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Identification and characterization of PAMPS/NAMPS released by nematodes

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DEDICATION

For you Dad

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LIST OF ABBREVIATIONS

PPN	Plant parasitic nematodes
PTI	Patter triggered immunity
ETI	Effector triggered immunity
PAMPS	Pathogen associated molecular patterns
NAMPS	Nematode associated molecular patterns
DAMPS	Damage associated molecular patterns
ROS	Reactive oxygen species
ADAM-10	A disintegrin and metalloproteinase domain-containing protein 10
qRT-PCR	quantitative real time polymerase chain reaction
dpi	days post infection

COVID-19 PREAMBLE

The main goal of the study was to screen for putative nematode associated molecular patterns involved in activating plant basal defense. Using the sugar beet cyst nematode (*Heterodera schachtii*) as model organism, we intended to check expression of different candidate genes selected from the NemaWater protein analysis data against the recently sequenced (*H. schachtii)* transcriptome data available at the host institute and validate the expression of these genes in nematodes using qRT-PCR. Further in the work package was to make transgenic plants with inducible promoter overexpressing some of the interesting candidate genes using the ß-estradiol-inducible expression with the *XVE/OlexA* inducible system. The next step would have been to perform nematode infection assays with the transgenic lines, phenotype screening and defense gene expression analysis. Furthermore, we were going silence the genes in the nematode using the RNAi silencing system and then use the J2s with silenced target genes for infection assays and phenotype screening. Due to Covid-19, the institute was closed and when it was opened again, we only managed to validate expression of four out of the seven candidate using qRT-PCR. The remaining three candidate genes needed primers to be redesigned. Therefore, this thesis presents an extended literature review on the thesis topic, transcriptomic and qRT-PCR data of selected candidate genes.

This preamble has been prepared in consultation with the student and the supervisor and approved by *both*

ABSTRACT

Plants defend themselves against pathogenic microbes including nematodes with the help of an evolutionary conserved innate immune system. Plant innate immunity relies on the recognition of conserved pathogen associated molecular patterns (PAMPS) by surface localized pattern recognition receptors which lead to activation of defense responses known as pattern triggered immunity or PTI. The role of PTI has been well described for many pathogens including bacteria, fungi, and in insect pests. However, little is known about PTI in plants-nematodes interaction. So-far, ascarosides, signalling molecule widely conserved among different types of nematodes are the only predicted nematode associated molecular patterns (NAMPS). Previous studies have described the activation of PTI responses in *Arabidopsis thaliana* by a nematode aqueous *diffusate* termed NemaWater. Plant roots treated with NemaWater activated defense responses such as reactive oxygen species (ROS-burst) generation, plant growth inhibition, and defense gene expression. A proteomic analysis of NemaWater revealed many proteins, mostly nematode surface-associated proteins which gives them a higher probability of being recognized by the plant as NAMPS. To further screen for putative elicitors, we selected proteins that were high in peptide counts and confidence score for reliability and researched on their function in different organisms as well as their role in host defense modulation. Candidates were Enolase, Cuticlin, Heat shock protein 90/Endoplasmin, 14-3-3 protein, ADAM-10 metallopeptidase, Tetraspanin, Heat shock protein 70/Actin 5C. The transcriptome data of these candidates at different time points of infection and *H. schachtii* life cycle is available and we determined whether the expression levels of candidate proteins correlated with the abundance of transcriptional expression levels. Furthermore, a gene expression analysis was done at 10 and 48 hours post infection using qRT-PCR. Transcriptome data showed that all candidates are expressed in nematodes in both pre and parasitic stages. Expression varied with time and the results of the qRT-PCR showed that the candidates are all expressed in J2, 10 and 48 hours post infection. In conclusion, the proteins found in NemaWater have coding genes in the nematode and are suitable for further characterization as NAMPs. Identification of NAMPS in nematodes and corresponding receptors in plant will serve as valuable information for engineering plants for broad resistance and increased yield.

Keywords: PAMPS, NAMPS, Enolase, transcriptome, *H. schachtii*, Plant parasitic nematodes

1. INTRODUCTION

1.1 Background

Food insecurity in the global populations has been increasing over the recent years and one of the main causes of this trend is a multitude of the crop pests and diseases. Besides reduction of food quantities, plant pathogens also decrease the quality of food hence an overall decline in human health. Like any other group of pathogens, plant parasitic nematodes (PPNs) lead to heavy and costly losses in agricultural crops (Bernard et al., 2017). Over 4,100 plant parasitic nematode (PPN) species (Decraemer& Hunt, 2006) have been discovered and these directly attack mostly plant roots preventing water and nutrients uptake. The overall consequence is a reduction in quality and quantity of crop yields. PPNs attack a wide range of commercially important crops including rice, maize, wheat, potato, tomato, cotton, soybean, sugar beet among others (Elling, 2013). Apart from direct damage on crops, PPNs form disease-complexes with other pathogens increasing the crop yield losses. Plant-parasitic nematodes have devastating effects on agricultural crops, being responsible for 10% of total losses (Savary et al., 2012). These losses can be translated to approximately $80–$118 billion dollars per year as reported by Nicol et al. (2011) while Singh et al. (2015) estimated the losses to $US157 billion annually worldwide. Another report estimated nematodes to cause an annual loss of roughly 100 billion dollars in crops, despite all the efforts invested towards nematode control and management (Coyne et al., 2018). Nonetheless, these are extremely high losses that need to be prevented by all means necessary to ensure global food security. Yield loss estimates are highly likely to be biased because most farmers particularly in developing countries are not aware of the problem due to the asymptomatic nature of nematode symptoms (Coyne et al., 2018).

The most important groups of nematodes among PPNS are root-knot nematodes belonging to *Meloidogyne* genus followed by cyst nematodes (Jones et al., 2013). Cyst nematodes originated from South America, but widely spread and a quarantine nematode in major potato growing regions (Hockland et al., 2012). Losses can exceed 90% in conducive environments for nematode infection and worldwide potato losses of 9% (Nicol et al., 2011). Success of PPNs is attributed to their occurrence in large numbers, ability to survive in soils for prolonged periods without host plant. For instance, potato cyst nematode stays for up to 20 years in the soil till environmental conditions become favourable (Grainger, 1964) consequently, control and eradication becomes difficult.

Plants are constantly challenged by many kinds of biotic agents such as bacterial, fungal, viral and nematode pathogen as well as insects; and therefore, have evolved constitutive and induced defense system against them. Plants have a complex and advanced defense system which ranges from simple mechanisms such as cell wall reinforcements, secretion of toxic metabolites, to induced or basal plant immunity activated by virulent pathogens on susceptible host plants (Bigeard et al., 2015). Host plants develop immune and defense mechanisms against plant pathogens. The system was summarized in the zig-zag model by (Jones & Dangl, 2006). Basal defense occurs when plants recognize pathogens by perceiving conserved pathogen-associated molecular patterns (PAMPS) through membrane localized pattern recognition receptors (PRRs) thereby inducing PAMP triggered immunity (PTI) (Dodds & Rathjen, 2010). Plants can also sense cellular damage caused by pathogens through recognition of plant derived damage-associated molecular patterns (DAMPs) by PRRs. These receptors are membrane-localized receptor-like kinases (RLKs), consisting of a ligand-binding ectodomain, a single-pass transmembrane domain and an intracellular kinase domain or receptor-like proteins (RLPs), without kinase domain. The recognition of conserved PAMPs by PRRs triggers a cascade of intracellular responses such as mitogen-associated protein kinases (MAPKs) activation, production of reactive oxygen species (ROS), Ca^{2+} burst, transcriptional reprogramming, hormones biosynthesis, and deposition of callose to strengthen the cell wall (Bigeard et al., 2015). However, in compatible interactions pathogens including plant parasitic nematodes release effector proteins to suppress PTI. These effectors are recognized by intracellular nucleotide-binding domain leucine-rich repeat (NLR) receptors, leading to effector-triggered immunity (ETI) and a consequent hypersensitive response-induced cell death (Jones & Dangl, 2006).

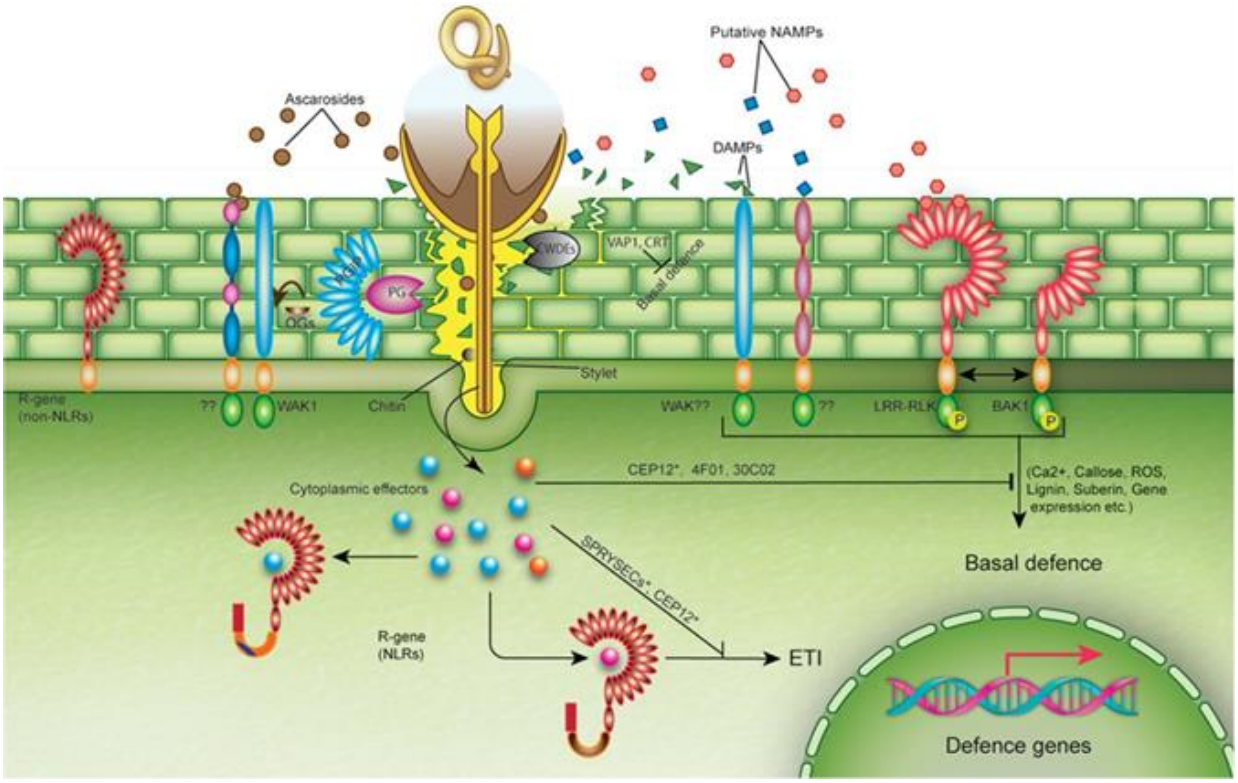

Figure 1.1 A summary of molecular players involved in plant immune responses after nematode attack (Holbein et al., 2016).

Invasion of the plant by nematodes causes damage of the cell wall which produces damage-associated molecular patterns (DAMPs). These DAMPS are recognized by DAMP-receptors leading to activation of plant defense responses. Nematodes can also secrete enzymes to degrade cell walls (e.g. polygalacturonase, PG) but the plant in turn produces polygalacturonase-inhibiting proteins (PGIPs) (Figure 1.1). The interaction of these two molecules promotes the formation of oligogalacturonides (OGs) which are DAMPS. Perception of PAMPs by plants occurs via LRR-RLKs and co-receptor Brassinosteroid Insensitive 1-Associated Receptor Kinase 1 (BAK1) as co-receptors (Chinchilla et al., 2007). Nematodes effectors can either be secreted via the apoplastic (e.g. VAP1, CRT), or the cytoplasmic pathway (e.g.CEP12) (Chronis et al., 2013; Jaouannet et al., 2013). Apoplastic effectors have the ability to interfere with PAMP/DAMP detection by PRRs while cytoplasmic effectors can hinder the downstream signalling response of PTI (Holbein et al., 2016).

To date, several other microbe PAMPS have been described and these include bacterial flagellin (Felix et al., 1999), lipopolysaccharide (Dow et al., 2000), peptidoglycan, elongation factor Tu, fungal chitin (Felix et al., 1993). Specifically, the molecules released by nematodes are called NAMPS for nematode-associated molecular patterns and so far, the only identified NAMP is ascaroside 18. It was recently reported that infected plants metabolize the NAMP ascr#18 into a mixture of shorter chain ascarosides via the peroxisomal β-oxidation pathway (Manohar et al., 2020). These shorter side-chained ascarosides repel nematodes thereby leading to reduced attack of the plant by nematodes (Figure 1.2).

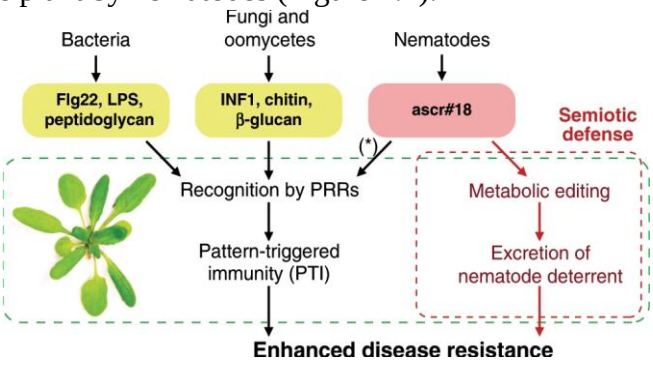

Figure 1.2 Pathogen associated molecular patterns produced by different pathogens, bacteria, fungi and nematode trigger PTI in plants but no information on their metabolism inside the plant is available (Manohar et al., 2020).

Plant early responses to nematodes have been studied using different sources for NAMPs such as crude tissue extracts of J2s which induced defense marker genes and root enlargement in *Arabidopsis thaliana* roots (Teixeira et al., 2016). Previous studies by Mendy et al. (2017) describe the activation of PTI responses in *Arabidopsis thaliana* by NemaWater (a mixture of currently unidentified nematode PAMPs - NAMPs). NemaWater was produced by incubating infective-stage juveniles of *H. schachtii* (~40000 J2s) in water for 24 hours at room temperature. Afterwards the nematodes were concentrated by gentle centrifugation and the resultant supernatant was termed NemaWater. *A. thaliana* plant roots treated with NemaWater activated defense responses such as reactive oxygen species (ROS-burst) generation, plant growth inhibition, and defense gene expression. Further, they reported that *Arabidopsis* seedlings treated with NemaWater were more resistance to cyst nematode *H. schachtii* infection compared to control as indicated by the reduced number of nematodes. The number of different NAMPS in NemaWater is still unknown but they are expected to be many as in the case of microbial pathogens. In addition, they proved that the elicitors in NemaWater may be proteins (Mendy et al., 2017). This was confirmed by experiments they performed after treatment of NemaWater with proteinase K enzyme and heating reduced the basal defense responses observed in untreated NemaWater samples. These indicate that the

elicitor/s contained in NemaWater is/are of proteinaceous nature and heat labile. A leucine-rich repeat receptor like kinase referred to as NILR1 (Nematode induce LRR-receptor 1) has been shown to be specifically expressed upon treatment with NemaWater in *A. thaliana* (Mendy et al., 2017). Mutants of NILR1 were shown to be hypersusceptible to several other nematode species and displayed impaired PTI responses induced by NemaWater. It's most likely that NILR1 forms a complex with BAK1 upon perception of nematode derived protein molecules present in NemaWater (Mendy et al., 2017). Although the actual proteins present in NemaWater are not yet known, treating rice plants with NemaWater at '133 nematode-equivalents/mL' for 4 hours followed by gene expression analysis using qRT-PCR showed upregulation of PTI related genes (De Kesel et al., 2020). Atighi et al. (2020) reported a global DNA hypomethylation upon treatment of rice and tomato plants with NemaWater from different nematode species and observed ROS production.

Identification and isolation of specific nematode proteins responsible for this triggering of PTI represents an exciting study on NemaWater with potential for enhancing the control of plant parasitic nematodes. A proteomic analysis of NemaWater revealed many proteins, mostly nematode surface-associated proteins which gives them a higher probability of being recognized by the plant as NAMPS.

To screen for potential NAMPs from NemaWater protein data, we selected proteins that were abundant in peptide counts, high confidence score, and were previously described in animal parasitic nematode studies to have a role in host immune modulation. Since we cannot screen all the proteins at the same time, we limited our search to seven candidates. These include Enolase, Cuticlin, Heat shock protein 90 (Endoplasmin homologue), 14-3-3 protein, ADAM-10 metallopeptidase, Tetraspanin, Heat shock protein 70 (Actin 5C). The transcriptome data of these candidates at different time points of infection and *H. schachtii* life cycle is available and accessible at the host institute. We determined whether the expression levels of candidate proteins correlated with the abundance of transcriptional expression levels. We used gene-specific primers for the genes encoding the proteins to investigate their expression at different time points using quantitative real-time PCR analysis. qRT-PCR was done for all the candidates at 10 hours post infection and 48 hours post infection. This data gives an overview of how conserved and consistent these selected candidates are, which will help in future NAMP prediction and further screenings in NemaWater.

1.2 Research rationale

Plant parasitic nematodes cause heavy and costly losses in agricultural crops (Bernard et al., 2017). From time immemorial, plant breeders' efforts to control nematodes in crops in an environmentally friendly approach/ way has been centred on resistance genes (Dodds & Rathjen, 2010). Discovery of NAMPS) which trigger pattern triggered immunity in plants and understanding the ability of plants to sense these nematode elicitors provides a potential to developing novel biotechnological tactics to crop protection. NAMPS could be used as priming agents in crops to activate the plant defense system and therefore providing an environmentally friendly solution to control nematodes and possibly other pathogens.

1.3 Objectives

Generally, the objectives of this study were to identify and characterize potential NAMPS present in NemaWater. The specific objectives were:

1. To assess expression of potential NAMP candidates using *H. schachtii* transcriptome analysis data.
2. To check differential expression of candidate genes at 10 and 48 hours post infection using qRT-PCR.
3. To further characterize the candidates and develop transgenic plants overexpressing the genes and perform phenotype screening, defense gene expression analysis and infection assays.
4. To knock down the candidate genes in the nematode using RNAi silencing and investigate the effects on nematode morphology and infectivity.

1.4 Hypotheses

We hypothesize that the candidate genes are expressed at different infection stages.

2. LITERATURE REVIEW

2.1 Life cycle of *Heterodera schachtii*

The beet cyst nematode (*H. schachtii)* eggs rely on host exudates for hatching and they produce extremely high numbers of juveniles in response to diffusates but also moderate numbers in water (Lee, 2002). Other environmental factors can also trigger hatching for instance soil moisture and temperatures. Host location by the invasive second stage juveniles J2 is facilitated by chemical gradients released by the host (Lee, 2002). J2 penetration usually occurs directly behind the growing root tip and the nematodes migrate towards the pericycle and selects a cell suitable for feeding site initiation (Lilley et al., 2005). The stylet delivers saliva from nematode pharyngeal glands into the cell and takes up cell content as food. A feeding tube is formed from nematode molecules and this serves as a filter for larger plant products. The selected cell further develops into a syncytium, a multinucleate feeding structure through lysis of the cell wall by nematode cell wall degrading enzymes (Gheysen & Jones, 2006). The nematode moults into J3, J4 and finally adult males and females (Figure 2.1) over a period of two weeks (Endo &Wyss, 1992). The size of the feeding site/syncytium determines the sex of the nematode. For example, those nematodes that form a larger feeding site usually develop into a female while those that form smaller sites develop into males. Vermiform male nematodes are non-feeding and they move outside the root, find females through pheromones, mate and live for a short period of time in the soil. Female nematodes become swollen, lemon-shaped with a short neck and terminal cone; in adult stages they are filled with roughly 200-250 eggs. Further growth of the females causes the root cortex to rupture and the anterior end stays inside the root while the posterior end is left outside (Perry & Wharton, 2011). They become sedentary feeders, later die and the cuticle tans forming a tough protective cyst through a process called cutinisation. The duration of the life cycle depending on environmental conditions is four to eight weeks.

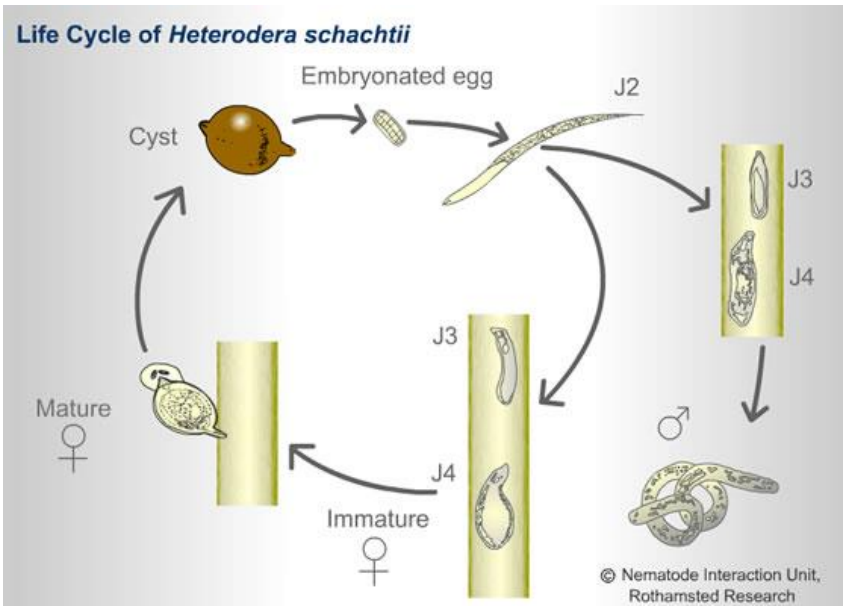

Figure 2.1 Illustration of the life cycle of *H. schachtii* showing eggs inside the cyst, juvenile stages, second stage juvenile (J2), third stage juvenile (J3), fourth stage juvenile (4) and the adult males and females. Illustration is courtesy of Nematode Interaction unit at Rothamsted Research.

2.2 The nematode cuticle

The nematode cuticle is covered with a surface coat (SC), highly glycosylated and made from hypodermal and specialized pharyngeal secretory glands secretions. It is the outermost interface of the nematode cuticle therefore has direct contact with the external environment (Davies & Curtis, 2011). The cuticle is an exoskeleton without cells, shed off between life stages and various biochemical processes occur on its surface. There is an incessant production and export of various surface components. Nematode surfaces are the first line of defense against external environment including host immune evasion, suppression and modulation therefore, biosynthesis and maintenance of nematode cuticle is crucial (Bird and Bird 1991). The cuticle has multiple roles ranging from protection against harsh environment to locomotion and movement. In-depth studies of cuticle in nematodes are mainly centred on *Caenorhabditis elegans* yet their suitability for modelling plant parasitic nematodes is questionable (Davies & Curtis, 2011). However, using it in understanding basic aspects of plant-nematode interactions involving the cuticle and the surface

coat is likely to provide insights on the role of cuticle-based genes in host defense inducement and feeding sites formation.

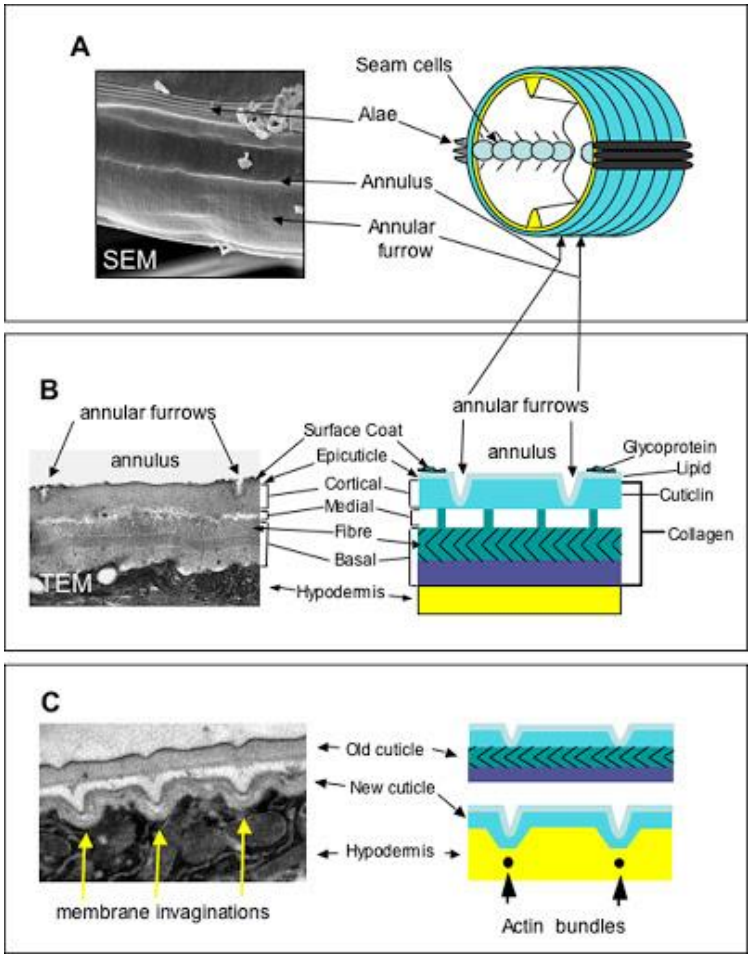

Figure 2.2 Cuticle structure of *Caenorhabditis elegans* showing scanning electron micrograph of an adult nematode and the corresponding schematic representation (A). Picture B is a transmission electron micrograph of the longitudinal cross-section of the cuticle highlighting the different structural layers and their respective composition. Panel C shows new cuticle synthesis and old cuticle detachment during molting (Page & Johnstone, 2007).

Collagens are found in all layers of the cuticle except for the epicuticle and the surface coat while cuticlins are constrained to the cortical region (Figure 2.2). Invaginations of the hypodermal membrane which forms the cuticular annules correspond to the circumferential actin bundles position. The glycoprotein-rich surface coat covers the epicuticle which contains lipids and proteins.

The surface cuticle of plant parasitic nematodes is negatively charged and approximately 5-30 nm thick containing countless proteins with varying molecular weights across different nematode

species (Spiegel & McClure, 1995). J2 surface coat is special because it is in contact with both soil, rhizosphere and host plant environments thereby most likely dynamic in protein composition. The surface coat duty in the soil is to protect nematodes from abiotic and biotic stress and once the nematode invades the plant roots it must avoid/overcome the plant immune response. Therefore, the surface coat must have adequate flexibility in order to cope with the two highly dissimilar environments. The surface coat proteins are the first to interact with the plant defense system.

The cuticle of *C. elegans* consists of glycosylated and sometimes lipid-modified proteins manufactured from the secretory epithelial and seam cells. Liégeois et al. (2006) were the first to report an apical secretion pathway in *C. elegans* that involves multivesicular bodies and release of exosomes at the plasma membrane.

2.3 Exosomes

Extracellular vesicles or exosomes are released as membrane-wrapped structures by microbial cells and several other eukaryotes. The term extracellular vesicles includes microvesicles, nanovesicles, ectosomes, exovesicles, exosomes, and exosome-like vesicles (Figure 2.4) (Colombo et al., 2014). Over the years, in most parasites, bacteria, fungi and metazoans, exosomes were believed to be active for excretion of unwanted cell components therefore not much research has been done on them. Researchers only became interested when exosomes were reported to be involved in host defense stimulation (Bobrie et al., 2011). As a result, exosomes in mammals and invertebrates described seem to be involved in various molecular, biological and cellular functions within the cell. Exosomes do not only contain proteins and lipids involved in their own genesis, signal transduction or cytoskeleton organization but also nucleic acids such as micro RNAs and as a result they become reservoirs of pathogen proteins as well as many other proteins with a role as membrane microdomains and enzymes (Schorey et al., 2015).

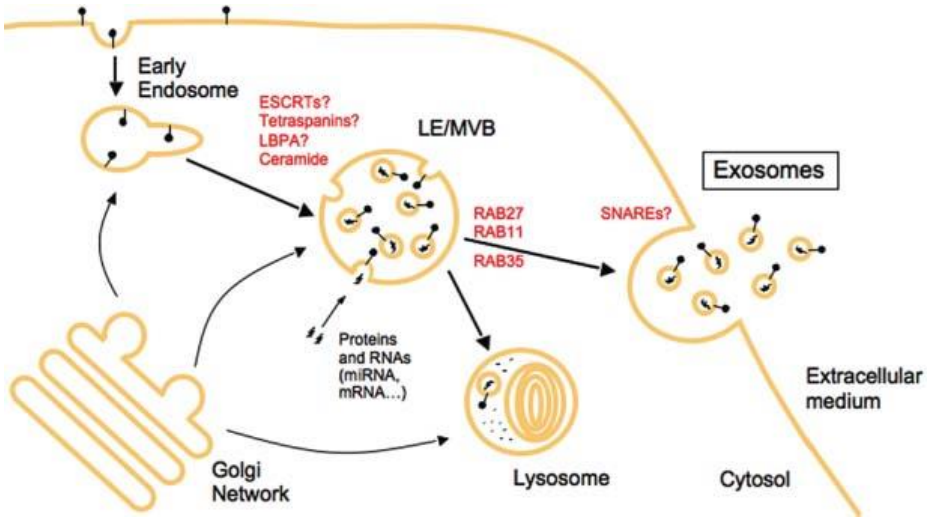

Fig 2.3 Formation of exosomes and vesicular trafficking (Bobrie et al., 2011).

Exosomes are formed from internal compartments called multivesicular bodies (MVBs) or late endosomes (LEs). The formation of MVBs requires endosomal sorting complex required for transport (ESCRT) proteins, tetraspanins and the lipid LBPA. Regulation of vesicular trafficking is carried out by various Arf/Sar and Rab small GTPases. Besides transport regulation GTPases are responsible for vesicle formation on the donor membrane and direct specific trafficking to target membranes. Fusion of the MVBs with plasma membrane to secrete exosomes is regulated by a complex of Soluble NSF attachment proteins 5 receptor (SNARE) proteins (Bobrie et al., 2011). In *C. elegans*, small GTPase RAL-1 is responsible for MVBs formation and subsequent fusion with the plasma membrane for exosome secretion(Figure 2.3) (Gillingham & Munro, 2007; Hyenne et al., 2018).

Within the endoplasmic reticulum, several proteins such as heat shock proteins (HSPs) and respective chaperones are essential for protein folding modes, secretion of exosomes and surface coat localizations of proteins (Park & Seo, 2015). Transport of lipids and proteins within the endomembrane system is mediated by membrane trafficking. The system consists of small vesicles and larger intracellular organelles such as the endoplasmic reticulum (ER), Golgi body, endosomes, lysosomes, and autophagosomes (Delic et al., 2013). The actual protein transport between different compartments is partly mediated by budding and fusion of small vesicles, fusion and fission of the

larger organelles, and subsequent maturation processes that alter organelle identity over time (Sato et al., 2018).

2.3.1 Exosomes in animal parasites

Exosome-like vesicles were observed in *Steinernema carpocapsae* an entomopathogenic nematode which parasitizes on insects and they were found to be localized on the nematode lateral field, alae and the cuticle. Mass spectrometry analysis of the exosomes identified cytoskeletal proteins such as actin, tubulin as well as several filament disassembly proteins and vesicle transport-related proteins. Many proteins were protein/carbohydrate binding including metalloendopeptidases. The authors concluded that exosomes could be one of the mechanisms by which entomopathogenic nematodes interact with their host to deliver molecular effectors (Toubarro et al., 2018). Another case of evidence for exosomes in nematodes was reported in a mouse colon nematode *Trichuris muris*. The parasite excretes various compounds during interactions with the host, ultracentrifugation and purification with density gradient followed by identification of proteins and RNAs, revealed about 364 protein molecules including tetraspanins (Eichenberger et al., 2018).

Zhu and colleagues, (2016) found vesicle biogenesis proteins such as HSPs 70, HSPs 90 and Rab GTPase family proteins in the exosomes of *Schistosoma japonicum*. These research findings indicate that exosomes/extracellular vesicle formation is highly conserved in eukaryotes. Studies in *S. mansoni*, a water-borne human parasite, demonstrated that schistosomes secrete extracellular vesicles containing tetraspanin a transmembrane domain, heat shock protein 70, aminopeptidases,14-3-3 protein and enolase (Sotillo et al., 2016). These proteins, for instance enolase and HSP-70, are expressed during several life-cycle stages, eggs included, thereby giving an indication that vaccines developed on the basis of these proteins have the potential to target a number of different life stages (Cass et al., 2007). These exosomes also possess the ability to suppress host defense *in vivo* (Riaz & Cheng, 2017).

Trematodes *Echinostoma caproni* and *Fasciola hepatica*, internal parasites of vertebrates and molluscs, both secrete exosomes or microvesicles (mv) that are taken up by the host cells, indicating a potential role in host-parasite communication. Further the identification of proteins present in the purified exosomes via proteomics and immune-transmission electron microscopy revealed glycolytic enzyme enolase, cytoskeletal actin 5C, stress related proteins HSP90, HSP70 and signalling molecules such as 14-3-3 protein. The most abundant proteins in the exosomes were enolase and actin which were shown by binding of specific antibodies (Marcilla et al., 2012).

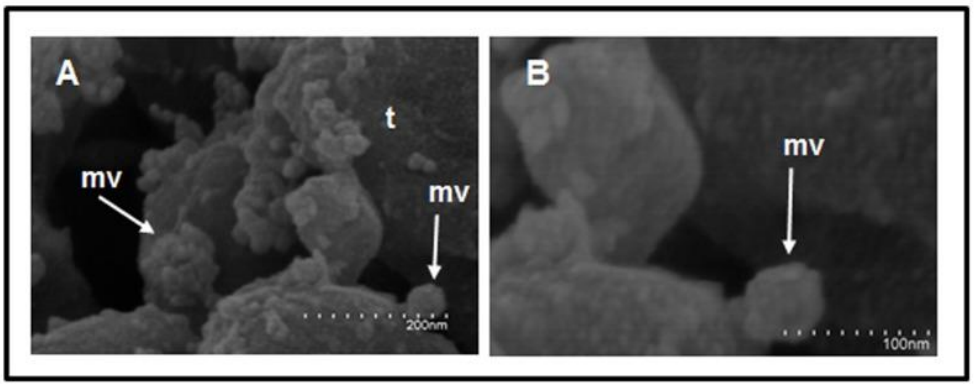

Figure 2.4 Exosomes at the surface/tegument of *Echinostoma caproni* visualized by scanning electron microscopy at different ×200000 (A), and ×350000 (B) magnifications. The surface or tegument is shown by the letter t and exosomes as mv/ microvesicles. Adapted from (Marcilla et al., 2012).

Heligmosomoides polygyrus secretes exosomes or vesicles enriched in proteins such as enolase, HSP70, and miRNAs that are all internalized by the host and can suppress the host immune system (Buck et al., 2014). Duguet et al. (2020) compared miRNA in exosomes of *C. elegans* and adult parasitic nematodes *Ascaris suum, Haemonchus contortus*, and *Trichuris muris* and found a significant similarity. However, no information is available currently on the role of exosomes or micro RNAs in plant-nematode interactions. One of the ways miRNAs are transported in animal cells is through extracellular vesicles. The evidence of such can be clearly seen in the case of *Heligmosomoides polygyrus*, a gastrointestinal parasite which secretes exosomes leading to transport of miRNAs into mammalian cells thereby suppressing host immunity (Buck et al., 2014).

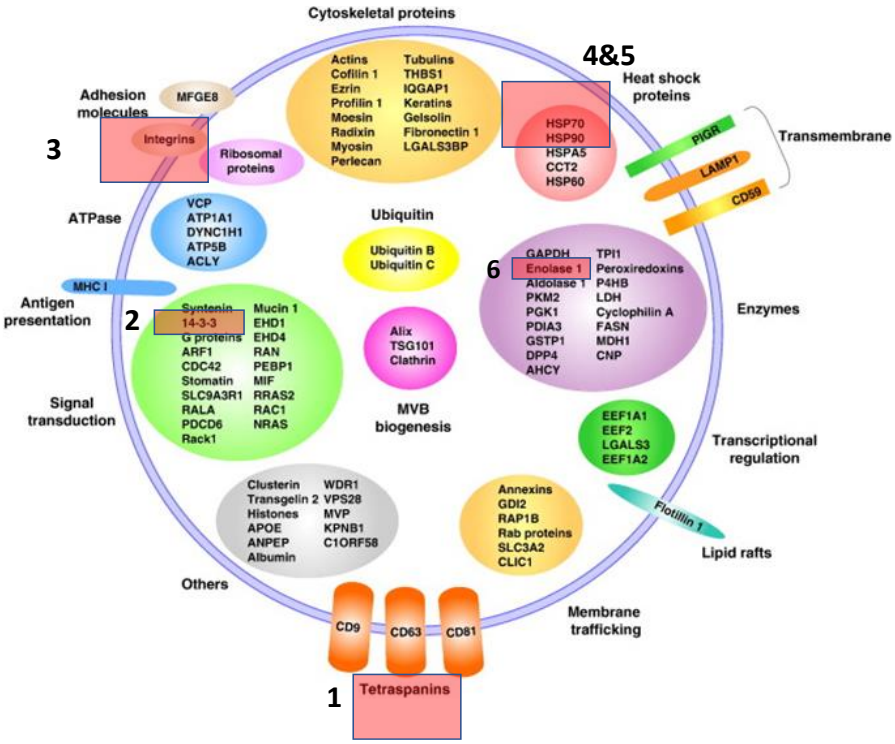

Fig 2.5 A graphical representation exosomal protein composition from various studies categorized by their function. Labelled red boxes from 1 to 6 show our candidate genes for this study (1) tetraspanins, (2) 14-3-3 protein, (3) ADAM-10 integrins, (4&5) heat shock proteins 70 and 90 and (6) enolase. Adapted from Mathivanan et al. (2010).

2.3.2 Exosomes in plant parasitic nematodes

Meloidogyne incognita secretes enolase, heat shock protein and actin as some of the most abundant peptides (Bellafiore et al., 2008). An interesting study of binding several surface proteins of *Meloidogyne javanica* with antibodies showed that these surface antigens are present in both J2 and J3 stages and possibly produced in the hypodermal region (Sharon et al., 2002). Fibrillar exudates were found by (Endo &Wyss, 1992) on J2 and early J3 of *Heterodera schachtii* attached to feeding sites. The exudates observed showed similarity to fibrillar patterns of the cuticle and secretion vesicles accumulated at the Golgi apparatus in the hypodermis later coalesce and form large vesicles in the cytoplasm. The authors were the first to propose transcuticular secretion of surface coat proteins in *H. schachtii* in which secretion vesicles transport cuticle associated proteins outside the nematode by exocytosis.

Most exosomes have been shown to have functional roles in mostly animal parasitic nematodes. An attempt to characterize exosomes in plant parasitic nematodes was done on *Heterodera glycines* and *Meloidogyne incognita* by Barnes, (2018) (unpublished). Most researchers study exosome

proteins by injecting the purified products into model organisms. Our study is the first attempt to characterize surface proteins of *H. schachtii* pre and post infection.

2.3.3 Role of exosomes in disease development

Exosomes have a significant role in antigen presentation for the activation of immune cells. Once the host cells are infected by microbials, exosomes can either strengthen innate immune responses thereby causing host resistance to the invading microbes or they can act as immunosuppression molecules during microbial infection(Figure 2.6) (Wang et al., 2018).

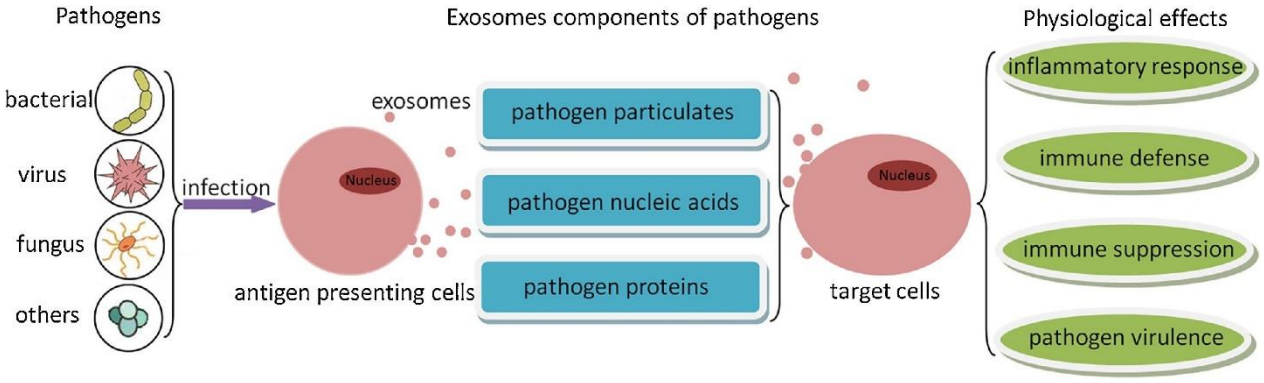

Figure 2.6 Modulation of host immune response by exosomes during pathogenic infection. (Wang et al., 2018).

Several parasites including, fungi, protozoa and helminths were shown to produce exosomes during parasitism thereby providing evidence that exosomes have a prominent role in host immune manipulation (Buck et al., 2014). For example, helminth *Trypanosoma cruzi* releases exosomes which alter the host immune system to enhance infection (Coakley et al., 2015). By contrast, *Heligmosomoides polygyrus*, an animal parasite, exosomes suppress host immunity (Buck et al., 2014).

2.4 Candidate proteins

The candidate genes in this study were obtained from protein analysis of NemaWater and the proteins were blasted against the *Globodera pallida* genome to obtain gene sequences. The sequences were further blasted to *H. schachtii* genome available to obtain the candidate sequences we worked with in this study.

2.4.1 Enolase

Enolase, sometimes called 2-phospho-D-glycerate (2-PGE) hydrolase is an abundant enzyme catalyzing the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate (PEP). Although enolase is multifunctional, its main biological function is its involvement in the glycolytic,

gluconeogenesis and other non-glycolytic pathways (Pirovich et al., 2019). At molecular level, enolase binds to magnesium ions for catalysis, and is found in various sugar metabolizing organisms. Several pathogens such as bacteria, fungi, helminths and protozoa possess surface localized enolase. However, the mechanism through which enolase is transported on cell surfaces is still unknown (Pancholi, 2001).

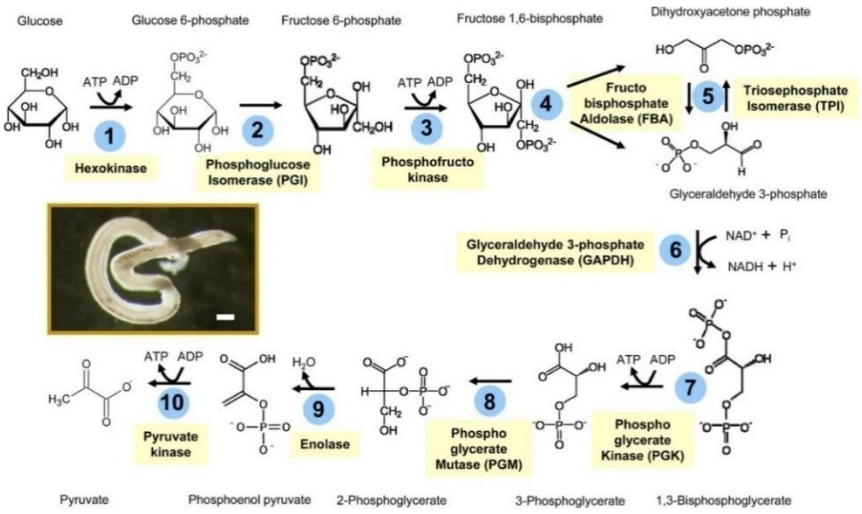

Figure 2.7 Glycolysis pathway with all the other involved enzymes highlighted resulting in net gain of two molecule adenosine triphosphate (ATP) molecules. The picture of an adult male *Schistosoma mansoni* as adapted from Pirovich et al. (2019).

The *C. elegans* enolase orthologue is involved in gene transcription and corresponding expression during normal nematode larval development (Huang et al., 2008). RNAi silencing of *Ascaris suum* enolase delays larval development as indicated by shorter larvae in the mutants (Chen et al., 2012). At elevated temperatures, HSP 48 in *Saccharomyces cervesiae* is identified as alpha-enolase and it is a product of the *eno1* gene supposedly involved in growth control and thermal tolerance in *S. cervesiae* (Ji et al., 2016).

There is some evidence that enolase has the potential to prevent Ascariasis caused by *Ascaris* which records as the common parasite problem in human beings and pigs across the world. The properties of inducing host immunity were shown in mice immunized with pVAX-Enol by a development of specific antibody responses against *Ascaris suum* (Chen et al., 2012). On the contrary, Liu et al. (2012) found enolase on the cuticle and surface coat of the entomopathogenic nematode *S. glaseri* termed Sg-ENOL. Furtherly, expression in *Escherichia coli* confirmed its glycolytic activity by converting 2-phospho-D-glycerate (2-PGE) to phosphoenolpyruvate (PEP). Detection of Sg-ENOL in the insect hemocoel of *G. mellonella* upon

infection *with S. glaseri*, showed that enolase is involved in host immune suppression. Another notorious nematode parasite of small ruminants, *Haemonchus contortus* possesses enolase with the same 2-PGE to PEP enzyme activity at optimum temperature of 7°C (Han et al., 2012). The study would have been more interesting if it had explored the effect of enolase on host immunity. Root-knot nematode *Meloidogyne incognita* was also reported to release enolase protein in a non-resistant sweet potato cultivar (Ha et al., 2017).

Enolase belongs to moonlighting protein families due to its ability to perform more than one function. The functions of enolase are related to sub-cellular localization and proteins which have other functions besides their classical activities are called moonlight proteins (Didiasova et al., 2019).

2.4.2 Cuticlin

The nematode cuticle is made up of three layers: cortical, median and basal zone. Cuticulin is found in the cortical zone and it's a highly resistant protein stabilized by cross linkages of dityrosine (Basyoni & Rizk, 2016). Cuticlins are insoluble remnants of the cuticle with cysteine-rich/zona pellucida-like domains expressed in the hypodermis. One of the reported functions of cuticlin is in stage specific formation of cuticular alae from the seam cells (Page & Johnstone, 2007). In *C. elegans*, there are six cuticlin genes (cut-1, cut-2, cut-3, cut-4, cut-5 and cut-6) reported to be involved in alae formation and cuticle (Sebastiano et al., 1991;Sapio et al., 2005). In plant parasitic nematode, a *cut-1* homologue was isolated in *Meloidogyne artiella* and in *cut-1* and *cut-2* were found in the animal parasites *Ascaris lumbricoides* and *Brugia pahangi*. This is evidence that cuticlin is highly conserved among nematodes (Lewis et al., 1994).

2.4.3 Heat shock protein 90/ Endoplasmin homolog

Heat shock protein 90 (HSP90) family is abundant in the cell cytosol and evolutionarily conserved in various organisms such as mammals and other eukaryotes. The HSP90 structure consists of three domains namely the N-terminal ATPase (ND), the middle domain (MD) vital for client binding and lastly the C-terminal dimerization domain (CD) (Kadota & Shirasu, 2012). Once an ATP molecule is bound, the "lid" segment of the N-terminal domain rotates to approximately 180° and encloses ATP. The two HSP90-NDs get closer together and form a closed conformation of the dimer thereby creating an active site for ATPase enzyme with the arginine residue from the MD catalytic loop. After the hydrolysis of ATP, the ND lid segment rotates back and the HSP90 dimer

forms another open conformation. Chaperoning functions of HSP90s is due to this conformational cycle (Kadota & Shirasu, 2012).

The functions of most of HSP90 proteins include a role in chaperone function, antigen presentation and cell cycle control. Endoplasmin is another member of HSP90 class found in the endoplasmic reticulum and acts as a molecular chaperone (Tsutsumi & Neckers, 2007). HSP90 associates with several intracellular proteins, such as calmodulin, tubulin, actin, kinases and receptor proteins (Gupta et al., 2010). In model plants such as *A. thaliana* and *Nicotiana benthamiana*, HSP90s have been reported to play a key role in regulation of growth and development. HSP90s also have significant roles in plant immunity by activating cytosolic R proteins containing the nucleotide-binding domain and leucine-rich repeat, which intermediates defense against many PAMPS (Kadota & Shirasu, 2012). In plant parasitic nematodes, *Heterodera glycines hsp*90 gene has been cloned and characterized (Skantar & Carta, 2004). Its function in parasitism is still unknown. *Meloidogyne artiellia* highly expresses *Mt-hsp*90 gene in all developmental stages (De Luca et al., 2009).

2.4.4 Tetraspanin

Tetraspanins are a diverse superfamily of transmembrane proteins with four membrane-spanning domains which occur ubiquitously in eukaryotic cells. Several members of this group have been discovered in mammals and model plant *A. thaliana* (Jimenez-Jimenez et al., 2019). Although tetraspanins traverse the cell membrane four times, unlike other four-pass membrane proteins they possess conserved charged residues in their transmembrane domains and the larger of the two extracellular domains (EC2) has a defining ‘signature’ motif (Hassuna et al., 2009). Tetraspanins associate non-specifically between themselves, and with other proteins to form specialized microdomains called tetraspanin-enriched microdomain (TEMs). Proteomic studies have proved that TEMs have an integral role as part of exosome membranes, cell-cell fusion, membrane recognition, motility, signalling as well as physiological processes such as sperm–egg fusion, tissue differentiation and antigen presentation (Boavida et al., 2013). Evidence from past research demonstrates that some tetraspanins participate in host-pathogen interactions for instance in human cells, viral pathogens need tetraspanins for entry and cell replication.

2.4.5 ADAM-10

ADAM-10 metalloproteinase disintegrins are single-pass transmembrane metalloproteases containing a disintegrin domain ADAMs and have been reported to exist in a wide variety of

animals, from protozoans to mammals. ADAM-10 is synthesized in the endoplasmic reticulum but in an inactive state and is only activated when proprotein convertases or furin removes its prodomain (Anders et al., 2001). Ectodomain shedding is generally understood to mean proteolytical cleavage of cell surface proteins at the juxta-membrane region resulting in the detachment of their ectodomain or extracellular region. ADAM proteins have been identified to mediate the various shedding events (Huovila et al., 2005). A recent study showed interesting evidence that Tetraspanin 15 is the central modulator of ADAM-10 in mammalian cells by accelerating the exit of ADAM10–TSPAN15 complex from the ER and also stabilizing the active proform of ADAM-10 at the surface (Prox et al., 2012).

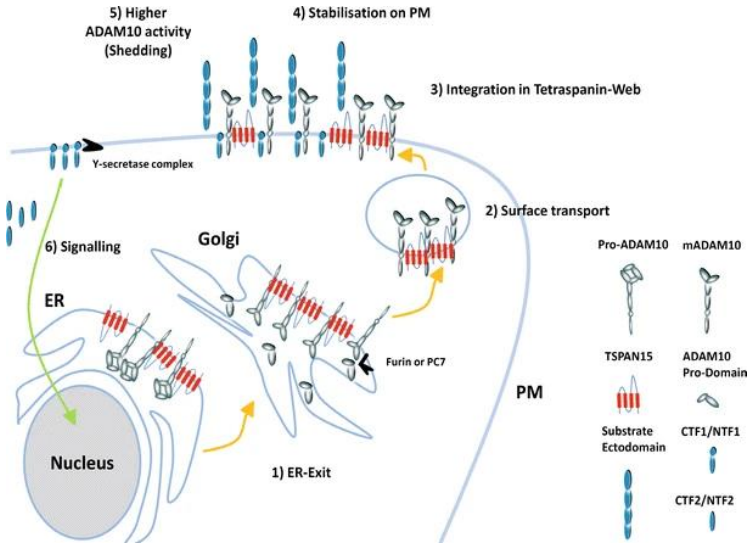

Figure 2.8 Interaction of TSPAN15 and ADAM-10 in mammalian cells. ADAM10 is produced in an inactive form (pro-ADAM10) from the ER (1) and its exit (2) is accelerated by TSPAN15. After the exit, the ADAM10 is activated when furin or a proprotein convertase removes the inhibitory prodomain then immediately transported towards the plasma membrane (PM) with TSPAN15 (3). The integration of ADAM-10 at the PM is facilitated by TSPAN15 in tetraspanin web. At step 4 and 5 ADAM-10 is stabilized and web composition aids ADAM-10 to access and cleave its substrates in the juxta-membrane. The remnant membrane-bound fragments are further cleaved by γ-secretase complex in the transmembrane region leading to the release of a soluble intracellular domain which has signalling functions (Prox et al., 2012).

2.4.6 Heat shock protein 70/Actin 5C

Actin belongs to the actin–heat shock protein70–sugarkinase superfamily and is a highly abundant and conserved protein. Excretory/secretory product analysis from *Trichuris muris*, a gastrointestinal nematode showed actin 5C as one of the components among other proteins such as enolase and 14-3-3 protein. The same actin 5C was previously reported to exist in secreted particles

of seven other parasitic nematodes and they all are exosomal proteins (Tritten et al., 2017). Characterization of actin from plant parasitic nematodes *H. glycines* and *G. rostochiensis* and a free living nematode *Panagrellus redivivus* was done by Kovaleva et al. (2005). Actin amino acid sequences from the two PPNs displayed high similarity to filarial nematodes. A comparison of *H. glycines* actin to six other *Heterodera* species showed that the actin gene is characteristic to a wide range of plant parasitic cyst nematodes.

2.4.7 14-3-3 protein

The 14-3-3 proteins are a seven-isoform (β, γ, ε, ζ, η, θ, σ) group of highly conserved dimeric cytosolic adaptor proteins. Each monomer comprises of nine antiparallel α-helices and the 14-3-3 dimer possesses a typical cup-like shape with a large channel at the center with two amphipathic binding grooves (Kaplan et al., 2017). The protein (14-3-3s) plays an important role in cell signaling, regulation of neuron development and axon guidance. In animal parasites, 14-3-3s were shown be secreted by the *Echinococcus granulosus* and *S. japonicum* (Zhang et al., 1999; Siles-Lucas et al., 2000). Jaubert et al. (2002), obtained two 14-3-3 protein peptides from *M. incognita* freshly hatched juveniles. Further study of the transcription of *Mi*-14-3-3-*a* and using qRT-PCR was done during the nematode life cycle (Jaubert et al., 2004). Transcription pattern analysis revealed that both isoforms were transcribed in unhatched J2s, infective J2s, both female and male adults. *In-situ* hybridization of J2s showed localization of *Mi*-14-3-3-*a* in the germinal primordium and Mi-14-3-3-*b* occurred in the dorsal esophageal gland.

The subcellular distribution of *Mi*-14-3-3-*b* after secretion into plant tissues is mainly located in the cytoplasm and faintly in the nucleus and its overproduction in BY2 cells induced no growth or reproduction defect. 14-3-3 was previously reported as part of the transcriptome proteins in other plant parasitic nematodes such as *H. avenae* (Cui et al., 2018). However, in another study, 14-3-3 located in the dorsal gland failed to suppress programmed cell death (Chen et al., 2018).The functional role of 14-3-3 proteins in host plant tissues after secretion by plant parasitic nematodes remains unclear.

3. METHODOLOGY

3.1 Nematode cultures and hatching

H. schachtii J2s used in the experiments were obtained from cysts reared on mustard (*Sinapis alba* cv.) plants grown in the dark under aseptic conditions on agar. Hatching was be stimulated by adding 3 mM $ZnCl_2$ on approximately 300 mature cysts collected in an autoclaved modified Baermann funnel. The funnel J2s were tightly sealed with aluminium foil and parafilm and placed in a dark growing room. After three days, J2 hatched and were sterilized by washing in incubating for three minutes 0.05% mercury chloride ($HgCl_2$) followed by washing three to four times in double distilled water. The J2s were collected in an Eppendorf tubes and used immediately for infection and some were frozen in liquid nitrogen and stored in -80°C for RNA extraction.

3.2 Plant material and seed sterilization

A. thaliana ecotype Col-0 was used for the experiments and the seeds were surface-sterilized to avoid contamination. Eppendorf tubes containing seeds were shook in a solution of 0.6% sodium hypochlorite ($NaOCl_2$) for five minutes. After carefully removing the $NaOCl_2$ supernatant, 70% ethanol was added and seeds shaken for further five minutes. The seeds were finally rinsed five times in double distilled and left to dry onto an autoclaved filter paper in a Petri dish.

3.3 Growing medium

Plants were grown on sterile agar in 9mm plastic Petri dishes plate containing 2% Knop's nutrient. The medium was made by a mixture of different stock solutions as shown in Table 3.1.

Table 3.1*: Chemical components used to prepare Knop medium (per litre)*

Composition	Quantity
Sucrose	20g
Daishin agar	9g
Stock solution I	2ml
Stock solution II	2ml
Stock solution III	2ml
Stock solution IV	0.4ml
Stock solution V	0.2ml
Vitamin B5 (added after autoclaving)	1ml
ddH2O	Filled up to 1L

The stock solutions were prepared by combining the chemicals listed on Table 2.

***Table 3.2**: Stock solution quantities per litre*

Stock solution	Chemical	[g L^{-1}]
Stock solution I	KNO_3	121.32 g L^{-1}
	$MgSO_4$ - $7H_2O$	19.71 g L^{-1}
Stock solution II	$Ca(NO_3)_2$ - 4 H_2O	120 g L^{-1}
Stock solution III	KH_2PO_4	27.22 g L^{-1}
Stock solution IV	FeNaEDTA	7.34 g L^{-1}
Stock solution V	H_3BO_3	2.86 g L^{-1}
	$MnCl_2$	1.81 g L^{-1}
	$CuSO_4$ -5 H_2O	0.073 g L^{-1}
	$ZnSO_4$ - 7 H_2O	0:36 g L^{-1}
	$CoCl_2$ - 6 H_2O	0.03 g L^{-1}
	H_2MoO_4	0.052 g L^{-1}
	NaCl	2 gL^{-1}

The materials were mixed in a 1 L bottle, filled to the mark with ddH_2O and dissolved thoroughly using a magnet stirrer. The pH of the solution was adjusted to approximately 6.4 using potassium hydroxide or hydrogen chloride when necessary. The solution was autoclaved, allowed to cool and 1 ml of vitamin B5 was added and the agar poured in Petri dishes on the same day.

3.4 Planting

Two seeds of *A. thaliana* were planted per Petri dish and placed in a growing room with controlled conditions. Petri dishes were tilted four days after planting to allow lateral root growth. The plants were grown in inverted plates with a 16hr light and 8hr dark cycle as described previously (Sijmons et al., 1991).

3.5 Inoculation of plants

Nematode inoculation was done on plants that were between 10-12 days. Approximately 100-150 of J2 nematodes were inoculated per plant. The plates were sealed and placed back to controlled environment with day and night temperatures of 22°C and 18°C respectively.

3.6 Sample collection

Nematodes in plant roots were collected by cutting the root segment containing the J2 at 10 and 48 hours post infection. The samples were frozen by placing them in 2ml Eppendorf tubes filled with

in liquid nitrogen and stored in -80°C for subsequent RNA extraction. Each time point comprises of three biological replicates.

3.7 Total RNA extraction and cDNA synthesis

Total RNA was extracted from samples using RNeasy Mini kit 50 and plant RNeasy Mini Plant kit 50 (Quiagen, Germany) according to the manufacturer's instructions. RNA concentration was determined by Nanodrop 2000c spectrophotometer (peqlab, Germany) and samples above 100ng/µl were used for corresponding cDNA synthesis. Treatment with DNase was done to remove genomic DNA using Turbo DNA-free^{TM} kit (Invitrogen, Germany). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied biosystems, Germany).

3.8 qRT-PCR method

qRT-PCR was done using fast SYBR^{TM} Green Master Mix (appliedbiosystems, Lithuania) on StepOne Plus Real-Time PCR System (Applied Biosystems, Germany). 18S was used as a reference gene and relative changes in the gene expression were quantified by $2^{-\Delta\Delta CT}$ method according to Pfaffl, (2001). Each 20 µL qPCR mixture included 10 µL SYBR green master mix, 0.5 µL of each primer, 1 µL of undiluted cDNA for the target gene or diluted 1:100 for the reference gene 18S and 8 µL of nuclease free water in 20 µL of total reaction volume. The reaction protocol was set at 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. The melt curve was generated at 95°C for 15 s, 60°C for 1 minute, and 95°C for 15s. Each treatment had three technical and biological replicates.

3.9 Primer design

Specific primers for q-PCR were designed using Prime 3 according to parameters of 59–63°C melting temperature, 18–23 bp primer length, 40–60% GC content and product length between 60–250 bp. The primers, their properties, amplification efficiencies and correlation coefficients are presented in Table 4.1.To assess primer efficiency (*E)* of primers and correlation coefficients (R^2), cDNA was serially diluted into a 3-fold (3×, 3^2×, 3^3× and 3^4× dilutions).

3.10 Data Analysis

Analysis variance was done in Sigma plot 13 on transcriptome and qPCR data after testing assumption of ANOVA. Graphs were drawn using Sigma plot 13. Annotation and gene description were done using Blast2go.

4. RESULTS

4.1 Gene ontology

The gene ontology (GO) terms of the candidate gene sequences, description and annotation of predictive cellular components, biological processes and molecular functions were determined using Blast2GO (Conesa et al., 2005). None of the proteins were unique to our study. GO terms *membrane* and *integral component of the membrane* were significantly overrepresented among the nematode proteins with four of the proteins being membrane proteins (ADAM-10, HSP90/endoplasmin, cuticlin and tetraspanin) (Figure 4.1).

Functional analysis revealed that the biological processes for the candidates include *glycolytic process* (enolase), *proteolysis* (HSP90/endoplasmin, ADAM-10), *regulation of cellular process and multicellular organismal development* (tetraspanin), *male tail tip morphogenesis* and *vulval development* (ADAM-10), *locomotion, embryo development, birth and egg hatching*, *cortical actin* and *cytoskeleton organization* and *mitotic and meiotic cytokinesis* (HSP70/actin 5C) (Table 4.1).

Figure 4.1 Cellular components of the candidate genes displaying of *H. schachtii* enolase, cuticulin, HSP90/endoplasmin, tetraspanin, HSP70/actin 5C and ADAM-10 metallopeptidase sequences. The node score is sum of sequences associated with the given GO term either directly or indirectly.

14-3-3 protein was blasted but no biological processes were found in Blast2Go. Blasting the 14-3-3 protein sequence in UniProt and annotation with SMART showed that 14-3-3 homologues are involved in signal transduction. They bind to phosphoserine-containing proteins and play a role in growth factor signaling. The predicted cellular role therefore is phosphoserine-binding and homodimer formation and more than 90% of this protein family are involved in the KEGG pathway cell cycle (Letunic & Bork, 2018).

GO term	Biological function	Molecular function
Enolase	Glycolytic process	Magnesium ion binding, phosphopyruvate hydratase
Tetraspanin family protein	Regulation of cellular process and multicellular organismal development	
HSP90/ Endoplasmin homologue	Proteolysis	Protein and ATP binding
ADAM-10/ disintegrin family protein	Proteolysis, regulation of cellular process and multicellular organismal development, embryo development, birth and egg hatching, male tail tip morphogenesis, vulval development	Protein binding, metalloendopeptidase
HSP70/Actin 5C	Locomotion, embryo development ending in birth or egg hatching, cortical actin and cytoskeleton organisation, mitotic and meiosis III cytokinesis	ATP binding, structural constituent of the cytoskeleton
Cuticlin/ zona pellucida-like domain		Structural constituent of cuticle
1433 protein family	Blasted but not annotated	

Figure 4.2 Gene ontology (GO) terms, description and annotation of predictive biological and molecular functions using Blast2GO

The predicted molecular functions were magnesium ion binding for enolase, ATP binding for heat shock proteins, protein binding (HSP90 and ADAM-10), structural constituent of the cytoskeleton (HSP70), metalloendopeptidase (ADAM-10) and cuticlin was annotated as a structural constituent of cuticle but no biological function was predicted (Figure 4.2).

4.2 Primer design and efficiency

Our primers were designed using primer 3 and two of them HSP70 and HSP90 had efficiencies of 1.90 and 1.93 respectively. Enolase and HSP90 had lower efficiencies as shown in Table 4.2. The efficiencies values for the four genes were ranging from 1.7 to 1.93 and R^2 values were all greater than 0.99.

Table 4.2 *Primer pair sequences, PCR product lengths, melting temperatures of primers, amplification efficiencies (E), and correlation coefficients (*R^2*).*

GO term	Primer sequence 5'-3'	length (bp)	Mt (°C)	GC%	product length (bp)	E%	R^2
	F: TGCCGGCTTTCAACATGATT	20	58.74	45			
Enolase	R: CGTAGCGTTCTTTGATGACCT	21	58.39	48	162	1.75	0.998
	F: TGTCATGGCTTGCAAAACCG	20	59.97	50			
Tetraspanin	R: TGCCGGATCGAGGTAGAAAA	20	58.81	50	225	1.71	0.999
HSP90/Endoplasmin	F: CATTAGCAGCGATTTCGCCC	20	60.0	55			
homologue	R: GTCAATGATTTCGTCCGCCG	20	60.0	55	80	1.93	0.994
	F: CGCATGCAGAAGGAGATGAC	20	59.1	55			
HSP70/Actin 5C	R: CTTGCTTGCTGATCCACATC	20	57.2	50	148	1.90	0.997

4.3 Differential expression of candidate genes during *H. schachtii* life cycle

In order to observe transcriptomic changes in nematodes candidate genes, transcriptome analysis was done in previous work at cysts, J2, 10, 40 hours post infection, 12 days (males and females) and 24 days post infection (unpublished data). The candidates were taken for further qRT-PCR analysis to validate transcriptome data and details on the primers, reference gene and control are provided in Table 4.2. Fold change was obtained by measuring relative expression between J2 (control), 10 and 48 hours normalized against 18s as a reference gene. Analysis of variance for qRT-PCR data was done on ΔCT values for the three technical and three biological replicates. We managed to perform qPCR for enolase, tetraspanin HSP90 and HSP70. The remaining three candidate namely ADAM-10, 14-3-3 protein and cuticlin only have transcriptome data but no qRT-PCR was done on them.

4.3.1 Enolase

Analysis of variance of enolase transcriptomic data showed that it is expressed in all stages with significant differences in levels of expression across infection stages (Tukeys test, $p<0,001$). Except for cyst and J2 stages in which expression remained unchanged, expression was the highest in females at 12 days post infection followed by 48 hours and 12dpi males, 10 hours and 24 dpi females. qPCR data showed expression of enolase at both 10 and 48 hours and same significant

increase (p=0.004) in expression of enolase from J2 to 10 hours post infection but surprisingly a decrease back to lower levels at 48 hours which was not the trend in transcriptome results (Figure 3.2). The fold change increased from 1 in J2 to 49 at 10 hours and then went down to 6 at 48 hours post infection.

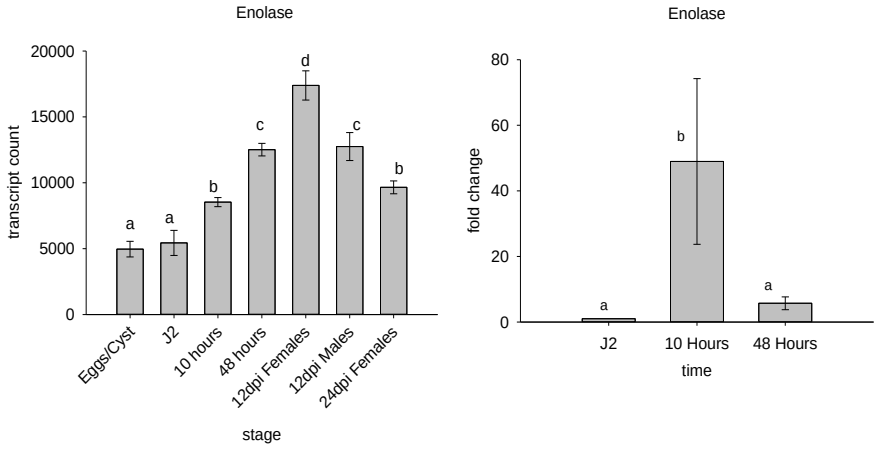

Figure 4.2 Differential expression of enolase in *H. schachtii* at different life stages shown by mean transcript count. Graph on the left shows' expression of enolase at egg, J2, 10- and 48-hours post infection, 12 days post infection females, males and lastly 24 days post infection. Graph on the right is the expression of enolase expressed as fold change in hatched invasive J2, 10- and 48-hours post infection. Analysis of variance of 3 biological replicates. Bars with different letters are significantly different at $p \leq 0.05$.

4.3.2 Tetraspanin

Transcriptome analysis showed expression of tetraspanin in all stages and no significant differences in expression of tetraspanin was observed in J2, 10 and 48 hours, and 12 dpi females while qRT-PCR showed significant increase in expression at 10 hours post infection. The fold change increase from J2 to 10 hours was 227 and 6 at 48 hours post infection. The expression level is generally similar between different stages except significant downregulation in J2 and upregulation in 12 dpi males, the highest transcript count is found in 12 dpi males (Figure 4.3).

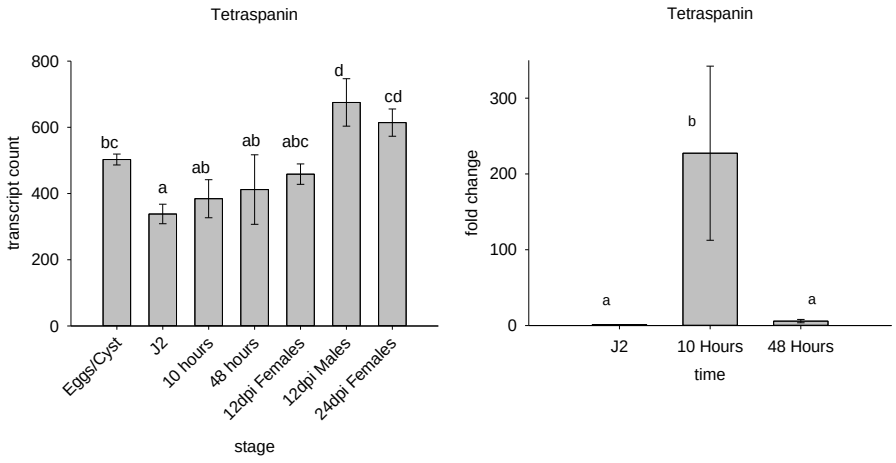

Figure 4.3 Differential expression of tetraspanin at eggs, J2s and different times post and qRT-PCR quantification of tetraspanin expression levels in *H. schachtii* infecting *Arabidopsis thaliana*.

4.3.3 HSP90/Endoplasmin

Endoplasmin showed variation in expression among the different stages, its expression was upregulated in earlier stages J2 and 10 hours post infection. At later stages of nematode development, gene expression levels start to go down to the same level as J2 as nematodes becomes adults from 12 days post infection. A similar trend is seen on qRT-PCR data with a significant increase of gene expression from J2 to 10 post infection. We did not expect a significant difference between J2s and 48 hours post infection. The magnitude of upregulation from J2 was twice-fold greater in 48 hours post infection. Fold change was 1.3 at 10 hours and 2 at 48 hours (Figure 4.4). Furthermore, we observed no difference between 10- and 48-hours post infection in qRT-PCR data collaborated with transcriptome data.

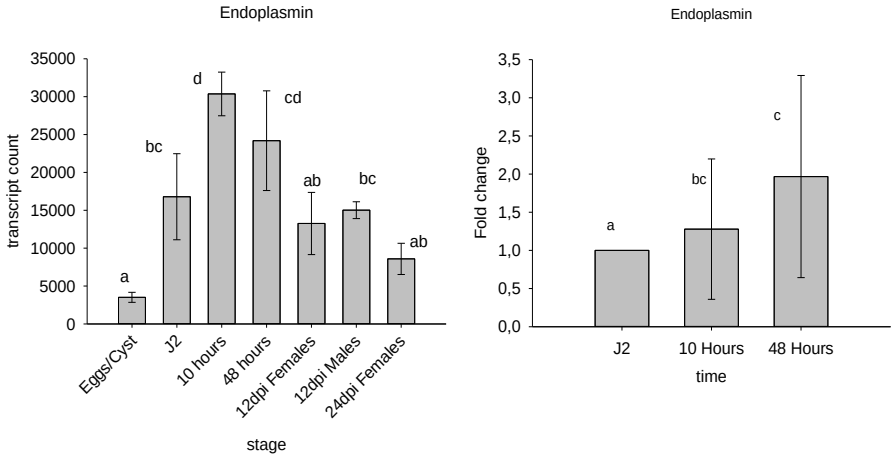

Figure 4.4 HSP90/Endoplasmin transcript count and fold change. The values of fold change represent the changes in mRNA levels in *H. schachtii* at J2, 10 and 48 hours post infection on *Arabidopsis thaliana* roots relative to 18s as the endogenous control.

4.3.4 HSP70/Actin 5C

Transcriptome analysis showed that HSP70 has a biphasic expression pattern, transcript count is significantly upregulated at early stages, eggs and J2. However, the expression is significantly down regulated in the rest of the stages and significantly up regulated again at 12 dpi in males. qRT-PCR data did not show a significant difference between J2, 10 and 48 hours yet we expected J2 to be significantly higher than the other stages. Although there were no statistical differences, fold change values were 0.006 for 10 hours and 7 at 48 hours post infection. Transcripts of the HSP70 in eggs, J2 and 12 dpi males displayed a 4-fold greater abundance when compared to the other stages (Figure 4.5).

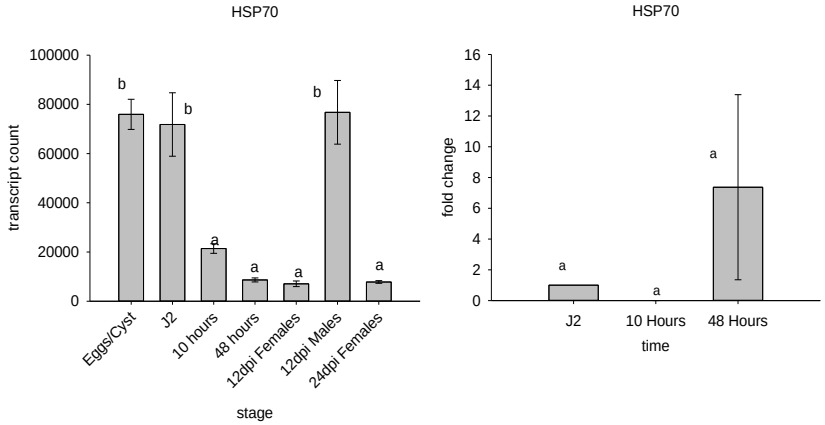

Figure 4.5 Expression of HSP70/Actin 5C shown by transcriptome and qRT-PCR data.

4.3.5 ADAM-10, 14-3-3 protein and Cuticlin

Expression of ADAM-10 metalloendopeptidase in transcriptome data shows non-significant differences between eggs, J2, 10 hours, 48 and 12dpi females after mean separation with Tukeys test (Figure 5.5). The highest expression was observed in 12 dpi females and 24 dpi females ($p<0.001$).

14-3-3 protein is present in all stages and significantly upregulated ($p<0.001$). The lowest expression level was found in eggs with less than 2000 transcripts. A five-fold increase was observed in 10 and 48 hours post infection. The lowest expression of less than 2000 transcripts was found in eggs. At 24 days post infection, expression starts to go down (Figure 4.5).

Cuticlin exhibits showed very low and biphasic expression with less than eight transcript counts but is significantly upregulated in adult nematode stages ($p<0.001$) at 12 and 24 days post infection (Figure 4.5).

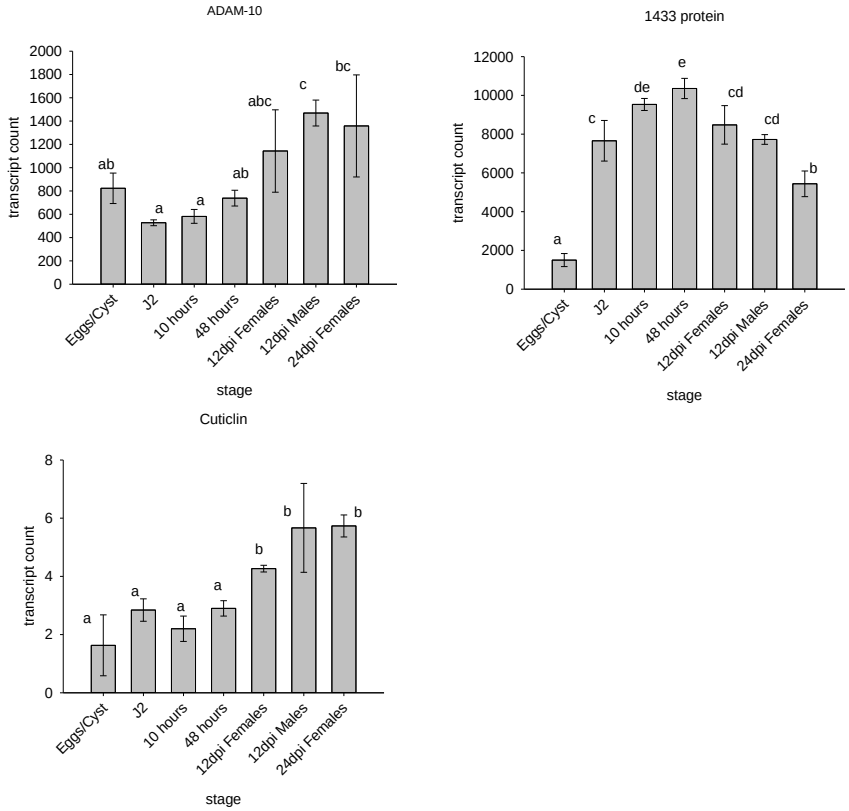

Figure 4.6 Transcript count of ADAM-10, 14-3-3 and cuticlin at different life stages in *H. schachtii*. Data was obtained from transcriptome analysis of nematodes at egg, J2s, 10, 48 hours post infection, 12 days post infection, females and males and lastly 24 days post infection females.

We did not manage to validate the expression of ADAM-10, 14-3-3 protein and cuticlin with qRT-PCR because the three primer pairs we tested were not efficient enough. We suspect that all the tested primers for cuticlin could not amplify the gene because of its extremely low expression levels as seen by the transcript count. ADAM-10 and 14-3-3 were amplified and produced the right product sizes but the melt curves showed presence of non-specific amplification products and therefore could not be further used for expression analysis.

5. DISCUSSION

Our study provides transcriptome data of selected *H. schachtii* genes which are potential NAMPS. Gene ontology and annotation showed that our candidate genes are major structural components of the cell membrane and are involved in basic metabolic pathways in the nematode. Although we expected qRT-PCR data to show a similar expression pattern to transcriptome data, which was not the case in some cases, the reasons could be due to differences in RNA quality as we did not use the same samples. Secondly, we did not measure RNA quality using a bioanalyzer which is crucial for accurate results in gene expression studies. We used 18S as the reference which might not have been the best to normalize RNA for an accurate measurement of relative expression in our samples. Experimental error in qRT-PCR studies can be caused by and poor quality of RNA and reference genes are essential for reliable gene expression results (Huggett et al., 2005). We also checked the expression of another possible reference gene actin in the transcriptome data, but the expression was not stable.

5.1 Enolase

Pathogens have evolved with their hosts and take advantage of host proteins for their survival (Ayón-Núñez et al., 2018). In our study results, we found significant differences in enolase transcriptome proportions across nematode infection stages. Enolase is an enzyme required for the glycolysis process for conversion of 2-phospho-D-glycerate to phosphoenolpyruvate (PEP) with an overall production of two ATP molecules. The results of this study showed that enolase is expressed in all stages from eggs until 24 days post infection females and therefore must be present at all stages for nematode growth and development. The results are consistent with reports that the *C. elegans* enol-1 orthologue is involved in gene transcription and corresponding expression during normal nematode larval development (Huang et al., 2008). RNAi silencing of *Ascaris suum* enolase delays larval development as indicated by shorter larvae in the mutants (Chen et al., 2012). The

observed higher expression of enolase in both migratory stage 10 hours post infection and later stages could be due to the nematode requirements for increased for metabolic processes. According to results obtained by Chen et al. (2011) survival rate of nematodes treated with RNAi for 72 hours is reduced by 20%. Enolase was reported to be a surface protein in various parasites such as entomopathogenic nematodes (Toubarro et al., 2018), human parasitic nematodes (Sotillo et al., 2016) and *Meloidogyne incognita* (Bellafiore et al., 2008). The role of enolase in most pathogens is for invasion host cells. In *Plasmodium falciparum*a, the human malaria parasite, surface enolase binds to and activates host plasminogen into plasmin through a plasminogen activator (Ghosh & Jacobs-Lorena, 2011; Didiasova et al., 2019). Comparable results were also reported in human cancer diseases, exosomes containing enolase enhance the migration and invasion of cancer cells (Didiasova et al., 2015).

Glycolysis and gluconeogenesis have crucial roles in supply of ATP, production of virulence and viability sugar molecules for the pathogen, therefore the pathways are potential targets for development of anti-parasite drugs. The potential of enolase as an antigen for vaccine development has been shown in various parasites such as *A. suum* (Chen et al., 2012) and *Streptococcus dysgalactiae* (Nguyen et al., 2020) in fish. Enolase could be a NAMP because it triggers an immune response which prevents *A. suum* larval migration (Chen et al., 2012). Furthermore, there is a high probability that it could also be involved in host immune suppression because similar findings were reported by Liu et al. (2012) where *S. glaseri* enolase was involved in immunity suppression of *Galleria mellonella*. Pathogens employ this mechanism through in order to protect themselves from the host defense system.

5.2 ADAM-10 and Tetraspanin

The high expression of ADAM-10 at eggs, adult males and females could be due to its function as revealed by BLAST2GO analysis that ADAM-10 is involved in hatching, male tail tip and vulva development. ADAM-10 and Tetraspanin have same expression pattern according to the transcriptome data and these two genes are functionally linked. Besides its classical role of interaction with adhesion proteins, tetraspanins also control cell membrane and metalloproteases. Tetraspanin 15 is the central modulator of ADAM-10 in mammalian cells by accelerating the exits of ADAM10–TSPAN15 complex from the ER and stabilizes the active proform of ADAM-10 at the surface membrane (Prox et al., 2012). The co-functionality of the two proteins could be the

reason why they are both expressed similarly. These two proteins were also predicted to have same biological function of regulation of cellular process and multicellular organismal development.

5.2 Heat shock proteins

HSP70/actin 5C is only seen upregulated in eggs, J2 and males. The reason could be due to involvement in embryo development ending in birth or egg hatching as predicted by Blast2Go biological functions. Males and J2 are the migratory stages with high expression of actin 5C, which makes sense because it is involved in locomotion.

HSP90/endoplasmin was significantly upregulated at early stages and the predicted biological functions were proteolysis, chaperone protein and ATP binding. Expression of HSP90 in various nematodes is highly expressed constitutively without disrupting normal growth and development (Perry & Wharton, 2011). HSP90 transcripts were detected in PPNs *M. incognita*, *H. glycines* and *M. artiellia* juvenile stages. Pre-parasitic J2s which is the most exposed development stage to adverse environmental conditions showed lower levels of HSP90 expression compared to parasitic juveniles. In our study result, a similar trend was observed as expression sharply increased in nematodes 10 hours post infection compared to J2 stage. These results suggest that plant defense mechanisms such as ROS are worse stressing conditions compared to soil environment hence the increase in need for HSP90 for its role in protecting PPNs from plant immunity (Lourenço-Tessutti et al., 2015). A subsequent knockdown of HSP90 in *M. incognita* resulted in delayed feeding site formation, 50% reduction in gall number and less egg production. HSP90 therefore may be involved in both plant defense evasion and nematode reproduction (Lourenço-Tessutti et al., 2015).

HSPs have immunologic functions, for instance HSP70, HSP90 and calreticulin, among other HSPs isolated from cancer tumor cells have the ability to induce specific immune responses (Basu & Srivastava 1999; Srivastava, 2002). Heat shock proteins are soluble intracellular proteins abundant in all cells. They bind to antigenic peptides and antigen presenting cells within cells therefore, HSPs have an essential role in stimulation of antigen presenting cells, cross priming or indirect presentation and peptide chaperoning during antigen presentation. HSPs may have been involved in basal/innate immunity since the evolvement and appearance of phagocytes in multicellular organisms of early existence. They became more specific over time and these attributes of HSPs allow them to be used in cancer and other infections immunotherapy (Srivastava, 2002; Colaco et al., 2013).

5.3 14-3-3 protein

14-3-3 protein sequences have been identified in more than a few unicellular and multicellular parasites as well as other non-parasitic but related organisms such as *C. elegans*. Nonetheless, their functions and expression patterns have not been fully investigated in most parasites. Jaubert et al. (2004) found that 14-3-3 protein is expressed in all stages; unhatched and invasive J2s, adult males and females of *M. incognita*. The same expression pattern was observed in our transcriptome data. 14–3-3 proteins are essential in the growth and survival of parasites therefore they are a potential vaccine candidates (del Mar Siles-Lucas & Gottstein, 2003). In *Schistosoma* and *Echinococcus* species, several 14-3-3 protein isoforms have been reported to enhance host immunity in experimental animals when used as vaccines. With this in mind, we cannot rule out that 14-3-3 protein in *H. schachtii* could also have possible plant immunity activation upon recognition by plant PRRs hence it is a NAMP. Five 14-3-3 proteins were identified in the secretome of *H. avenae* by Cui et al. (2018). The potential roles of these proteins secreted into host plant cells are still unclear because they have more than one function. It might be possible that they are involved in feeding site formation (Jaubert et al., 2004).

5.4 Cuticlin

Cuticlin has generally low transcript counts compared to other genes and is upregulated in adult stages. One of the reported functions of cuticlin is in stage specific formation of cuticular alae from the seam cells and only the first stage juveniles and adult nematodes possess alae (Page & Johnstone, 2007). This could be the reason why we observed an upregulation in adult stages. Cuticlins are highly conserved in nematodes as they occur in PPNs such as *M. artiella* and other human nematodes including *Ascaris lumbricoides* and *Brugia pahangi* (Lewis et al., 1994). Model organism *C. elegans* possesses six cuticlin genes which are also involved in dauer alae formation and cuticle formation in adult nematodes (Sapio et al., 2005).

5.5 Perspectives on NAMPS

NAMP recognition is an early warning sign to the plant for presence of harmful pathogens. According to both transcriptomic and qRT-PCR data, all the seven candidates in our study are expressed in all stages of infection therefore they are potential NAMPS. Their expression varies with time and is due to requirements of the nematode for that time. NAMPS do not need be differentially expressed because their mere presence is enough to alarm the plant to activate the innate immunity through PRR recognition. However, the quantity of a NAMP/PAMP available for

perception by PRRs may affect the intensity of the PTI induced. Even though some of the proteins exist in *Arabidopsis*, careful discrimination between self and those of nematode origin is crucial to prevent unnecessary or precocious defense eliciting (Vidhyasekaran, 2014).

It is of paramount importance to note that PAMPS instead of being produced and perceived singly in plant, they are presented as a cocktail that act synergistically or antagonistically to elicit defense (Aslam et al., 2009). PAMPs also evolved to evade recognition by PRRs as shown by the *Xanthomonas campestris* pv. *Campestris* flg22, which has a within-species polymorphism. *A. thaliana* is incapable of recognizing the 'disguised' epitope of flg22 (Sun et al., 2006). Therefore, our candidate proteins could also have within-species polymorphisms which could affect recognition by PRRs (Schwessinger & Zipfel, 2008).

According to Nürnberger and Brunner (2002), PAMPs are conserved components of microbial surfaces that are directly bound to by plant PRRs and cause an induction of defense responses. All our candidate genes have been reported as surface/exosomal proteins in various other parasites (Mathivanan et al., 2010). Exosomes can either act as immune activators or as immunosuppression molecules during microbial infection (Wang et al., 2018).While we are convinced that our candidate proteins found in NemaWater genes are most likely NAMPS which trigger PTI once perceived, we cannot dismiss the possibility of some of them having dual functions. Previous studies revealed the same phenomena in PAMPS of several pathogens such as INF1 and AVR3a, PAMPs secreted by potato pathogen *Phytophtora infestans* (Bos et al., 2010). The dual action is governed by presence/absence of individual amino acid residues or different domains within the pathogen protein (Bos et al., 2009). In plant-nematode interactions, effector proteins are released by nematodes to suppress PTI. Ethylene inducing xylanase (EIX) a *Trichoderma viride* effector possesses a five amino acid PAMP perceived by PRRs on its β- strand exposed to the surface (Mackey and McFall 2006).

Although most of the studied surface proteins are in helminths, remarkable similarities in PAMP perception have been discovered in animals and plants. Plants possess receptors that resemble mammalian cytoplasmic nucleotide-binding domain and LRR proteins. Therefore, the same mode of action that our candidates employ in helminths is most likely the same in *Arabidopsis* (Nürnberger et al., 2004). Thus, our results provide enough justification for further characterization of the candidates as NAMPS.

6. CONCLUSIONS AND RECOMMENDATIONS

All our candidate genes are differentially expressed in *Heterodera schachtii* at different times post infection. Expression check with qRT-PCR gave similar trends in some cases but most importantly they are expressed before and after infection on plants. We can therefore conclude that our candidate proteins found in NemaWater are indeed produced by corresponding genes in the nematodes. Further studies are needed to investigate how these candidate genes act as elicitors of plant defense.

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