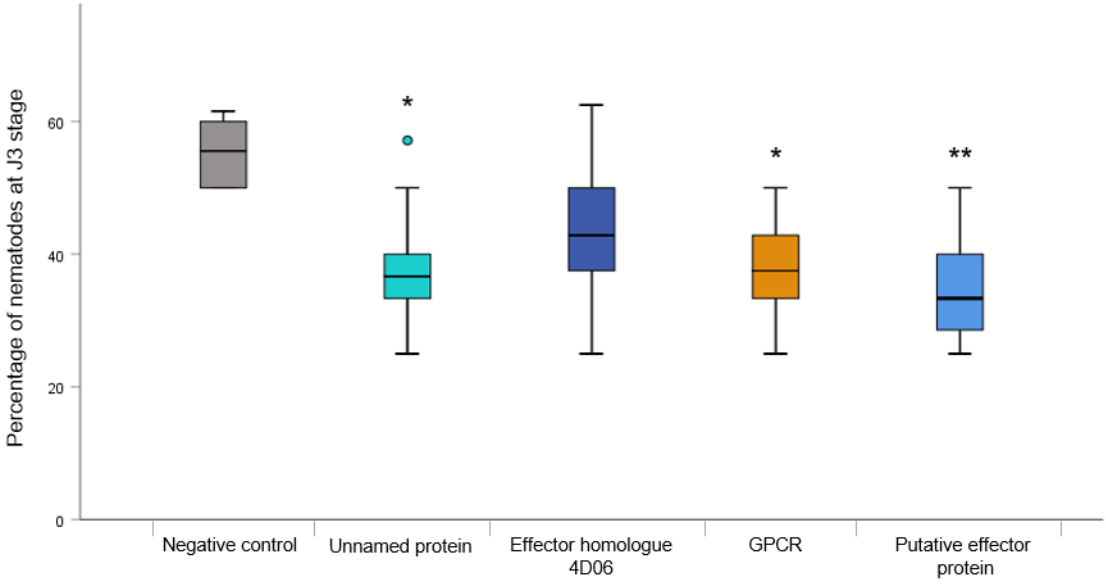

Figure 5.13- Knock-down of genes of interest by RNAi reduces the percentage of *G. pallida* progressing to the J3 stage in potato roots.

Genes of interest were knocked down in separate pools of *G. pallida* J2s using RNAi. Subsequent infection of potato seedlings with these nematodes revealed that those treated to knock-down the expression of genes encoding the unnamed protein, the GPCR and putative effector protein showed significantly reduced proportions of J3 stage nematodes in the roots of potato plants compared to the control. Populations treated to knock-down the expression of the 4D06 effector homologue showed no significant differences in the percentage of nematodes at the J3 stage in the roots compared to the control. The dark line in the centre of the boxplot represents the median percent of nematodes at J3 stage and the coloured box illustrates the interquartile range of the data set. The ends of the whiskers show the minimum and maximum percentage of nematodes at J3 stage, while the coloured circle (o) represents an outlier. Values are means (negative control n=8, genes of interest n=7) +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** $p \leq 0.01$, *** $p \leq 0.001$) (One-way ANOVA, Dunnett's post hoc test).

5.4.5 Gene knock-down causes reduced numbers of *M. incognita* in adzuki bean roots

To determine the role that the genes of interest played in parasitism, the expression of each one was knocked-down in separate *M. incognita* J2 pools using RNAi followed by infection assays. Compared to the negative control, significantly reduced numbers of nematodes were seen in the adzuki bean roots that had been infected with *M. incognita* J2s treated to knock-down the expression of the genes encoding the expansin-like protein and the GPCR (Figure 5.14). Reduced numbers of nematodes were also present for the other two genes tested but, likely due in part to high variability between plants, this reduction was not significant.

As with *G. pallida,* morphological differences were also observed in the roots of adzuki bean plants infected with *M. incognita* (Figure 5.15). Nematodes that were causing swelling in the root were clearly distinguishable under the microscope (Figure 5.15) and as a result the numbers of nematodes inducing this early gall formation were recorded. The proportion of nematodes causing the root to swell was compared between groups treated to knock-down the expression of genes of interest and the control group (Figure 5.16). A significantly lower proportion of nematodes that had been treated to knock-down the expression of all four of the genes tested caused root swellings in the roots of infected adzuki bean plants compared to the control (Figure 5.16). This was the case particularly for the avirulence protein and astacin genes, where RNAi did not cause a significant reduction in numbers of nematodes.

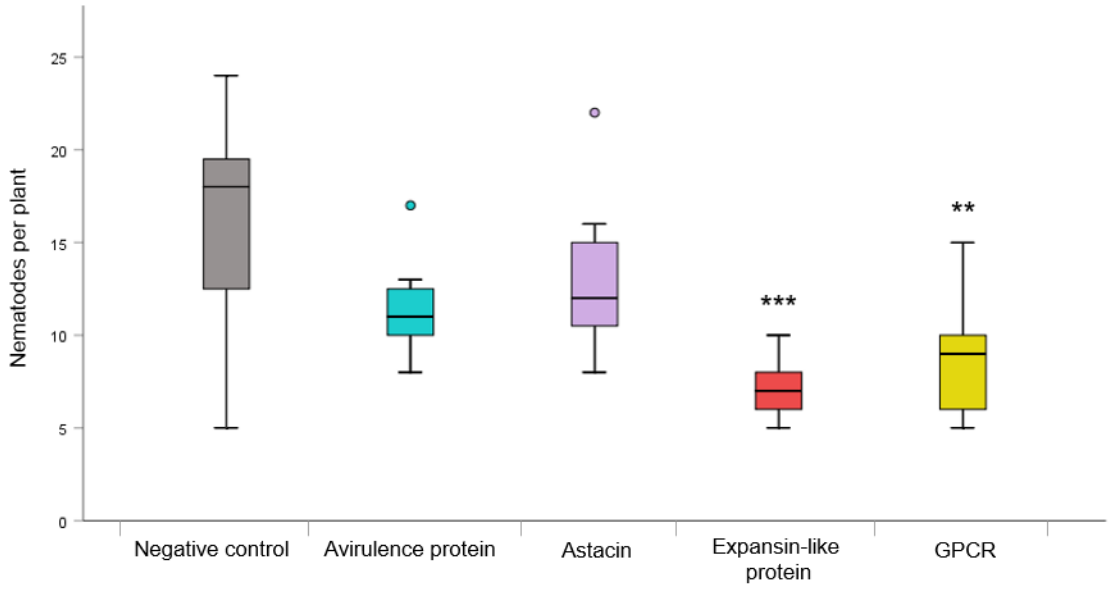

Figure 5.14- Knock-down of genes of interest by RNAi affects the ability of *M. incognita* to infect adzuki bean roots.

Genes of interest were knocked down in separate pools of *M. incognita* J2s using RNAi. Subsequent infection of adzuki bean seedlings revealed that nematodes treated as J2s to knock-down the expression of genes encoding the expansin-like protein and the GPCR were significantly reduced in number in the roots of plants after seven days compared to the control. Knock-down of the genes encoding the putative avirulence protein and astacin did not have a significant influence on the number of nematodes counted in the roots compared to the control. The dark line in the centre of the boxplot represents the median number of nematodes per plant and the coloured box illustrates the interquartile range of the data set. The ends of the whiskers show the minimum and maximum number of recorded nematodes per plant, while the coloured circles (o) represent outliers. Values are means (negative control n=7, effector homologue 4D06 n=9, unnamed protein, GPCR and putative effector protein n=10) +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** p≤0.01, *** p≤0.001) (One-way ANOVA, Dunnett's test).

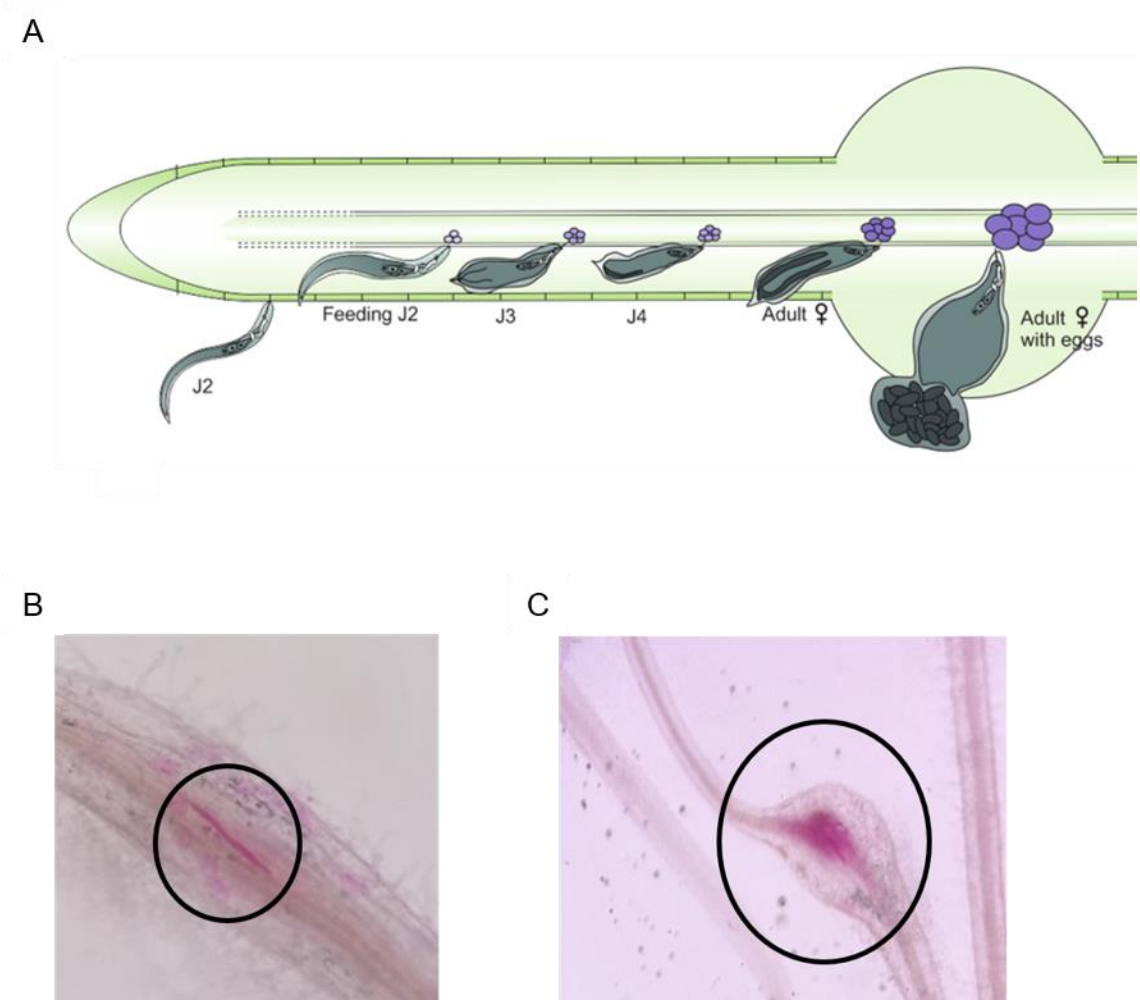

Figure 5.15- Differences in root morphology or *M. incognita* infected adzuki bean plants.

A) Root-knot nematode life-cycle. When inside the host root, *M. incognita* J2s migrate to the vascular cylinder where they set up their permanent feeding sites. Here, they moult into J3 and J4 stages before becoming adults.

B) J2 stage *M. incognita* in the root of adzuki bean. Nematode matching the size and shape of a typical parasitic-stage J2 *M. incognita* in the root of an adzuki bean plant. No swelling observed in the root.

C) *M. incognita* causing swelling in the root of adzuki bean. Nematode in the root of an adzuki bean plant matching the size and shape of a typical parasitic-stage J2 *M. incognita,* causing obvious swelling of the root indicating initiation of a feeding site.

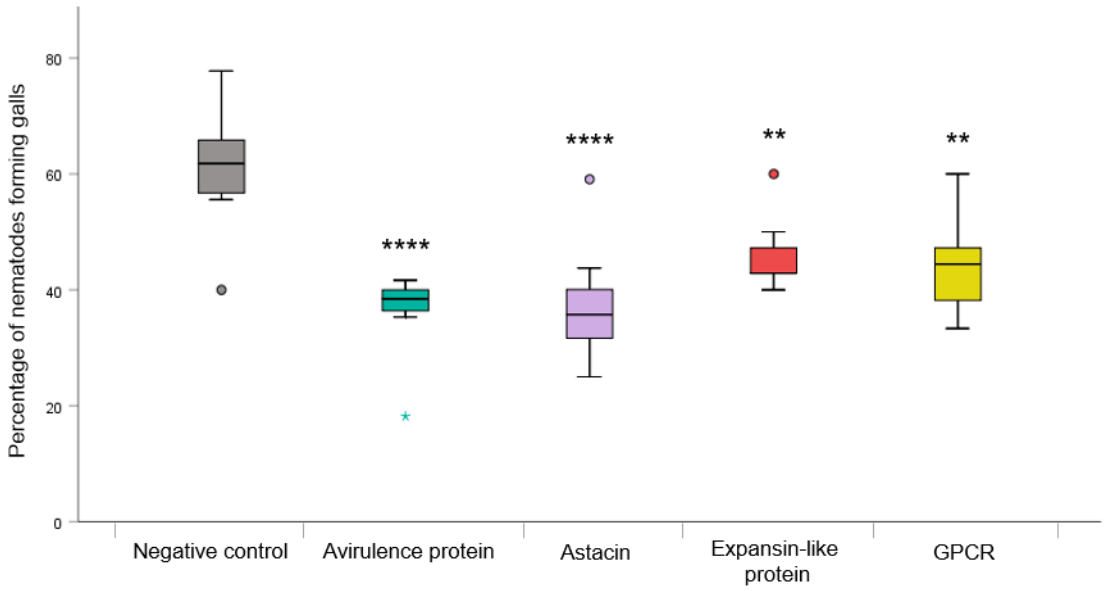

Figure 5.16- Knock-down of genes of interest by RNAi reduces the percentage of *M. incognita* forming root-knots in adzuki bean roots.

Genes of interest were knocked down in separate populations of *M. incognita* J2s using RNAi. Subsequent infection of adzuki bean seedlings with these nematodes revealed that populations that had been treated to knock-down the expression of genes encoding the putative avirulence protein, astacin, expansin-like protein and the GPCR showed significantly reduced proportions of nematodes forming root-knots or swellings in the roots of adzuki bean plants compared to the control. The dark line in the centre of the boxplot represents the median percentage of nematodes forming galls and the coloured box illustrates the interquartile range of the data set. The ends of the whiskers show the minimum and maximum percentage of nematodes forming galls, while the coloured circles (o) and coloured asterisks (*) represent outliers. Values are means (negative control n=8, genes of interest n=7) +/- SEM with asterisks (*) corresponding to significant differences between genes of interest and the negative control (** p≤0.01, *** p≤0.001) (One-way ANOVA, Dunnett's test).

5.5 Discussion

The results suggest that each of the genes of interest, with the exception of the putative oesophageal gland cell secretory protein in *M. incognita,* have potential roles in parasitism in *G. pallida* or *M. incognita.*

5.5.1 Spatial expression of genes of interest

Genes expressed within the pharyngeal glands of plant parasitic nematodes typically encode effector proteins which are subsequently secreted into the roots of their hosts (Vieira and Gleason, 2019). Here, they play an important role in manipulating the host to the nematode's advantage. The results in this chapter revealed that the genes encoding the unnamed protein and the putative effector protein are expressed in the sub-ventral pharyngeal glands of *G. pallida*. Although not as exclusive, there was also staining in this region associated with the 4D06 effector homologue gene. As spatial expression relates to gene function, these results suggest that the genes of interest are likely to encode effectors and as such, have roles in parasitism.

Similarly, the gene encoding avirulence protein in *M. incognita* was also found to be expressed in the sub-ventral pharyngeal glands of these nematodes which suggests that this gene may also act as an effector. Interestingly, the gene encoding astacin was found to be expressed mainly in the amphids of *M. incognita*. There is evidence to suggest that some effectors are expressed in the amphids (Rehman *et al*., 2009, Semblat *et al*., 2001), so this does not necessarily rule out the astacin as having an effector function in *M. incognita.* It is presumed that effectors produced in the amphids are not introduced into plant cells, however, they could function within the apoplast to suppress plant immunity or defence responses, as is the case in apoplast targeting effectors in the fungal pathogen, *Cladosporium fulvum* (Karimi-Jashni *et al*., 2022).

Nematode effectors have been shown to have a range of different functions from facilitating cell wall degradation to suppressing host immune responses, and enabling feeding site formation (Ali *et al*., 2015, Goverse and Smant, 2014, Rai *et al*., 2015a, Lilley *et al*., 2018). While spatial expression alone cannot determine the precise function of these genes of interest in *G. pallida* and *M. incognita*, it has provided insights into their overarching roles as effectors.

A clear hybridisation signal was not detected for certain target genes within both *G. pallida* and *M. incognita.* Specifically, the spatial expression of genes encoding GPCRs in both species remained undisclosed. This is presumably attributed to their expected dispersion across various regions within the nematodes. The visualisation of the spatial expression of the other genes may have been obscured either by their low

expression levels, or by their expression in tissues that are challenging analysis using *in situ* hybridisation.

5.5.2 RNAi using DsiRNA is effective in *G. pallida* and *M. incognita*

RNAi using Dicer-substrate small interfering RNAs (DsiRNAs) was utilised to knock-down the expression of the genes of interest in *G. pallida* and *M. incognita*. In this instance, DsiRNAs were employed as an alternative to conventional small interfering RNAs (siRNAs) or long double-stranded RNA (dsRNA) for RNAi, as they are reportedly more efficient (Snead *et al*., 2013). The mechanism of action for these 27-nucleotide DsiRNAs involves their direct interaction with the Dicer enzyme, facilitating the direct entry of DsiRNA into the RNA-induced silencing complex (RISC). This strategic interaction takes advantage of the inherent processing pathway, thereby enhancing the effectiveness of gene knock-down (Kim *et al*., 2005). In contrast, conventional 21-nucleotide siRNAs achieve gene knock-down without interacting with the Dicer enzyme. Instead, they mimic the products resulting from Dicer processing. This bypass of direct interaction with the enzyme leads to a reduction in the efficacy of the gene knock-down (Kim *et al*., 2005).

The expression of three of the genes of interest in *G. pallida* and four in *M. incognita* was successfully down-regulated using this technique. Gene knock-down by RNAi has been achieved in these nematodes before (Niu *et al*., 2012, Shingles *et al*., 2007, Sindhu *et al*., 2009, Urwin *et al*., 2002, Rosso *et al*., 2005), but this is the first time it has been achieved utilising DsiRNA in these species. The successful utilisation of this method provides a foundation for employing DsiRNA-mediated RNAi for gene knock-down in *G. pallida* and *M. incognita* in the future to investigate functional roles of many other genes.

5.5.3 Gene knock-down causes reduced numbers of nematodes in roots

Plant parasitic nematodes depend on effectors for the successful infection of their host plants. Many previous studies have demonstrated that knocking down the expression of genes encoding effectors in cyst and root-knot nematodes results in diminished parasitic success. This reduction is evidenced by fewer nematodes in the roots, degreased gall formation, lower numbers of adult females, and reduced egg mass production (Bakhetia *et al*., 2007, Banakar *et al*., 2020, Hada *et al*., 2020, Kumar *et al*., 2022, Kumar *et al*., 2017, Mani *et al*., 2020, Somvanshi *et al*., 2020, Tian *et al*., 2019). For example, host-induced gene knock-down of known effector genes in *M. incognita, Mi-msp10* and *Mi-msp23* led to 61 % and 51 % reduction in gall formation on Arabidopsis roots, respectively (Kumar *et al*., 2022). The knock-down of *Mi-msp10* also led to a 59 %

reduction in adult females and 76 % reduction in egg masses which subsequently reduced the reproductive factor of these nematodes by 92 % (Kumar *et al*., 2022).

The findings presented in this chapter indicate that knocking down the expression of the genes of interest in *M. incognita* also leads to a decrease in parasite success, by either reducing the numbers of nematodes in the roots, delaying nematode development, or both. Similar results were also seen when knocking down the expression of the genes of interest in *G. pallida.* This could highlight the importance of these genes in various parasitic processes. However, it is important to acknowledge that the knock-down of these specific genes may not fully explain the observed effects. In the case of *M. incognita,* direct RNAi-mediated knock-down of effectors *Mi-msp-1* and *Mi-msp-20* resulted in significant alterations in the expression of 20 and 409 other transcripts, respectively (Somvanshi *et al*., 2020). This cascading effect on the expression of other genes could potentially contribute to diminished parasitic capabilities of the nematode. A similar mechanism may underlie the reduced parasitic success observed in the results of this work with *M. incognita* and *G. pallida.*

It is not yet understood exactly how sensing of root exudate components leads to the altered gene expression in the pharyngeal gland cells that has been observed in *G. pallida* and *M. incognita*. As previously mentioned, serotonin signalling is involved in nematode behaviour and it is hypothesised that differential gene expression is also most likely attributed to neurological pathways. Stimuli are probably perceived by sensory neurones in the amphids and phasmids, and signalling pathways are activated to induce the changes observed. It is credible to suggest that G-protein coupled receptors might be involved in subsequent signal transduction as, compared to ionotropic receptors, the binding of neurotransmitters to G-protein coupled receptors, mediates slower and longer lasting effects, such as changes to transcription (Koelle, 2018). As this type of receptor responds to external stimuli to generate intracellular responses, it is likely that they are involved in the responses to the detection of root exudates observed in previous studies.

5.5.3.1 *G. pallida*

Numbers of *G. pallida* in the roots of potato plants were significantly reduced upon infection with nematodes whose gene expression had been targeted through RNAi. Specifically, knocking down the expression of genes encoding the unnamed protein, the 4D06 effector homologue, putative effector protein and the GPCR in *G. pallida* resulted in significantly diminished nematode numbers in addition to notable reductions were in the proportion of nematodes reaching the J3 stage. The decreased numbers observed may be attributed to a comprised infection ability, which could stem from reduced root penetration and impaired migration towards the vascular cylinder, or it could arise from

an impaired ability to establish or maintain the feeding site. These results contribute to the evidence provided by the spatial gene expression that the genes encoding the unnamed protein, as well as the 4D06 effector homologue, and putative effector protein could be effectors or are involved in the parasitism of *G. pallida*. Additional investigations are warranted to uncover the precise roles of these genes.

The knock-down in expression of the 4D06 effector homologue gene causes reduced numbers of nematodes in the roots without impacting the proportion of these nematodes developing to J3s. These findings suggest its exclusive involvement in the initial root penetration process. However, this gene is likely to belong to a family of 4D06 effectors, which were first identified in *H. glycines* (Gao *et al*., 2003). Genes from this family have previously been documented in *G. pallida* (Thorpe *et al*., 2014) and *G. rostochiensis* (Chen *et al*., 2023). While little is known about the function of these genes, their proposed role is in the formation and maintenance of syncytia (Eves-van den Akker *et al*., 2014a). This hypothesis gains further support from the observation that Gr29D06 genes, part of the 4D06 gene family in *G. rostochiensis,* are known to be expressed in the dorsal pharyngeal glands (Chen *et al*., 2023). Effectors expressed in this gland are known to be involved in the later stages of the parasitic process (Mitchum *et al*., 2013). Furthermore, genes in the 4D06 family are also present in *Nacobbus aberrans* (Eves-van den Akker *et al*., 2014a). Once this nematode reaches adulthood, it becomes a sedentary endoparasite, establishing a permanent feeding site within the roots of its host in the form of a syncytium (Jones and Payne, 1977, Vovlas *et al*., 2007). The absence of genes belonging to this particular gene family in root-knot nematodes, which do not form syncytia, provides additional evidence supporting their putative involvement in syncytium formation. This is interesting since the results of this work suggest that the knock-down of that particular 4D06 effector homologue in *G. pallida* impairs root penetration.

The gene annotated as an unnamed protein exhibits characteristic attributes of an effector protein. This is evidenced by its expression in the pharyngeal glands of *G. pallida* and the impact that knocking down its expression had on the number of nematodes in the roots of potato plants. The reduced number of nematodes, along with the lower proportion of J3s provides evidence to support the notion that this unnamed protein has a role in host infection. This protein lacks any known domains, and there are no known orthologues in other plant parasitic nematodes. Therefore, further investigations are required to reveal the precise molecular mechanisms that underlie the effector-like properties of this gene and its role in host-parasite interactions.

5.5.3.2 *M. incognita*

Knocking down the expression of genes encoding the expansin-like protein and the GPCR in *M. incognita* reduced the number of nematodes in the roots of infected adzuki bean plants. These findings suggest that these genes may have roles in the initial stages of infection, with the expansin-like protein likely acting to weaken the bonds in cellulose to facilitate host entry (Qin *et al*., 2004). As GPCRs are known to be involved in cellular signalling, the gene encoding the GPCR of interest in *M. incognita* is expected to contribute to signalling pathways associated with host infection and migration. The decrease in nematode population within the roots and the reduced formation of swellings following the knock-out of this gene suggest a potential disruption in the associated signalling pathway. However, further investigation is necessary to fully elucidate the specific functions of this gene.

Conversely, knock-down of the expression of the putative avirulence protein and astacin genes had no effect on the numbers of nematodes within the roots of infected adzuki bean plants after one week, implying that the products of these particular genes do not contribute to the initial penetration of the root. However, it is noteworthy that the knock-down of these genes does have an impact on the proportion of nematodes that successfully form swellings, or knots within the adzuki bean roots. In fact, knocking down the expression of all four of the genes of interest has this impact. This suggests that these genes could have potential roles in parasitism, encompassing functions from facilitating root migration to the suppression of host defences and the formation or maintenance of the feeding site. An alternative explanation is that the knock-down of these genes does have an effect on root invasion, but the effect of the RNAi treatment is not long lasting. In human embryonic kidney cells, gene knock-out using DsiRNA was demonstrated to last for up to 10 days (Kim *et al*., 2005). However, this duration may not be directly applicable in plant parasitic nematodes. It is conceivable that some J2 nematodes may recover from the knock-out after a few days, enabling them to subsequently initiate root invasion. Consequently, these nematodes might exhibit an apparent delay in their developmental progression. An indicator of whether or not this is the case could be obtained by assessing the nematode’s position in the root relative to the root tip.

The expansin-like protein is expected to lack enzymatic activity but may play a role in facilitating the weakening of cellulose bonds during infection. This action makes the plant cell wall more susceptible to degradation by cell wall-degrading enzymes. It is plausible that this protein also assumes a similar role during nematode migration through the roots towards the vasculature. As a result, the observed reduction in the number of nematodes

forming swellings upon knocking down of the expression of this gene could be attributed to their impaired ability to migrate towards the site where the syncytium is established.

In *C. elegans* and other nematode species, astacins are involved in cuticle biosynthesis, moulting and hatching (Davis *et al*., 2004, Möhrlen *et al*., 2003, Suzuki *et al*., 2004, Park *et al*., 2010, Martín-Galiano and Sotillo, 2022). It is possible that knocking down the expression of the gene encoding an astacin in *M. incognita* affects cuticle biosynthesis and therefore impacts the moulting process which could explain why fewer nematodes are observed forming swellings in the roots. Through the disruption of the moulting process, nematodes are unable to progress into J3 and J4 stages which are typically advanced enough to initiate the formation of swellings. However, the *in situ* hybridisation suggested that this particular astacin gene is expressed mainly in the amphids of the J2, so it is likely to have other roles earlier on in the parasitic process, such as sensing the presence of a nearby host, or suppressing host immunity in the apoplast. Further research is necessary to pinpoint the precise function.

The knock-down of the avirulence protein in *M. incognita* also leads to reduced proportions of nematodes forming swellings in the roots but does not seem to have a significant impact on the initial infection ability of the nematodes. Although this gene is annotated as a putative avirulence protein precursor in the *M. incognita* genome assembly (Blanc-Mathieu *et al*., 2017), further investigations aimed at elucidating its functions have revealed that it remains uncharacterised on WormBase Parasite. Blast searches using the amino acid sequence as the query sequence revealed that the avirulence protein shares its highest degree of homology with genes that also lack annotation. Nevertheless, it displayed some homology (40.0 % sequence ID) with *Meloidogyne* avirulence gene, *Mi-map-1.* Phylogenetic analysis suggests that *Mi-map-1* is closely related to genes encoding expansin-like proteins (Danchin *et al*., 2010). While the avirulence gene exhibits a certain degree of homology with *Mi-map-1,* it cannot be concluded that they are functionally equivalent based on this information. Further characterisation is needed to elucidate its precise function. This gene does however encode a signal peptide and displays characteristics consistent with a gland-secreted effector, suggesting its anticipated involvement in *M. incognita* parasitism.

The experimental RNAi-mediated knock-down of secretory protein 40 did not successfully down-regulate the expression of this gene in this work. In contrast, previous work has successfully knocked down its expression using *in-planta* RNAi (Niu *et al*., 2016). These discrepant outcomes may be attributed to the impact of the life stage on the susceptibility to the knockdown. In this work, pre-parasitic J2 nematodes were targeted experimentally, whereas in the work by Niu *et al*. (2016), the nematodes had already entered the feeding stage before being subjected to targeting. Examination

of RNA-Seq data concerning life stage expression (Choi *et al*., 2017) indicates that this particular gene displays relatively low expression levels in J2s compared to much higher expression levels in J3s and J4s. This observation may offer a plausible explanation for the observed absence of significant down-regulation in this study, as the initial expression levels may have already been inherently low.

5.5.4 Conclusions

The results confirm that a subset of the genes of interest chosen from the RNA-Seq analysis in both *G. pallida* and *M. incognita* have probable roles in parasitism. In *G. pallida* the spatial expression and broad function of a previously unnamed protein has been characterised. Further experiments such as protein purification and enzyme activity testing could be conducted in the future to determine its precise function. In addition, a role in signal transduction from sensing host presence to influencing gene expression has been eluded to for the chosen up-regulated GPCRs in both *G. pallida* and *M. incognita.*

It is important to functionally characterise genes as it provides insights into their biological roles and in this case, their roles in nematode parasitism. This contributes to the understanding of host-parasite interactions and the molecular mechanisms underlying this which could be utilised to inform nematode management strategies.

5.6 Summary

- Spatial expression of an unnamed protein, effector homologue 4D06 and putative effector protein was confirmed in the pharyngeal glands of *G. pallida.*

- Spatial expression of avirulence protein and expansin-like protein was confirmed in the pharyngeal glands of *M. incognita.* The expression of an astacin gene was seen in the amphids.

- The expression of the effector homologue 4D06, putative effector protein, the GPCR and putative effector protein in *G. pallida* was successfully knocked-down using DsiRNA-mediated RNAi.

- The expression of the avirulence protein, astacin, expansin-like protein and the GPCR in *M. incognita* was successfully knocked-down using DsiRNA-mediated RNAi.

- Knock-down of the genes of interest in both *G. pallida* and *M. incognita* caused reduced parasitic success.

- The spatial expression and broad function of a previously unnamed protein in *G. pallida* has been characterised.

Chapter 6
General discussion

To effectively parasitise their hosts, plant parasitic nematodes must first sense, and then respond to host-derived signals in the soil (Gang and Hallem, 2016). This work has shown that *G. pallida* and *M. incognita* both exhibit behavioural and transcriptional alterations in response to host-derived signals and that the observed responses are tailored to each host species. It has also characterised some of the genes that are differentially regulated in response to host-derived signals and uncovered their potential roles in parasitism. The enhanced knowledge of the host-parasite interactions of *G. pallida* and *M. incognita* could be utilised in the future to inform novel targeted control mechanisms against these economically important pests.

Previous studies that have investigated gene expression in *G. pallida* and *M. incognita* in response to host plants have not explored the impact of varying hosts and/or do not provide any gene-specific data (Kooliyottil *et al*., 2019, Kud *et al*., 2021, Teillet *et al*., 2013). This work provides comprehensive comparisons across different root exudate treatments and also provides detailed catalogues of differentially regulated genes within each treatment group.

Although not all of the differentially regulated genes could be analysed in detail during this project, a comprehensive repository has been established cataloguing the differential gene expression patterns in *G. pallida* when subjected to carrot, maize, and potato root exudates, and in *M. incognita* when subjected to cabbage, carrot, maize, potato, and tomato root exudates. This resource now provides the opportunity for more extensive investigations to be carried out into the host-specific responses observed.

6.1 Transcriptional responses of *G. pallida* and *M. incognita* in response to host-specific cues

The analysis of altered gene expression in J2s exposed to varying root exudates has provided new insights into the transcriptional responses of *G. pallida* and *M. incognita* to host detection. Expanding on the results of Bell *et al*. (2019), where the host-specific expression of two genes encoding cell wall-degrading enzymes was reported in *P. coffeae*, *G. pallida* and *M. incognita* have been shown here to exhibit differential regulation of similar genes in a host-specific manner. In a broader gene expression analysis, root exudate from varying host plant species was found to elicit a unique transcriptional response in both *G. pallida* and *M. incognita*, implying that these nematodes tailor their responses to host root exudates.

Notably, *G. pallida* showed a very strong transcriptional response to root exudate from its favoured host, potato, while displaying comparatively minor responses to maize and carrot root exudates. This may suggest that *G. pallida* possesses the ability to distinguish between host and non-host environments by detecting the distinct composition of root exudates across different plant species (Barber and Martin, 1976, Bertin *et al*., 2003). Interestingly, no biological processes were significantly over-represented among the genes that were differentially regulated in response to potato root exudate. This could imply that host detection does not significantly influence any specific biological process and highlights the individual importance of certain genes in infection for this species.

In contrast, although there was very little overlap in the genes that were differentially regulated, the scale of the transcriptional responses induced by individual root exudate treatments in *M incognita* were much more comparable. Like *G. pallida,* potato root exudates also induced the strongest transcriptional response in *M. incognita.* This observation was unexpected, given that among the various root exudates to which this nematode was exposed, tomato is considered its most favourable host (Sikora and Fernández, 2005). Possible explanations for this include the presence of distinctive chemical compounds within potato exudates, that are not present in the root exudates of the other plants, capable of triggering a strong nematode response. For example, the root systems of potato plants encompass a diverse range of secondary metabolites including glycoalkaloids, calystegine alkaloids, protease inhibitors, lectins, phenolic compounds, and chlorophyll (Friedman, 2006). While some of these compounds may be found in other plant roots to some extent, the specific combination and concentration of the secondary metabolites in potatoes are unique to this plant species. Similar to the requirement of a combination of root exudate components for significant hatching of cyst nematodes (Bell *et al*., 2021), the distinct combination of compounds in potato root exudate could be responsible for eliciting the robust responses observed in response to this root exudate.

Alternatively, it could be the result of *M. incognita* J2s being sourced from the roots of tomato plants, potentially masking the true extent of the impact exerted by this root exudate on the nematodes. *M. incognita* J2s have previously demonstrated habituation towards potato host root exudates, as evidenced by their reduced attraction towards this root exudate in subsequent bioassays (Fleming *et al*., 2017). It is therefore plausible that the *M. incognita* J2s used in this work have developed a habituation response towards specific tomato cues during their rearing on tomato roots, resulting in a transcriptional response, that was not as robust as initially anticipated, when exposed to root exudate from the same species. The constant exposure to tomato-specific cues may have allowed the nematodes to become habituated. Habituation to the compounds within the

tomato root exudates could involve changes in the nematode's neurophysiology, such as alterations in the sensitivity of chemosensory receptors or neurotransmitter levels. Further investigations could reveal the exact mechanisms through which plant parasitic nematodes detect, and orchestrate their responses towards, root exudates.

6.2 Regulation of host-specific gene expression

The mechanisms by which the transcriptional plasticity observed in these plant parasitic nematodes is achieved remain elusive. It is plausible that signalling pathways, comprising receptors, kinases, and other signalling molecules, relay host cues to the nucleus, where they activate or repress transcription factors. In this context, it is plausible that GPCR signalling mediates differential gene expression, as these receptors are at the interface of neurons and intercellular responses. In *C. elegans,* endogenous neuropeptide ligands binding to G-protein coupled pigment dispersing factor (PDF) receptors play an important role in both sensing external stimuli and in governing locomotion (Janssen *et al*., 2008). This observation suggests that GPCRs could be involved in the sensing of host-derived signals from the external environment by plant parasitic nematodes. Further evidence comes from studies on the root-knot nematode, *M. graminicola,* where the disruption of neuropeptides involved in GPCR-mediated signalling resulted in impaired behavioural patterns and migration, particularly in the context of infection (Bresso *et al*., 2019). This suggests that GPCR signalling could play a role in the nematode infection process. Among the significantly up-regulated genes in both *G. pallida* and *M. incognita* in response to various root exudates were numerous genes encoding GPCRs and neuropeptides. Examples include genes annotated as neuropeptide receptors, FMRFamide receptors, or neuropeptide-like peptides. This suggests that neuronal signalling pathways are triggered after root exudate detection in these nematodes, which reinforces the notion that these signalling pathways could play an important role in regulating gene expression during infection.

The gene-specific data indicated that a substantial portion of the up-regulated genes in response to the various root exudate treatments in both *G. pallida* and *M. incognita* comprised genes responsible for producing cell wall-degrading enzymes and other effectors. Genes of this nature are involved in nematode parasitism and they are typically expressed in the dorsal or sub-ventral pharyngeal glands (Rehman *et al*., 2016, Vieira and Gleason, 2019). Specific DNA motifs in the promoter regions of effector genes are believed to regulate the coordination of their expression, at least with regards to tissue-specificity. These include the dorsal gland (DOG) box in *G. rostochiensis* (Eves-van den Akker *et al*., 2016), the STATAWAARS promoter motif in *B. xylophilus* (Espada *et al*., 2018), and the Mel-DOG

in root-knot nematodes (Da Rocha *et al*., 2021). Given the roles of these genes, their promoter motifs may also serve as targets for transcription factors activated by signalling pathways triggered by a number of external cues. This could be the detection of a nearby host, or the recognition of the necessity to initiate the formation of feeding the site. Indeed, a transcription factor responsible for regulating sub-ventral gland expression (SUGR) in cyst nematodes, and that responds to root extract, has been recently identified (personal communication, S. Eves-van den Akker).

Most genes are likely to have several transcription factor binding sites or conserved motifs in their promoter regions, that are responsible for different aspects of their expression. For example, an effector gene could plausibly have one motif within its promoter region that interacts with a transcription factor to direct gland cell expression, and another that interacts with a different transcription factor to respond to environmental or developmental cues.

In the context of this work, the detection of specific root exudates could be activating signalling pathways that subsequently target transcription factors to the promoter motifs of genes in order to facilitate the coordinated up-regulation of the numerous effector genes observed in *G. pallida* and *M. incognita.* However, only a subset of the genes that were found to be differentially regulated in *G. pallida* and *M. incognita* are likely to be expressed in the pharyngeal gland cells. Future work could therefore focus on identifying other common promoter motifs in the differentially regulated genes in these nematode species. The likelihood of a single universal promoter motif governing the coordinated up- or down-regulation of all differentially expressed genes is low. It is plausible that distinct promoter motifs control the expression of effector genes, those associated with neuronal signalling, and the various other biological and molecular processes influenced by the detection of root exudates. Uncovering these distinct common promoter motifs would provide valuable insights into the mechanisms underlying the transcriptional responses seen in *G. pallida* and *M. incognita.*

6.3 Transcriptional adaptations of other plant associated organisms in response to different plants

This work showed that *G. pallida* and *M. incognita* undergo distinct changes in gene expression upon the detection of host-specific cues, with their responses being specific to the particular host detected. Transcriptional plasticity is also seen in a range of other eukaryotic organisms associated with plants when they colonise their hosts. Research suggests that on average, these organisms, including both parasites and mutualists, alter the expression of roughly 11 percent of their genes upon recognising a host plant (Petre *et al*., 2020). This phenomenon is widespread, occurring in insects, fungi and

even parasitic plants. Many of the genes that undergo differential regulation encode effector proteins, which modify the host's structure to benefit the organism itself (Petre *et al*., 2020). The intricate molecular mechanisms that underpin transcriptional plasticity in response to host cues are largely unknown. In addition to the mechanisms previously discussed, it can be speculated that transcriptional alterations could stem from epigenetic modifications, including DNA methylation and histone modification, as well as hormone signalling or potential metabolic re-programming. Understanding these mechanisms is essential for understanding how plant associated organisms, including plant parasitic nematodes, adapt and respond to changing environmental conditions, such detecting the presence of nearby host plants.

Like plant parasitic nematodes, insects have developed dependencies on plants for their survival. Aphids are highly specialised herbivores that cause damage to various plant species by feeding on their sap. A number of aphids have been shown to exhibit significant changes in gene expression in response to different plants which may be attributed to their ability to colonise a wide variety of plant species (Eyres *et al*., 2016, Lu *et al*., 2016, Mathers *et al*., 2017). Similarly, the significant and varied transcriptional responses observed in *M. incognita* upon exposure to different host plant root exudates could also be attributed to this nematode's ability to infect a wide range of hosts. This nematode may be employing distinct sets of genes to overcome the diverse physical barriers presented by different hosts, or could be priming itself for adaptation within the varying root systems of these hosts, potentially contributing to the distinctive responses observed in each treatment.

In the generalist aphid, *M. persicae,* rapid transcriptional alteration of aphid-specific multi-gene families, potentially driven by epigenetic mechanisms, was observed when it was transferred to a different host plant (Mathers *et al*., 2017). In various parasitic species, including nematodes such as *Strongyloides*, expansions of lineage-specific gene families have been associated with the broadening of their host range (Hunt *et al*., 2016). Previous literature searches conducted in the context of this research revealed that *M. incognita* possesses at least 21 genes encoding cell wall-degrading enzymes that belong to the GH5 family (Abad *et al*., 2008, Bozbuga *et al*., 2018). In contrast, *G. pallida* has only 15 genes belonging to this same family (Thorpe *et al*., 2014). The increased number of GH5 family genes in *M. incognita* may signify a gene family expansion within this species, which could serve as an alternative explanation for its ability to adopt a larger host range. A larger gene family can encompass a broader range of enzyme variants, each potentially optimised for different substrates or conditions. This diversity could allow *M. incognita* to selectively target the various cell wall components that it is faced with, providing another explanation for the

ability of this nematode to parasitise a broad spectrum of hosts. Notably, several genes belonging to the GH5 family exhibited significant up-regulation in both *G. pallida* and *M. incognita* in response to varying root exudate exposures. This observation highlights their important roles parasitism and provides an insight into why an extensive repertoire of such genes is crucial for nematodes that infect broad spectrum of host ranges. Similarly, the leaf rust fungus, *Melampsora larici-populina,* is also known to differentially regulate genes within the glycosyl hydrolase families in response to detection of its hosts (Lorrain *et al*., 2018). This implies that genes involved in cell wall degradation play important roles in targeting host plants, spanning multiple species.

Fungi also form a range of mutualistic and pathogenic interactions with plants. Mutualistic fungi, such as mycorrhizae, form beneficial partnerships with plants, aiding nutrient uptake. In contrast, pathogenic fungi like rusts and mildews cause plant diseases, impacting agricultural yields. Many fungal pathogens also differentially express genes when infecting their host plants (Allan *et al*., 2019, Enguita *et al*., 2016, Shahid, 2020). For example, a filamentous fungal pathogen of maize and soybean, known as *Fusarium virguliforme,* demonstrates distinct gene expression patterns that are influenced by the specific host it interacts with (Shahid, 2020). Little overlap was found in the genes that are differentially expressed in the fungus when it infects maize compared to soybean roots (Shahid, 2020). This mirrors the findings in *G. pallida* and *M. incognita,* which also display distinct gene expression patterns when they encounter to different host species. This suggests that tailoring transcriptional responses to specific hosts is not exclusive to plant parasitic nematodes but is likely a widespread strategy employed by most plant-associated organisms.

A number of pathogenic insects and fungi have been shown to differentially regulate genes that encode detoxification proteins in response to host detection or colonisation. For example, *Zymoseptoria tritici,* a fungal pathogen of wheat was found to differentially express genes encoding detoxification proteins and proteins responsible for maintaining redox balance during infection of its host (Kellner *et al*., 2014). In addition, insects including the Oleander aphid (*Aphis nerii*), the Monarch butterfly (*Danaus plexippus*) and the spider mite (*Tetranychus urticae*) have also demonstrated up-regulation of genes that encode detoxification proteins when encountering hosts that produce potentially harmful compounds (Birnbaum *et al*., 2017, Grbić *et al*., 2011, Tan *et al*., 2019). Furthermore, the pinewood nematode, *B. xylophilus,* was shown to up-regulate genes involved in detoxification metabolism in response to the secondary metabolite, β-pinene, found in the roots of pine trees, (Li *et al*., 2020).

In the context of this research, *M. incognita* was shown to significantly up-regulate genes that may be involved in the nematode stress response upon exposure to potato, but not

other root exudates. This was presumed to allow these nematodes to overcome the toxic effects of glycoalkaloids and/or protease inhibitors which are exclusive to potato roots (Dunse *et al*., 2010, Sivasankara Pillai and Dandurand, 2021, Turrà *et al*., 2020). The up-regulation of these types of genes in fungi, insects and nematodes is presumed to facilitate their continued ability to establish and thrive within the preferred host environments.

As discussed in Chapter 4, protease inhibitors and secondary metabolites including potato-specific glycoalkaloids potentially present in potato root exudates could be responsible for inducing the stress responses in these nematodes that are not seen in response to other root exudates (Dunse *et al*., 2010, Sivasankara Pillai and Dandurand, 2021, Turrà *et al*., 2020). Beyond these specific compounds, potato roots also contain multiple other specific secondary metabolites that are not commonly found in the roots of other plants. The responses seen could potentially be the result of the detection of any number of these. Patatins, for example are a family of soluble glycoproteins present in potatoes (Mignery *et al*., 1988) and they are known to play a role in defence against insect herbivores (Srivastava, 2002, Shewry, 2003, de Souza Candido, 2011). These glycoproteins have been shown to be present in moderate concentrations in the roots of potato plants (Pikaard *et al*., 1987), and could therefore also play a role in defending against other pests. Therefore, they could be responsible for triggering the up-regulation of some of the genes involved in the stress responses seen in plant parasitic nematodes upon detection of potato root exudate.

6.4 Future research opportunities

The opportunity for further exploration of the molecular mechanisms involved in host-parasite interactions of plant parasitic nematodes could not be pursued as extensively as was desired during this project. However, this has led to exciting prospects for future research endeavours.

In Chapter 3, a subset of cellulase and xylanase genes in *G. pallida* and *M. incognita* were selected for investigation. These genes were chosen because of the study's focus on investigating genes that were similar to those found to be up-regulated in *P. coffeae* J2s in response to root exudate (Bell *et al*., 2019). The majority of the selected genes exhibited their highest expression levels during the J2 stage of the nematode's life-cycle. However, GPLIN_000755200 displayed its peak expression during the male stage of *G. pallida's* life-cycle. In this species, males are known to utilise cell wall-degrading enzymes for exiting the root. Had time constraints not been a limiting factor, the expression of this, and other genes with peak expression in male nematodes, would have been analysed in response to root compounds during this project. This analysis

would have aimed to explore variations in gene expression among different life stages in response to the root environment. This could have also been extended to encompass other life stages, such as J3 and J4 stage nematodes, which encounter varying conditions in the root environment during the formation and maintenance of the feeding site. Generating root extract to expose these nematodes to, rather than root exudate, would provide a more accurate representation of the natural conditions experienced by these nematodes. In the future, this could be extended to other species, perhaps focussing first on *M. incognita* and *P. coffeae*.

In addition, differential gene expression patterns were observed in response to exposure to root exudates from varying plant species. Given the unique elicited responses exhibited by each nematode species when exposed to each root exudate, it would be interesting to determine the precise bioactive components responsible for these effects. To pursue this, root exudates could be fractionated using filtration, followed by the quantitative assessment of gene expression in J2 nematodes, exposed to these fractions, by qRT-PCR. Fractions responsible for differential regulation could then undergo metabolite analysis, such as untargeted metabolomics or mass spectrometry, to determine the specific constituents responsible for observed the priming effects. This may then offer insights into the underlying reasons for the differential responses of nematodes to the exposure of root exudates from different host plants.

Whilst the results discussed in the previous chapters only consider the responses of *G. pallida* and *M. incognita* to root exudate detection, it would be valuable to extend these investigations to encompass other species of plant parasitic nematodes. Broadening the scope of study to include other economically important nematode species, perhaps with different parasitic lifestyles, could offer insights into the commonalities and distinctions within their interactions with host plants. Identifying these similarities and differences would increase our understanding of host-parasite interactions and could go on to inform species-specific control strategies.

6.5 Conclusions

The molecular mechanisms of the host-parasite interactions of *G. pallida* and *M. incognita* have been explored in detail. Here, the results show that when exposed to root exudates from varying host and non-host plants, both *G. pallida* and *M. incognita* show altered behaviour and gene expression patterns. These changes observed appear to be host-specific, with each plant eliciting a unique response. These results align with the previous findings in *P. coffeae* (Bell *et al*., 2019), which provided the foundation for this research. The knock-down of selected up-regulated genes in each of these species affected the nematode's ability to infect its host plant by either reducing the number of

nematodes able to infect the plant, influencing nematode development, or both. This suggests that nematodes differentially regulate their genes in order to maximise parasitic success.

Understanding the molecular mechanisms that underpin the host-parasite interactions of these nematode species is important as it provides insights into the fundamental biology of both nematodes and plants which could have broader applications in areas such as plant breeding and crop improvement. In addition, gaining insights into these mechanistic processes could provide valuable information for the development of strategies aimed at controlling and managing these pests. This could ultimately lead to the mitigation, and in some cases, the eradication of nematode infestations, thereby mitigating their detrimental impacts on agricultural yields.

Chapter 7

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