Nematode Defenses: Characterization of the Superoxide Dismutase (SOD) from the Potato Cyst Nematode *Globodera pallida*

A Thesis
Presented in Partial Fulfillment of the Requirements for the
Degree of Master of Science
with a
Major in Plant Science
in the
College of Graduate Studies
University of Idaho
by

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December 2019

Authorization to Submit Thesis

This thesis of Nejra Solo, submitted for the degree of Master of Science with a Major in Plant Science and titled "Nematode Defenses: Characterization of the Superoxide Dismutase (SOD) from the Potato Cyst Nematode *Globodera pallida*," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Potato cyst nematodes (PCN), such as *Globodera pallida* and *Globodera rostochiensis*, are some of the most agriculturally and economically important pests of potato. If no measures are used to control this pathogen, then it has the potential to cause up to 80% yield loss. *Globodera pallida* is a sedentary endoparasitic nematode that infects the roots of a susceptible plant in order to establish a feeding site as a sole source of nutrition for its development. Plants are not defenseless when exposed to pathogens and are able to fight off most of them by activating an innate immune system. An oxidative burst, along with the production of the reactive oxygen species (ROS), is an important part of plant defenses. Reactive oxygen species, such as superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are highly toxic molecules that cause damage to pathogens. In order to successfully infect the plant, nematodes protect themselves from ROS through the activation of antioxidant metabolism and ROS scavenging enzymes. One of these enzymes is a superoxide dismutase (SOD; EC 1.15.1.1), which prevents cellular damage by catalyzing conversion of the superoxide radical ($O_2^{-\cdot}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The main goals of this study were to: 1) isolate and localize the expression of the *SOD* gene from *G. pallida*, and 2) study its expression after infection of the susceptible potato cv. Desiree, resistant potato cv. Innovator, and immune host (*Solanum sisymbriifolium*). We have isolated a putatively secreted isoform of the superoxide dismutase from *G. pallida* and localized the expression of this gene in the posterior region of the nematode, to the hypodermal tissue. However further analysis is required to determine if this enzyme is being secreted to the surface of the nematode. The *SOD* gene was significantly upregulated when nematodes infected roots of a host plant, but significant differences in expression during the infection of susceptible, resistant, or immune hosts were not observed. Our study suggests that this gene may be responding to stress regardless of whether the nematode is experiencing a compatible or an incompatible interaction.

Acknowledgements

First and foremost, I would like to thank my major professor, Dr. Louise-Marie Dandurand, for introducing me to the beauties of the nematode research during my undergraduate studies, as well as for giving me an opportunity to complete my master's degree in her lab. I am very thankful for Dr. Dandurand's supervision and support in my graduate studies at the University of Idaho. I would like to acknowledge my committee members, Dr. Allan Caplan, Dr. Joseph Kuhl, and Dr. Fangming Xiao, as well. I really do appreciate all of their help and insight during my time at the University of Idaho. I am deeply grateful for the entire PCN lab because without their help it would be incredibly difficult to turn this research project into a reality. I am not sure what I would do without the help from Dr. Joanna (Asia) Kud. Thank you for being such a patient listener, mentor, and for supporting me both professionally and personally. I would also like to thank Dr. William Price for his help with the statistical analysis.

This research was funded by the *Globodera* Alliance (GLOBAL) project under award number 2015-69004-23634, the APHIS PCN Immunity project under award number AP19PPQFO000C495, and the Potato commission (NPRC) PCN immunity project.

Dedication

Most importantly, I would like to thank my family. In particular to my parents Hajrudin and Zehra Solo, who have made incredible sacrifices to support my dreams. Thank you for showering me with love and support throughout my life. I would have never been where I am today or the person I am today without the help and encouragement from my brother, Mahir Solo. Thank you for being such a great role model for me, and for showing me that giving up is never an option. Of course, a special thank you goes to the University of Idaho Women's Basketball program, all of the coaches and my teammates. Thank you for all of the great opportunities and memories. Lastly, I would like to thank all the good people that have been with me through this journey. In particular, I am very thankful for the entire Winger family for being my family away from family, especially to Susanna Winger for constantly making me a better person.

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Chapter 1: Introduction

Nematodes

Nematodes are morphologically simple organisms that belong to the phylum Nematoda and can be found in a wide range of environments (Perry & Moens, 2011). Many species of nematodes are pests of plants, animals, and humans, where some of the plant parasitic nematodes have detrimental effect on a variety of crops in the world. It is estimated that plant parasitic nematodes (PPNs) cause annual crop loss of 157 billion dollars worldwide (Abad et al., 2008; Ali et al., 2013). Plant parasitic nematodes are classified based on their feeding habits into endoparasites and ectoparasites (Hussey, 1989). Ectoparasitic nematodes, such as the dagger nematode *Xiphinema index*, feed on the roots from the outside (Hao et al., 2012). On the other hand, endoparasitic nematodes are further separated into two groups: migratory and sedentary. Migratory endoparasitic nematodes, such as the lesion nematode *Pratylenchus*, do not form a permanent feeding site, but are constantly moving through the roots while feeding on the plant (Moens & Perry, 2009). Sedentary endoparasitic nematodes are able to invade a host to establish a close relationship with their host plant, by setting up the feeding site in the root tissue to feed internally (Perry & Moens, 2011). This group includes some of the plant parasitic nematodes that have the greatest impact on agriculture, such as root-knot nematodes (RKN), *Meloidogyne* spp., and cyst nematodes in the genera, *Globodera* spp. or *Heterodera* spp.

The Potato Cyst Nematodes *Globodera pallida* and *Globodera rostochiensis*

The potato cyst nematodes (PCN), *Globodera pallida* and *Globodera rostochiensis*, are some of the most damaging plant parasitic nematodes worldwide. Both *G. pallida* and *G. rostochiensis* have a very narrow host range including potatoes (*Solanum tuberosum*) and several other Solanaceae species (Nicol et al., 2011). *Globodera pallida* is a serious problem for potato production. It is assumed that this nematode originated in South America and was spread to Europe via new world potato breeding material in the 19th century (Thorpe et al., 2014). In the United States, *G. rostochiensis* was detected for the first time in 1941 on Long Island, New York State, and because of the restrictive measures that were put in place shortly after its discovery, its spread remained limited

to New York State (Brodi & Mai, 1989). In contrast, in the US *G. pallida* was detected much later, in April of 2006, in eastern Idaho (Hafez et al., 2007). Presence of *G. pallida* in Idaho is a threat to the nation's leading potato producer. The development of effective control measures is essential to protect Idaho's potato industry (USDA, 2016). Potato cyst nematodes are able to survive in a wide variety of environments as encysted eggs. *Globodera pallida* and *G. rostochiensis* will hatch as second stage juveniles (J2) following stimulation by different chemical signals released from roots of a suitable host plant (Widdowson & Wiltshire, 1958; Byrne et al., 2001). After it finds a root, the J2s use a combination of physical and chemical methods to penetrate and invade the host (Sobezak & Golinowski, 2011). Penetration and migration of J2s in host roots is aided by a specialized mouth part known as a stylet. The stylet is a hollow, protrudable structure which is an essential structure found in all PPN needed for parasitism (Williamson & Hussey, 1996). Second stage juveniles use this stylet to continuously strike and mechanically disrupt the cell wall, as well as to secrete different plant cell wall degrading proteins to chemically disrupt the cell wall (Ali et al., 2013). Furthermore, small proteins, called effectors (discussed below), will be secreted through the stylet as well during both migration and syncytium formation. Migration through the roots occurs intracellularly until J2s reach the inner cortex layer (Sobezak & Golinowski, 2011). Here nematodes form a syncytium in order to complete the rest of their life cycle. The feeding site is formed by partial dissolution of the wall of cells that surround the initial feeding cell (Jones et al., 2013). Infective J2s use their stylet to probe the cell walls of plants, and this is repeated until a suitable plant cell is found. A suitable plant cell becomeS the initial syncytial cell (Jones et al., 2013). At this point, the syncytium will incorporate up to 200 adjacent cells by localized cell wall dissolution and protoplast fusion (Cotton et al., 2014). The second stage juvenile uses this syncytium for the following 3 to 6 weeks in order to finish its life cycle. Sexual differentiation to males or females occurs depending on the size of the syncytium and the amount of nutrients available (Sobezak & Golinowski, 2011). Once PCN stops feeding, the syncytium deteriorates indicating the importance of the nematode stimulation in the maintenance of the feeding site (Hütten et al., 2015). Females assume a spherical shape and remain in the plant roots, while males become vermiform and exit the roots in order to find females (Sobezak & Golinowski, 2011). After fertilization, adult females die to form a cyst, which holds and protects the eggs until they hatch. *Globodera pallida* encysted eggs remain viable in soil for up to 20 years in the absence of a host (Evans & Stone, 1977).

Secretions of Potato Cyst Nematodes

Secretions of the potato cyst nematodes contribute to the nematode infection process by delivering cell wall modifying and cell wall degrading enzymes, antioxidant and detoxifying enzymes, or effectors, to the extracorporeal space (Davis et al., 2011; Robertson et al., 2000; Dubreuil et al., 2007; Smant & Jones, 2011). Among all of these secreted molecules, a lot of attention has focused on understanding the role of the effectors in the infection process, since they can potentially be used as a target to control this pest. Effectors have a wide variety of roles, from modifying the plant cell wall during migration and formation of the feeding site, to changing the host cell cycle and gene expression (Quentin et al., 2013). Furthermore, they have an important role in suppressing the host defense response (Gardner et al., 2015), as well as in protection of the nematode during the infection process (Haegeman et al., 2012). Effector proteins are produced in specialized esophageal gland cells, whose activity is developmentally regulated (Quentin et al., 2013). Two subventral glands are particularly active during root invasion and J2 migration, while the dorsal gland becomes bigger and more active during the formation of syncytium (Haegeman et al., 2012). Besides secretions through the esophageal gland cells, secreted molecules can be delivered by the nematode through other specialized organs including the cuticle and the amphids (Fenoll et al., 1997).

Regulation of *G. pallida*

Development of effective control measures is essential to protect Idaho's potato industry. Idaho is the nation's main producer of potato with a total value of $1.03 billion for 2018 (USDA-NASS, 2019). If no measures are applied towards the control of PCN, then it has a potential of causing up to 80% yield loss (Brodie, 1984; Singh et al., 2013). *Globodera pallida* is a quarantined pest, regulated by the Animal and Plant Health Inspection Service (APHIS) and the Idaho State Department of Agriculture (ISDA). Potato cyst nematodes are hard to control because they form cysts which protect eggs allowing them to survive in the soil for a very long time, have high genetic diversity, and farmers lack efficient eradication methods (Tran, 2016). Fumigation with methyl bromide (MeBr) has been shown to be a highly successful control measure for PCN, but since 2014, MeBr is no longer in use due to it destructive effect on the environment (Montreal Protocol of 1987; EPA, 2018). Another potential measure is use of resistant potato varieties, however no commercially grown russet varieties in Idaho carry resistance to *G. pallida*. Crop rotations are not a useful method

of control because PCN will hatch only in response to the hatching factors released from a suitable host plant (Widdowson & Wiltshire, 1958; Byrne et al., 2001). However, *Solanum sisymbriifolium*, which is resistant to *G. pallida*, is an effective trap crop for this nematode (Scholte & Vos, 2000; Dandurand & Knudsen, 2016). Previous studies have shown that *S. sisymbriifolium* has the ability to reduce PCN progeny to 1/1000 of its initial population (Dandurand & Knudsen, 2016) confirming that it can effectively control this pest. This is in part because *S. sisymbriifolium* causes PCN to hatch at relatively high frequencies, yet J2s cannot complete their life cycle in the roots of *S. sisymbriifolium* and die (Scholte, 2000; Timmermans et al., 2006). Understanding the defense mechanisms occurring in *S. sisymbriifolium* could help protect susceptible plants from *G. pallida.*

Plant Innate Immune Response to the Nematode Infection

Plants are continuously under various biotic and abiotic stresses. However, despite the fact that plants do not have an immune system comparable to that of a vertebrate, plants are still able to respond to and withstand pathogens and pests to which they are exposed (Felix et al., 1999). Successful pathogen recognition during a plant pathogen interaction will mostly depend on MAMP-triggered immunity (MTI) and effector triggered immunity (ETI). MAMP-triggered immunity is activated upon recognition of conserved molecules, known as microbial-associated molecular patterns (MAMPs), by a group of receptors called pattern recognition receptors (Muthamilarasun & Prasad, 2013). One example of nematode associated molecular patterns would be the female pheromone ascaroside, which was found to be highly conserved across different nematode species (Choe et al., 2012; Manosalva et al., 2015). Invading pathogens and pests overcome PTI with the help of small proteins, effectors, which work towards suppression of plant defenses (Gardner et al., 2015). For instance, *G. pallida* secretes a group of effectors, known as SPRYSEC effectors, to effectively disrupt PTI (Diaz-Granados et al., 2016). In order to manage this, plants produce specific proteins, resistance (R) proteins, which recognize effectors and trigger ETI (Bigeard et al., 2015); ETI is a more specific and much stronger defense response than PTI (Jones & Dangl, 2006). There are several R genes available that confer resistance to the PCN. *Gro1-4* and *Gpa2* provides resistances against certain pathotypes of *G. rostochiensis* and *G. pallida*, respectively (Paal et al., 2004; Van Der Vossen et al., 2001). One of the effectors from G. *pallida*, known as *Gp-RBP-1*, that belongs to the SPRYSEC group of effectors is recognized by *Gpa2* (Sacco et al., 2009; Eoche-Bosy et al., 2017). Moreover, the tomato R gene known as *Hero* provides broad resistance to *G. rostochiensis*, but only partial

resistance to *G. pallida* (Ernst et al., 2002). Activation of either PTI or ETI will signal the plant to deploy a wide variety of defense mechanisms, such as production of secondary metabolites, antimicrobial compounds, defense-related proteins, changes in ion flux, oxidative burst, as well as fortification of the cell wall by callose deposition (van Loon *et* al., 2006). The plant is also able to activate defenses that are more specific for PPNs. Effectors and R proteins are under strong selective pressure. A pathogen can evade ETI and infect the plant by producing new effectors to suppress it, modifying an already existing effector, or losing that effector all together (Anderson et al., 2010). In order to keep up with this, plants produce a new or modify an already existing R gene (Anderson et al., 2010). Because of this evolutionary interplay between effectors and R genes, there is a great amount of genetic diversity present in both.

Hypersensitive Response (HR) and the Oxidative Burst

One of the most effective plant defense mechanisms is directed by ETI and involves a type of local programmed cell death known as the hypersensitive response (HR) (Bigeard et al., 2015). A hypersensitive response is useful against biotrophic pathogens, such as *G. pallida*, and is a crucial part of the three specific ways that the plant responds to the nematode infection (Goverse & Smant, 2014). These differ based on the progress of the infection and involve: a) rapid local HR that causes host cell death upon start of a formation of the syncytium, b) prevention of syncytium spread by forming a sheet of dead host cells around the nematode feeding structure, and c) prevention of a formed feeding structure to provide sufficient nutrient for the nematode, which can occur with or without the occurrence of the HR (Goverse & Smant, 2014). Different R genes act through different modes to combat nematode infection, for example the R gene *Hero* functions by signaling production of a sheet of necrotic host cells around the nematode feeding structure which separates it from the rest of the plant tissue and leads to the degradation of the syncytium (Sobczak et al., 2005). In contrast, the R gene *Gpa2* acts in a delayed fashion where it allows formation of the feeding site and maturation of some nematodes, but it halts their ability to reproduce (Lozano & Smant, 2011). The hypersensitive response appears to be an important aspect of the defense process elicited by the litchi tomato (*Solanum sisymbriifolium*) (Timmermans, 2005; Kooliyottil et al., 2016). Some early signs of the initiation of the HR are rapid influxes of free calcium (Ca^{2+}), production of the reactive oxygen species (ROS), nitric oxide, and changes in the phytohormone expression (Garcia-Brugger et al., 2006; Lozano & Smant, 2011). Among several important roles during the plant defense response,

rapid influxes of the Ca^{2+} are also considered to be crucial for the activation of the NADPH oxidase found on the membrane of the plant cell (Kadota et al., 2015). This oxidase produces extracellular ROS which initiates a cascade of events leading to an oxidative burst (Lozano & Smant, 2011).

An oxidative burst along with the production of ROS is also an important part of the plant defense, since ROS create a cytotoxic environment for the pathogen or pest, as well as act as signaling molecules for local and systemic defense responses (Rosso, 2009; Gillet et al., 2017). Reactive oxygen species, highly toxic molecules such as superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) cause great damage to nucleic acids, proteins, and membrane lipids, and are potentially lethal to the pathogen and the host alike (Henkle-Dührsen et al., 1995). Out of all of these, the hydroxyl radical ($OH^{\cdot}$) has the potential to cause the most damage. The hydroxyl radical is formed from the superoxide radical ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) through Haber-Weiss or Fenton reaction (Robertson et al., 2000). However, ROS are not only formed during a defense response, but tare also byproducts of processes such as photosynthesis and respiration (Dat et al., 2000). In aerobic organisms, such as nematodes, ROS are produced as a part of normal aerobic metabolism (Van Voorhies & Ward, 2000). Nematode response to the oxidative stress, resulting from exposure to either self- or plant-derived ROS, is mostly regulated by two transcription factor genes, *daf*-16 and *skn-1* (Gillet et al., 2017). These transcription factor genes are responsible for the activation of several different antioxidant and detoxifying pathways, which function to avoid formation of the hydroxyl radical, control and neutralize levels of other ROS, as well as to prevent cellular damage due to oxidative stress (Callahan et al., 1988; Rosso, 2009; Gillet et al., 2017). Antioxidant and detoxifying pathways employ a wide variety of ROS scavenging enzymes. Some of the major ones have been found in plant parasitic nematodes, including superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase/glutathione peroxidase system, as well as thioredoxin peroxidase/ thioredoxin reductase system (Robertson et al., 2000; Mittler, 2002).

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD, EC 1.15.1.1) is a metalloenzyme that prevents cellular damage by catalyzing conversion of the superoxide radical ($O_2^{-\cdot}$) to the hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). In this way SOD controls the amount of hydroxyl radical ($OH^{\cdot}$) being formed.

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Figure 1.1. The reaction catalyzed by the superoxide dismutase (SOD).

There are three distinct classes of SOD in eukaryotic cells, a cytosolic Cu-ZnSOD, or sometimes referred to as *sod-1*, a mitochondrial MnSOD, or *sod-2*, and an extracellular Cu-ZnSOD, or *sod-3*; these enzymes share the same function, but differ based on the type of the metal ion at their active site and the localization in the cell (Hunter et al., 1997). While there are some structural similarities between the cytosolic Cu-ZnSOD and extracellular Cu-ZnSOD, MnSOD appears to be distinctly different from the other two (Miao & St. Clair, 2009). Interestingly, previous studies have found that in the free living nematode *Caenorhabditis elegans*, there are five SODs, referred to as *sod-1, sod-2, sod-3, sod-4,* and *sod*-5 (Landis & Tower, 2005). *Sod-1, sod-2*, and *sod-4* correspond to the cytosolic Cu-ZnSOD, mitochondrial MnSOD, and the extracellular Cu-ZnSOD, respectively, while *sod-3* was found to be in the mitochondria and *sod-5* in the cytoplasm (Giglio *et la*., 1994; Hunter et al., 1997; Van Raamsdonk & Hekimi, 2009). This suggests that *C. elegans* has additional cytoplasmic and the mitochondrial classes of SODs, which are usually present at the low levels (Schaar et al., 2015). In addition, it was shown that in *C. elegans* and *Drosophila* extracellular SOD class has two isoforms, one anchored in the membrane and the other secreted into the medium, which are a result of alternative splicing (Fuji et al., 1998; Blackney et al., 2014).

The significance of SOD has been shown in a wide variety of organisms, including the yeast, fruit fly, mouse, and plants (van Loon et al., 1986; Blackney et al., 2014; Carlsson et al., 1995; Gupta et al., 1993). Furthermore, SOD was shown to play a very important role during host-pathogen interactions, where on the pathogen side, it is assumed to be protecting it from the host derived ROS and promoting its virulence. Most of the studies confirming this are focused on the highly studied SOD1 and SOD2 classes of enzymes. Thus, deletion of a cytoplasmic Cu-ZnSOD in the pathogenic fungus *Botrytis cinerea* caused impaired lesion formation on the leaves of the susceptible *Phaseolus vulgaris* host plant (Rolke et al., 2004). Knocking out SOD1 in the enterobacterium *Erwinia chrysanthemi* strain 3937 significantly reduced its pathogenicity on the susceptible host *Saintpaulia ionantha* (Santos et al., 2001). Since it is the most recently described class of SOD, less is known about the potential importance of the SOD3 in the host-pathogen interaction. However, there are some indications that SOD3 might be an important virulence factor, as well as the protector for the pathogens during the infection. For example, it was shown that SOD3 protects bacteria *Nocardia asteroides* during infection in mice (Beaman & Beaman, 1990). Deletion of SOD3 in the fungus *Histoplasma capsulatum* prevented it from causing a successful respiratory infection (Youseff et al., 2012). Moreover, secreted extracellular SOD was found in the secretomes of different plant and animal parasitic nematodes, such as *Onchocerca volvulus, Brugia malayi, Haemochus contortus,*

Bursaphelencus xylophilus, Meloidogyne incognita, and *G. rostochiensis*, indicating that it might play a significant role in the parasitism of these animals (Henkle et al., 1991; Ou et al., 2002; Yatsuda et al., 2003; Shinya et al., 2013; Bellafiore et al., 2008; Robertson et al., 1999). After SOD catalyzes conversion of the superoxide radical into the H_2O_2, different enzymes will further act upon hydrogen peroxide. More specifically, it will be converted into water and molecular oxygen by catalases, glutathione peroxidases/reductases, and the thioredoxin peroxidases/thioredoxin reductases (Robertson et al., 2000). Different organisms will use different combinations of these enzymes, but all of them will function towards conversion of the H_2O_2 into a compound that is non-toxic to the cell.

Other Protection Mechanisms

Beside the antioxidant pathway, nematodes also upregulate xenobiotic metabolism for protection during the infection process. Xenobiotic metabolism is activated against internal or external toxic compounds (Gillet et al., 2017), and has a role in the detoxification and excretion of these compounds (Lindblom and Dodd, 2009). Upon exposure to oxidative stress xenobiotic metabolism in nematodes will be activated to reduce lipid hydroperoxides or to regenerate the S-thiolated protein (Sheehan et al., 2001; Babu et al., 2012). There are three phases of xenobiotic metabolism. Phase I metabolism, which mainly involves cytochrome P450, makes xenobiotics and endobiotics more soluble, while phase II metabolism is a detoxification step. In this phase enzymes, such as uridine dinucleotide phosphate glucuronosyl transferases (UGT) and glutathione S-transferases (GST), catalyze conjugate formation of xenobiotics and endobiotics with glutathione, amino acids, acetate, sulfate, propionate, or phosphate marking them for excretion (Kurutas, 2015; Laing et al., 2014). Most commonly this involves conjugation to glutathione (GSH), which is a tripeptide (γ-Glu-Cys-Gly) that has a major role in the processes of detoxification and redox buffering. In its reduced form it acts as a nucleophile that attacks electrophilic carbon, nitrogen, or sulfur atom on the toxic nonpolar compound (Edwards et al., 2000; Islam et al., 2017). Together with other antioxidants, such as ascorbate, α-tocophoral, and cysteine, it is an important aspect of non-enzymatic protection against oxidative stress (Kurutas, 2015). In animals, including nematodes, phase III involves excretion of these conjugates by ATP-binding cassette (ABC) transporters, which do not belong to the family of detoxifying enzymes (Lindblom and Dodd, 2009). However, plants lack effective excretion pathways, so ABC transporters selectively import these conjugates into vacuoles (Reinemer et al., 1996).

Goal of the Study

The *Globodera pallida* gene encoding Cu-Zn superoxide dismutase was identified in a recent study that looked at the transcriptome of this nematode when it was infecting susceptible plant (*Solanum tuberosmu*) vs. immune host (*Solanum sisymbriifolium*) (Kooliyottil et al., 2019). This gene was upregulated in the immune host, *S. sisymbriifolium*, compared to the susceptible potato plant. Although this upregulation was not statistically significant, due to the protective role of the SODs in other organisms we hypothesized that it might have a similar role during the nematode infection process. Our goal was to isolate and localize the expression of the *SOD* gene transcript from *G. pallida*, as well as to study its expression at the early stages of the infection when the nematode is infecting a susceptible potato cultivar (Désirée), a resistant potato cultivar (Innovator), and an immune host (*S. sisymbriifolium*). Understanding processes that are occurring in the nematode itself during the infection process is very important, since in this way we can gain insight into the underlying host-pathogen relationship, as well as novel control methods for this pest.

General Methods and Materials

Nematode Populations

Globodera pallida populations that were used to obtain second stage infective J2s were originally from an infested field in Shelley, ID. These populations were reared in a greenhouse under standard conditions on a susceptible potato cultivar ‘Désirée’. Standard greenhouse conditions included maintaining plants at a range of 10^{o}C night-time temperature to 18^{o}C day-time temperature, and a 16:8-h (day:night) photoperiod. Species confirmation was done based on the method described by Skantar et al., 2007. Fenwick Can method was used to extract cysts from the soil after 16 weeks (Fenwick, 1940) and the number of eggs contained in the cysts was determined as described bt Dandurand et al., (2017). Cysts were kept at 4^{o}C for a minimum of 16 weeks preceding any experimental use.

Hatching of G. pallida Eggs

Juveniles (J2s) for further experiments were obtained from hatched eggs. Approximately 150 cysts were placed on a meshed PVC tube and surface sterilized by exposing them to 0.5% NaOCl

(v/v) solution for 5 minutes, following by rinsing with sterile deionized (DI) H_2O. Cysts were crushed with sterile tweezers and placed into a 1:1 solution of antibiotic gentamicin sulfate (37.5 mg/ml; Sigma Aldrich, St. Louis, MO, USA) and the potato root diffusate and placed in a 23ºC incubator for 7 days without any collection, after which J2s were collected every 3-4 days over a 2 week period. Following each collection, cysts were again exposed to the 1:1 solution of antibiotic and potato root diffusate. Collected J2s were washed three times with sterile DI H_2O using centrifugations at 860 rpm for 3 minutes (Centrifuge 5810 R, Eppendorf, NY, USA). Washed J2s were either used immediately or stored at -80ºC until needed.

RNA Isolation and Reverse Transcription

Total RNA was extracted from approximately 1000 *G. pallida* J2s. Collected J2s were frozen in liquid nitrogen for 1 minute. Next, one 6mm grinding satellite (OPS Diagnostics, NJ, USA), 0.2ml of 1.0mm acid washed zirconium beads (OPS Diagnostics, NJ, USA), and a solution of 350 µl of Buffer RLT Plus (RNeasy® Plus Micro Kit, Qiagen, Hildren, Germany) containing β-mercaptoethanol were added to the tube. Nematode tissue was disrupted in a Biospec 3110Bx Mini-Beadbeater-1 (Biospec, Bartlesville, OK, USA) for three intervals each lasting 20 seconds. Samples were centrifuged for 3 minutes at 13000rpm (Centrifuge 5424, Eppendorf, NY, USA), and the rest of the RNA isolation was completed following manufacturer's protocol (RNeasy® Plus Micro Kit, Qiagen, Hildren, Germany). Nanodrop ND-2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to check the concentration and the purity of the isolated RNA before storing it in -80ºC. Subsequently, SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) kit was used for the reverse transcription of the total RNA into cDNA using the Anchored Oligo$(dT)_{20}$ Primer (Invitrogen, Carlsbad, CA, USA) and by following the protocol provided by the manufacturer.

Chapter 2: Isolation, Cloning, and the Localization of the Expression of the *Gp-SOD3* Gene from the Potato Cyst Nematode *Globodera pallida*

Introduction

Globodera pallida is a sedentary endoparasitic nematode that infects roots of suitable host plants to establish a feeding site as a source of nutrition for its development (Davis et al., 2000). This biotroph has a relatively narrow host range and a lifestyle that allows it to endure harsh environmental conditions (Nicol et al., 2011). Second stage juveniles (J2s) will use a specialized mouth structure, stylet, to mechanically disrupt the cell wall, as well as to secrete compounds that aid in the infection process (Davis et al., 2000). Molecules with diverse range of functions have been found in the secretions of plant parasitic nematodes, including cell wall degrading enzymes, cell wall modifying enzymes, antioxidant enzymes, detoxifying enzymes, as well as effectors (Davis et al., 2011; Robertson et al., 2000; Dubreuil et al., 2007; Smant & Jones, 2011). Secreted molecules are released by the nematode through several specialized organs including the esophageal gland cells, the hypodermis, and the amphids (Fenoll et al., 1997). *Globodera pallida* has three specialized esophageal gland cells which are active at different times during the infection process (Vanholme et al., 2004).

Plants are not defenseless when exposed to various pathogens and are able to fight off most of them by activating a very efficient plant innate immune system (Felix et al., 2002). Activation of either PAMP-triggered immunity (PTI) and the effector triggered immunity (ETI) will involve many changes to the cell, such as the activation of defense-related genes, anti-microbial proteins, secondary metabolites, oxidative bursts, and fortification of the cell wall (Nürnberger et al., 2004). The hypersensitive response (HR) is a highly effective defense response against biotrophs, such as *G. pallida*, and is specifically directed by ETI to initiate programmed plant cell death (Bigeard et al., 2015). As well as during more general responses, an oxidative burst plays an important role during the HR, where two bursts occur at a different time and place during the defense response (Kaloshian et al., 2011). The oxidative burst leads to the production of reactive oxygen species (ROS), such as superoxide radical ($O2^{-\cdot}$), hydrogen peroxide (H2O2), and hydroxyl radical ($OH^{\cdot}$), which are potentially highly damaging molecules (Sharma et al., 2012). In order to successfully infect the plant, pathogens need to be able to protect themselves. This is especially important for the pathogens with endoparasitic lifestyles, such as *G. pallida*, which are exposed to the plant defense response for a

considerable amount of time during their life cycle (Robertson et al., 2000). This protection involves activation of the antioxidant metabolism and recruitment of various ROS scavenging enzymes.

In general, the first line of defense against ROS and oxidative stress is an enzyme called superoxide dismutase (SOD). This metalloenzyme (EC 1.15.1.1) prevents cellular damage by catalyzing conversion of the superoxide radical ($O_2^{-\cdot}$) to the hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). There are three distinct classes of SOD in eukaryotic cells, a cytosolic Cu-ZnSOD, sometimes referred to as *sod-1*, a mitochondrial MnSOD, or *sod-2*, and an extracellular Cu-ZnSOD, or *sod-3*. They all have shared function, but differ in the type of the metal ion at their active site and the localization in the cell (Hunter et al., 1997). Studies examining secretomes of numerous animal and plant parasitic nematodes detected presence of the SOD, suggesting that this enzyme might be an important aspect for the parasitism for these animals (Henkle et al., 1991; Ou et al., 2002; Yatsuda et al., 2003; Shinya et al., 2013; Bellafiore et al., 2008; Robertson et al., 1999).

A recent study in our lab examined the transcriptome of the potato cyst nematode *Globodera pallida* when it was infecting a susceptible host (*Solanum tuberosum*) compared to an immune host (*Solanum sisymbriifolium*). Once inside the roots of a plant, the nematode encounters various plant defense responses, including ROS, so we were interested in finding genes that might have important roles in the protection of the nematode when exposed to the oxidative stress. A search of the *G. pallida* transcriptome revealed that a gene homologous to superoxide dismutase (SOD) was expressed in the nematode during infection (Kooliyottil et al., 2019). Transcriptome analysis showed that this gene was slightly, but not significantly upregulated when the nematode was in the resistant compared to the susceptible environment. However, based on the confirmed protective role of this enzyme against oxidative stress in different organisms (Santos et al., 2001; Beaman & Beaman, 1990; Youseff et al., 2012), we proposed that it may serve in a protective role for *G. pallida* during the infection process. In addition, *G. pallida* genome analysis showed that this parasite has undergone an expansion of the SOD enzyme family compared to other nematodes, further suggesting that SOD might have an important role for the life and *G. pallida* parasitism (Cotton et al., 2014). Since this gene has high homology to genes corresponding to Cu-Zn SOD in free living, animal, and plant parasitic nematodes, we referred to it as *Gp-SOD3*. Here we focused on the isolation, cloning, and the localization of expression of this *Gp-SOD3* gene, in order to unravel some of the putative roles that it might have during the infection process.

Methods and Materials

DNA Isolation for the gDNA Amplification

Total DNA was extracted from approximately 3000 J2s of *G. pallida* according to the modified protocol described in Tran, (2016). Collected J2s were frozen in liquid nitrogen for 1 minute. One 6mm grinding satellite (OPS Diagnostics, NJ, USA), 0.2ml of 1.0mm acid washed zirconium beads (OPS Diagnostics, NJ, USA), a solution of 150 µl of lysis buffer (250mM NaCl, 200mM Tris-HCl (pH. 8.5), 25mM EDTA, 0.5% SDS, 120µg/ml proteinase K), and 75µl of 3M sodium acetate (pH 5.2), were added to the tube. Nematode tissue was disrupted in a Biospec 3110Bx Mini-Beadbeater-1 (Biospec, Bartlesville, OK, USA) for three intervals each lasting 20 seconds. Samples were incubated at -20^{o}C for 10 minutes and centrifuged for 5 minutes at 13000rpm (Centrifuge 5424, Eppendorf, NY, USA). Precipitation of gDNA was completed using isopropanol and centrifugation at 13000rpm for 25 minutes. Pellet was washed twice with 70% ethanol, followed by a wash with 100% ethanol. Next, pellet was air-dried and dissolved in 30µl of ddH_2O. Nanodrop ND-2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to check the concentration and the purity of the isolated gDNA before storing it at -20^{o}C.

Amplification of the Gene, Cloning, and Sequencing Analysis

The *G. pallida* transcriptome was screened for transcripts that were differentially expressed in *G. pallida* during early stages of infection in the susceptible plant vs. *S. sisymbriifolium,* and were homologous to genes important for the antioxidant pathway, by using the BLAST2GO tool (Kooliyottil et al., 2019). This search indicated several different genes whose corresponding sequences were obtained from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/). Further homology searches were done using the BLASTn and BLASTp tools. The SignalP 5.0 server was used to check the presence of the signal peptide (Armenteros et al., 2019) and TMHMM algorithm was used to check for any transmembrane domains (Krogh et al., 2001). Based on the results of this initial scan we decided to focus on the *Gp-SOD3* (GPLIN_000385400) in our study. The *Gp-SOD3* gene sequence was used to design gene specific primers (Table 2.1). A forward primer, SODF, aligned with the 5' end of the sequence and it contained restriction site corresponding to *Bam*HI. A reverse primers, SODR2, aligned with the 3' end, and contained a restriction site corresponding to *Xba*I. The gene was amplified using this primer combination in a 50µl PCR reaction containing 100ng of gDNA template

(see DNA Isolation for the gDNA Amplification), 10X Standard *Taq* Reaction Buffer (NEB, Ipswich, MA), 100µM of each dNTPs, 10µM of each primer, and 0.625 units of Standard *Taq* polymerase (NEB, Ipswich, MA). PCR amplification reaction was carried out in Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA) using the following program set up, initial denaturation at 95ºC for 30 seconds, 30 cycles at 95ºC for 30 seconds, 62ºC for 45 seconds, and 68ºC for 135 seconds, followed by a final extension at 68ºC for 5 minutes and a hold at 4ºC. The obtained PCR product was tested for correct size by agarose gel (1%) electrophoresis and purified using QIAquick PCR Purification Kit (Qiagen, Hildren, Germany). Purified PCR product was ligated into the pCR™8 TOPO® TA vector using the pCR™8/GW/TOPO® TA Cloning Kit (Sigma Aldrich, St. Louis, MO, USA). Following ligation, the products were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) using heat-shock transformation by following the protocol provided by the manufacturer. We selected 5 positive colonies and cultured them in order to extract plasmids (QIAprep Spin Miniprep Kit, Qiagen, Hildren, Germany) for sequencing with the universal M13F and M13R primers (Table 2.1). Plasmids were sequenced by the GenScript (Piscataway, NJ, USA) sequencing service in order to ensure the correct sequences of the insert.

Amplification of the Coding Sequence, Cloning, and Sequencing Analysis

Next, this gene was amplified from the cDNA in order to obtain the coding sequence of the gene. The same primer set was used as above (SOD F and SOD R2) to amplify this gene in a 50µl PCR reaction containing 200ng of cDNA template (see RNA Isolation and Reverse Transcription; Chapter 1), 10X Standard *Taq* Reaction Buffer (NEB, Ipswich, MA), 100µM of each dNTPs, 10µM of each primer, and 0.625 units of Standard *Taq* polymerase (NEB, Ipswich, MA). PCR amplification reaction was carried out in Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA) using the following program set up, initial denaturation at 95ºC for 30 seconds, 30 cycles at 95ºC for 30 seconds, 62ºC for 45 seconds, and 68ºC for 1 minute, followed by a final extension at 68ºC for 5 minutes and a hold at 4ºC. Obtained PCR product was tested for correct size by agarose gel (1%) electrophoresis and purified using QIAquick PCR Purification Kit (Qiagen, Hildren, Germany). Purified PCR product was digested using *Bam*HI and *Xba*I restriction enzymes (NEB, Ipswich, MA) following the protocol provided by the manufacturer. Next the digested product was ligated into a binary vector pBTEX carrying a 5X FLAG tag at the 3' end. Following ligation, the product was transformed, sequenced, and analyzed as described in Amplification of the Gene, Cloning, and Sequencing Analysis. pBTEX F2 and pBTEX R2 primers (Table 2.1) were used to sequence the plasmids.

Table 2.1. Primers used in this study.

Primer name	Sequence (5'-3')
SODF	ACGGATCCATGGCTTTTTTCCTCTCTCCGCT
SODR2	AGTCTAGATTAAAGCTCTTTGATGATGCCG
pBTEX F2	GGAGCATCGTGGAAAAAGAAGACGTTC
pBTEX R2	CATAAAAATACGATAGTAACGGGTGAT
M13F	TGTAAAACGACGGCCAG
M13R	GTCATAGCTGTTTCCTG
SODF4	TTTCAATCCGCACAACAAAA
SODR4	CCTTCTTTTCGTGCAAGACC
SODqF6	CTGAATGGCAGCATCAACGG
SODqR6	CGTGCTTTTTGTTGTGCGGA
EiF-F	AACATCTCTFTFAAGGACATTCG
EiF-R	TCTCCTTAAGTTCGGCGAATTTGC
GSTF2	GCATGGTCCAATACAAATTG
GSTR2	CAATCGGTTTTCTCCATCGT
GSTF3	GCTTGTCCATGGTGGACTTT
GSTR1	ACGATGGAGAAAACCGATTG

In situ Hybridization

In order to localize the transcript of this gene, we used an *in situ* hybridization experiment with antisense digoxigenin (DIG)-labeled DNA probes (Roche, Basel, Switzerland). The cDNA fragment library was amplified from the plasmid expressing *Gp-SOD3* using primers SODF4 and SODR4 and was used in an asymmetric PCR to synthesize the 293bp sense and antisense cDNA probes (Table 2.1). The *in situ* hybridization experiment was performed as reported in De Boer et al., 1998. Approximately 60,000 *G. pallida* J2s were fixed, cut, and permeabilized. After the permeabilization, DIG-labelled probes were added to the J2s and hybridization was performed at 50^{o}C overnight. In order to detect the cDNA probes, J2s were incubated with the alkaline-phosphatase conjugated anti-digoxigenin antibody (Roche, Basel, Switzerland), followed by an overnight incubation at 4^{o}C in the 4-Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche, Basel, Switzerland) staining solution. Results were visualized under an inverted light microscope (Leica Microsystems, model DMi8).

Results

Identification of the Gp-SOD3 from G. pallida

Using the BLAST2GO scan of the *G. pallida* transcriptome (Kooliyottil et al., 2019), we tried to find genes that might be upregulated or downregulated when the nematode is placed in a susceptible compared to a resistant environment to identify whether the gene may be associated with the antioxidant or the detoxifying pathway of the nematode. Our search did not produce any genes that were significantly differentially expressed when the nematode was exposed to these two environments. However, we were able to isolate several genes that might encode for the antioxidant or the detoxifying enzymes in *G. pallida*. Nucleotide and amino acid sequences for these genes were obtained from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/) in order to do a further investigation. Obtained gene sequences do not vary from the current version (WBPS14) of the *G. pallida* genome. One of the genes isolated in this analysis was GPLIN_000385400.

Amplification of the Gp-SOD3 from G. pallida

Specific primer set (Table 2.1) was designed based on the *Gp-SOD3* (GPLIN_000385400) sequence and was used to amplify the entire coding region of this gene from the cDNA library obtained from *G. pallida* pre-parasitic J2s. Initially, we designed a primer set combination that covers the sequence corresponding to the entire coding sequence of the GPLIN_000385400 found in the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/). However, we were unable to obtain any amplification with this primer set. Next, we used genomic DNA extracted from *G. pallida* pre-parasitic J2s to try to detect the presence of this gene with the same primer set combination. This step did not result in any amplification as well. We suspected that the *Gp-SOD3* sequence we were basing our investigation on was somehow misassembled or incomplete, so we looked at the ortholog of this gene, classified as GROS_g10871, from the closely related golden cyst nematode *G. rostochiensis* (https://parasite.wormbase.org/Globodera_rostochiensis_prjeb13504/Info/Index/). Nucleic acid of this ortholog had 94% identity, while amino acid sequence had 96% identity with the *Gp-SOD3* sequence. In spite of this high homology, GROS_g10871 sequence covered only 22% of the *Gp-SOD3* sequence, corresponding mostly to the predicted SOD binding domains. Since the 5’ ends of these two sequences were identical, we have designed a new reverse primer (Table 2.1), aligning to the 3’

end of the GROS_g10871 sequence. Using this primer we were able to amplify a sequence of approximately 600 bp from the cDNA library, as well as a sequence of approximately 2000bp from the *G. pallida* gDNA library. This corresponded to the cDNA and gDNA sequence sizes encoded by the GROS_g10871, not the sequence sizes predicted by the *Gp-SOD3* sequence in the *G. pallida* genome database. Further, we decided to clone and sequence this gene in order to obtain sequence of this gene.

Cloning and Sequencing of the Gp-SOD3 from gDNA

The amplified product from genomic DNA was cloned into a pCR™8 TOPO® TA vector. Positive clones were sequenced using Sanger sequencing platform. This revealed that *Gp-SOD3* gene sequence differed to the sequence predicted by the *G. pallida* genome database. We obtained gene sequence that encoded for a product of 2186 bp compared to >10kb sequence predicted by the *Gp-SOD3*. As well, we were able to show positions of introns and exons in the obtained gene sequence (Fig 2.1).

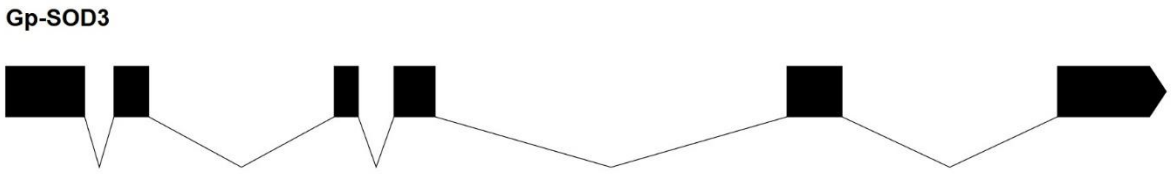

Figure 2.1. The gene sequence for the *G. pallida Gp-SOD3*. Exons are represented by boxes, while introns are represented by thin lines.

Cloning and Sequencing of the Gp-SOD3 Coding Sequence

Amplified product was cloned into a pBTEX vector with a 5x FLAG tag fused to the C terminus of the gene. Subsequent sequencing revealed that the coding sequence of the *Gp-SOD3* was very different to what was predicted by the *G. pallida* genome. We obtained coding sequence that encoded for a product of 642 bp (Fig 2.2) contrasting the 2835bp coding sequence predicted by the *Gp-SOD3* sequence available in the *G. pallida* genome database.

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atgcttttcttacttttcttgctccttcaattagccccttcggtcgaaaatgatggctgt
 M  L  F  L  L  F  L  L  L  Q  L  A  P  S  V  E  N  D  G  C
ggctatgggccaattggttacggttatgggcaaatcgggcacgggcatgggcgaccgccg
 G  Y  G  P  I  G  Y  G  Y  G  Q  I  G  H  G  H  G  R  P  P
ccacattccggaaattgtgtaacaagagtggtccaggctcgtgtgctaatgtttaaggca
 P  H  S  G  N  C  V  T  R  V  V  Q  A  R  V  L  M  F  K  A
accaataatacggactcgacgccatcacaaccgattggtgtggtggacatattcgagagt
 T  N  N  T  D  S  T  P  S  Q  P  I  G  V  V  D  I  F  E  S
gcttatgggactacgagcctgaatggcagcatcaacggcctcacgccgggcgaccatggc
 A  Y  G  T  T  S  L  N  G  S  I  N  G  L  T  P  G  D  H  G
tttcactttcaccagtttggcgacctttcgcaggcgtgcgcaggggcgggtgcgcatttc
 F  H  F  H  Q  F  G  D  L  S  Q  A  C  A  G  A  G  A  H  F
aatccgcacaacaaaaagcacggggcaccagacgcgaccgaacggcacgtgggcgatctg
 N  P  H  N  K  K  H  G  A  P  D  A  T  E  R  H  V  G  D  L
ggcaatttggtggccaatgctcacggagtggccgttgtgaacactcatgaccacatggtg
 G  N  L  V  A  N  A  H  G  V  A  V  V  N  T  H  D  H  M  V
acactgaacaacaacggcgtgaacagtgtggtcgggcgggcattggtcttgcacgaaaag
 T  L  N  N  N  G  V  N  S  V  V  G  R  A  L  V  L  H  E  K
aaggacgacctgggcctggccgccaacgaggaaagcctgaagacggacaattcgggtaaa
 K  D  D  L  G  L  A  A  N  E  E  S  L  K  T  D  N  S  G  K
agggtcgcctgcggcttggtcggcatcatcaaagagctttaa
 R  V  A  C  G  L  V  G  I  I  K  E  L  -
```

Figure 2.2. The coding and translated amino acid sequence for the *G. pallida Gp-SOD3* sequence (GPLIN_000385400).

We translated this nucleotide sequence into an amino acid sequence (Gasteiger et al., 2003) to obtain a putative full length open reading frame that encodes for a protein of 213 amino acids in size. The translated amino acid sequence was used to conduct a further *in silico* analysis. Our gene of interest was predicted to have 6 paralogs, so first we determined the homology of the obtained amino acid sequence with the amino acid sequences of the paralogs. All of these paralogs were predicted to contain a Cu-Zn SOD binding domain (PF00080), and comparisons showed that most of the similarity to our gene was shared in the region that encoded for this domain. Only one of these paralogs (GPLIN_000150100) was predicted to have a secretion peptide at the N-terminus (Fig B.2). The alignment of the amino acid sequence of the GPLIN_000150100 with the amino acid sequence we have obtained showed that there was 69% homology between the two, with the sequence disparities occurring mostly at the N-terminus of the encoded proteins.

Next, we did a homology search of our amino acid sequence by using several available databases. The database search revealed homology with the amino acid sequences of the predicted Cu-Zn SOD enzymes from different nematodes. The highest level of homology (69%) was shared with a putative effector protein from *Heterodera avenae* (Genbank accession number AVA09666.1) (Fig 2.3). This putative effector from *H. avenae* was assumed to contain a Cu-Zn SOD domain. The

predicted Cu-Zn superoxide dismutases from *Dictyocaulus viviparus* (ABS12246.1), and *Ditylenchus destructor* (AGI95995.1), shared 43% and 45% amino acid sequence similarity, respectively, to the *G. pallida* sequence of interest (Fig 2.3). Furthermore, it shared 40% homology with the extracellular SOD from the free living nematode *Caenorhabditis elegans* (Fig 2.3). Predicted binding sites for Cu and Zn metal ions, as well as the cysteine cross-linking sites are conserved amongst all of the compared sequences (Fig 2.3).

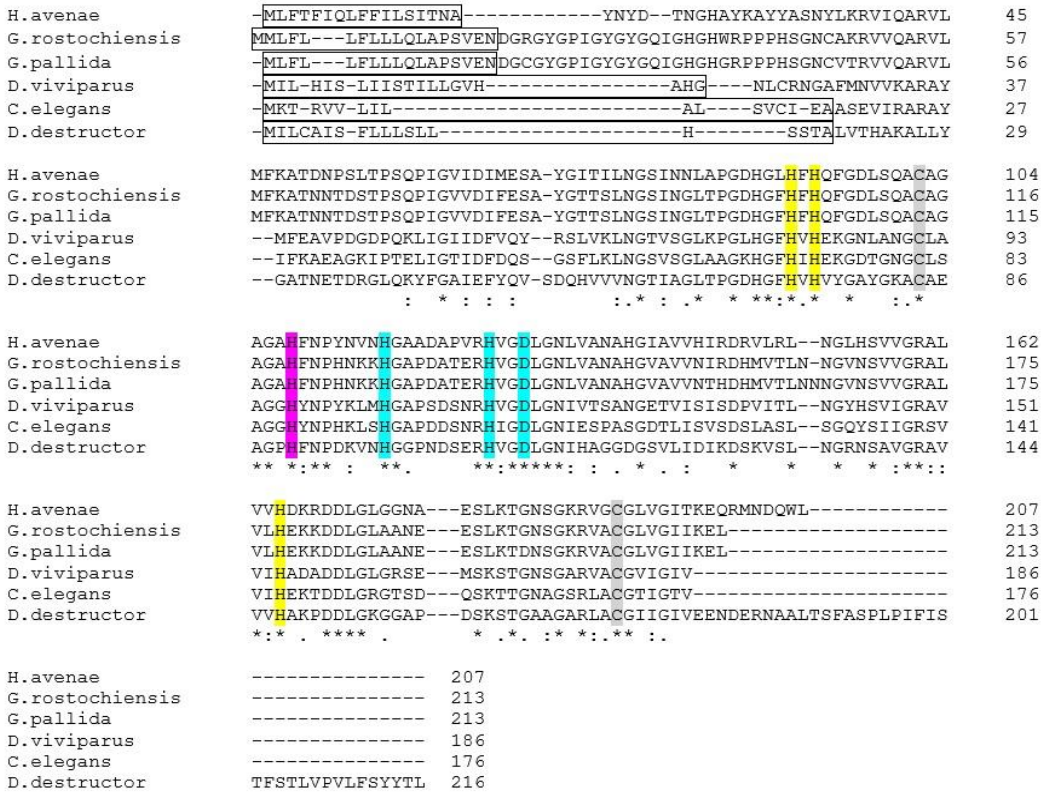

Figure 2.3. Alignment of the *Gp-SOD3* amino acid sequence with homologs from other nematode species (*Heterodera avenae, Globodera rostochiensis, Dictyocaulus viviparous, Caenorhabditis elegans, Ditylenchus destructor)*. Fully conserved regions are marked with (*); regions with the conservation between the groups of strongly similar properties are marked with (:); regions with the conservation between the groups of weakly similar properties are marked with (.). Binding sites for Cu are shaded in yellow; disulphide bond sites are shaded in light gray; binding sites for Cu and Zn are shaded in purple; binding sites for Zn are shaded in blue. Boxed regions at the N-terminus correspond to the predicted secretion peptides. Alignment was conducted using the Clustal Omega (Sievers, 2011) server.

The first 17 amino acids at the N-terminus of the Gp-SOD3 amino acid sequence were predicted to represent a signal peptide sequence using the SignalP 5.0 server (Fig B.1) (Armenteros et al., 2019) and no transmembrane domains were detected with the TMHMM algorithm (Krogh et al., 2001), which indicates that this enzyme could possibly be secreted by *G. pallida*.

In situ Hybridization

Based on the predicted signal peptide at the N-terminus of the Gp-SOD3, we hypothesized that this enzyme might be secreted by the nematode. We amplified a partial conserved region of the *Gp-SOD3* sequence from the *G. pallida* pre-parasitic J2s cDNA. The amplified product was used to generate antisense cDNA probes. *In situ* hybridization localized the expression of this gene in the posterior region of the nematode, which appears to be the hypodermal tissue of the nematode (Fig 2.4).

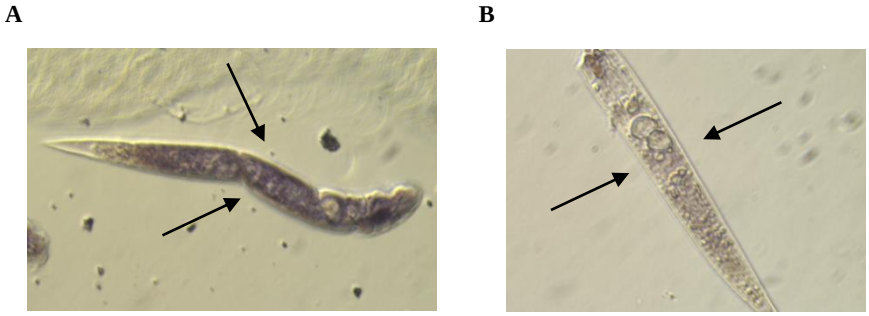

Figure 2.4. *In situ* hybridization of the pre-parasitic second stage juveniles (J2s). Antisense probe is binding to the tissues in the posterior region of the nematode (A). No signal was observed with the sense probe (B).

Discussion

Plants are sedentary life forms, and as such need to endure the wide array of challenges that they are exposed to. This pressure has allowed plants to evolve a very sensitive and complex immune system, which is crucial in the identification and the recognition of invading pathogens or the pests (Andersen et al., 2018). One part of the defense response that may play a significant role during the interaction between the plant and the pathogen is the oxidative burst (Mehdy, 1994). Oxidative bursts lead to the production of ROS, such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), which are toxic molecules that have several important roles during the defense response (Sharma et al., 2012). Some of these roles include reinforcement of the cell wall, prevention of the growth or spread of the pathogen, signaling activation of defense related genes, as well as contributing to the HR (Peters et al., 2017). In order for pathogens to cause a successful infection when challenged with the plant defense response, it is necessary for them to be able to overcome it. One way pathogens achieve this is by producing antioxidant enzymes, which specifically scavenge the ROS and convert them into compounds less toxic to the cell. SOD is the only antioxidant enzyme that can convert the superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2)

(Gupta et al., 2015). Eukaryotic cells have three types of the SOD enzyme, a cytosolic Cu-ZnSOD, a mitochondrial MnSOD, and an extracellular Cu-ZnSOD (Hunter et al., 1997).

In this project we have focused on the characterization of the Cu-ZnSOD enzyme from the potato cyst nematode, *G. pallida*, which we referred to as *Gp-SOD3*. This gene was isolated as one of the putative detoxification and antioxidant enzyme in a study that looked at the differences in the transcriptome of the PCN at the early points of the infection in a susceptible and a resistant plant (Kooliyottil et al., 2019). We have decided to focus on the *Gp-SOD3* specifically because the *G. pallida* genome was shown to have undergone an extensive expansion of SOD enzymes compared to the free living *C. elegans* or the root-knot nematode *Meloidogyne incognita* (Gregory, 2019; Cotton et al., 2014), suggesting that this family of enzymes may be important for the physiological processes in *G. pallida*. Most of the SODs predicted in the *G. pallida* genome are thought to belong to the Cu-Zn SOD class. A contributing factor to this expansion might be the intracellular migration of the cyst nematodes through the roots of the plant, which triggers increased oxidative response by the plant, compared to the intercellular migration of the root-knot nematodes (Cotton et al., 2014).

At the start of our experiment, we obtained the sequence for this gene from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/), but we were unable to amplify this gene from the pre-parasitic (non-infectious) J2s cDNA or the genomic DNA (gDNA) library. Since the genome database of *G. pallida* is fragmented and has much lower coverage compared to the genome of the closely related golden cysts nematode *G. rostochiensis*, we used the sequence of the *G. rostochiensis* ortholog as our reference sequence for the *Gp-SOD3* (Cotton et al., 2014; Eves-van den Akker et al., 2016). Using the sequence of this ortholog has allowed us to amplify *Gp-SOD3* from the *G. pallida* pre-parasitic J2s cDNA and gDNA library. This was followed by cloning and sequencing using the Sanger DNA sequencing platform. Sequencing has revealed that the sequence for the *Gp-SOD3* predicted by the *G. pallida* genome was misassembled and that it contained fragments of other genes. The sequence we have obtained only covered a portion of the predicted sequence, however it encoded what appeared to be an ORF without any premature stop codons. Further *in silico* analysis revealed high homology to genes predicted to encode for a Cu-Zn SOD or to contain a Cu-Zn SOD binding domain. This strengthens our assumption that *Gp-SOD3* might encode for a Cu-Zn SOD enzyme.

Since *G. pallida* genome database has predicted that *Gp-SOD3* has 6 paralogs, we wanted to look at their specific relationships to each other. Most of the amino acid sequence similarity was shared at the regions of the protein where the Cu-Zn SOD domain was predicted to be. Considering

that, in addition to the *Gp-SOD3*, only one of the paralogs has a signal peptide at the N-terminus, we assumed that all of the other paralogs are probably encoding for the cytoplasmic Cu-Zn SOD class. This would explain the homology we have observed between the *Gp-SOD3* and its paralogs, since it is assumed that the cytoplasmic Cu-ZnSOD gave rise to the extracellular Cu-ZnSOD through the addition of a secretion peptide (Landis & Tower, 2005). Nonetheless, the presence of two *SOD* encoding genes that are predicted to have a signal peptide, suggests that *G. pallida* may have two extracellular SODs. Multiple isoforms of the extracellular SOD enzyme have been found in other organisms such as the *C. elegans* and *Drosophila*, where one is anchored in the membrane and the other secreted into the medium (Fuji et al., 1998; Blackney et al., 2014). However, these isoforms are a product of the alternative splicing, where the membrane bound isoform is predicted to carry a hydrophobic region at the C-terminus allowing it to be embedded in the membrane (Fuji et al., 1998; Blackney et al., 2014). In our case, neither the *Gp-SOD3* nor its paralog were predicted to have multiple splice variants, or to encode for a hydrophobic region in the C-terminus. This could suggest that the expansion of the SOD enzymes in *G. pallida* has allowed it to evolve two extracellular superoxide dismutases that could be expressed at different parts of the life cycle of this nematode. For instance, a similar phenomenon is seen in the nematode *C. elegans*, where besides having three main classes of SOD enzyme, it also has an additional mitochondrial and cytoplasmic class that are usually present at low levels and induced at different stages of development (Hunter et al., 2015, Schaar et al., 2015). On the other hand, it could also be that besides predicting secretion outside of the nematode, the signal peptide found at the N-terminus of these two enzymes could be causing the secretion of one of the enzymes within the nematode, where it might be protecting the nematode from internal oxidative damage (Ou et al., 1995).

Figure 2.3 shows the amino acid sequence comparisons of the *Gp-SOD3* with the sequences of the predicted Cu-Zn SOD enzymes from *G. rostochiensis*, the cereal cyst nematode *H. avenae*, the bovine lungworm *D. viviparus*, the potato rot nematode *D. destructor*, and the free living nematode *C. elegans*. All of the sequences we have compared with Gp-SOD3 were predicted to have a secretion peptide at their N-terminus, suggesting that they are putatively secreted forms of the SOD enzyme. Furthermore, this alignment showed that most of the sequence similarity was seen at the C-terminus of the protein, where the SOD copper/zinc binding domain is predicted to be encoded, suggesting functional conservation of this protein. In contrast, the N-terminus of the encoded protein was highly divergent between these different nematode species. This is understandable since this part of the protein has a potential role in determining the cellular or the extracellular localization of SOD enzyme. In addition, it was shown that besides some general domain arrangement conservation, the

signal peptide is mostly highly heterogeneous from species to species (von Heijne, 1990). It was interesting to see that the Gp-SOD3 amino acid sequence shared high homology with a putative effector from *H. avenae*. This gene was isolated as one of the highly expressed genes in parasitic J2s from the transcriptome of *H. avenae*, and it was shown to be suppressing BAX-triggered plant cell death in the leaves of *Nicotiana benthamiana,* suggesting that it could have some putative role in a suppression of plant defenses (Chen et al., 2018). However, none of the subsequent analysis, such as localization of the transcript expression via *in situ* hybridization or the systemic transient expression in *N. benthamiana,* has been done with this gene, making it hard to make any further assumptions about its possible function.

Studies have shown that secretions from plant parasitic nematodes are crucial for a successful infection, which for *G. pallida* involves both migration through host roots and formation of a feeding site (Siddique & Grundler, 2018). A major component of PPN secretions are effector proteins. Effector proteins have many different roles in nematode parasitism, including evasion of the host defense system, modification of the cell wall, and maintenance of the feeding site (Rehman et al., 2016). Indications that a molecule is an effector are as follows: it has a signal peptide, it contains no transmembrane domains, and it is localized in the esophageal gland cells of the nematode. *In silico* analysis of the *Gp-SOD3* sequence predicted for it to have a secretion signal and no transmembrane domains (Armenteros et al., 2019; Krogh et al., 2001). Since secretions of both animal and plant parasitic nematodes have revealed the presence of a SOD enzyme (Henkle et al., 1991; Ou et al., 2002; Yatsuda et al., 2003; Shinya et al., 2013; Bellafiore et al., 2008; Robertson et al., 1999), we decided to look if the transcript of *Gp-SOD3* is being localized in the esophageal gland cells *G. pallida*. We found that the *Gp-SOD3* was localizing in the posterior region of the nematode body, to what appears to be the hypodermal tissue. The lack of the signal localization in the glands of the nematode does not entirely eliminate the possibility that *Gp-SOD3* is secreted. Secreted extracellular SOD has been reported in the secretions of *G. rostochiensis* indicating that PCN could secrete this enzyme through the use of another secretory organ, including the cuticle, amphids, excretory/secretory system, or the rectal gland (Robertson et al., 1999; Jones & Robertson, 1997; Rehman et al., 2016). The hypodermis is a tissue found right beneath the cuticle; transcript localization in the hypodermal tissue could suggest secretion of the protein to the cuticle of the nematode (Prior et al., 2001; Jones et al., 2004). The transcript of glutathione peroxidase, an enzyme of the antioxidant pathway, was localized in the hypodermis of *G. rostochiensis*, as well as detected on the surface of this nematode (Jones et al., 2004). Presence of the antioxidant enzymes, such as SOD, in the cuticle would most certainly be very beneficial for the nematode during the interaction

with the host. Here SOD could act directly on the superoxide radical produced by the plant and in this way protect the surface of the nematode from the potential oxidative damage (Jones et al., 2004). The extracellular SODs from the human parasitic nematodes *Brugia malayi* and *Onchocerca volvulus* were found in the hypodermal tissue, as well as in the secretomes collected from these nematodes (Ou et al., 1995; Henkle-Dührsen et al., 1997). However, in order to confirm if the Gp-SOD3 is being secreted to the outside cuticle of the nematode, additional experiments are required. One future approach would be to raise antibodies specific to this SOD enzyme to test if the antibodies would be able to detect presence of the enzyme on the cuticle of the nematode.

In summary, we present the isolation and the identification of the copper-zinc class of the superoxide dismutase enzyme from the potato cyst nematode *G. pallida*. We show that this enzyme shares high homology to the Cu-Zn SOD domain containing protein from the golden cyst nematode *G. rostochiensis*, as well as similar proteins from several other nematode species. Our results indicate that the expression of this gene is localized in the hypodermal tissue of the nematode, however additional analysis is required to confirm that this protein is being secreted to the outer surface of the nematode body.

Chapter 3: Expression of the *Gp-SOD3* at the Early Stages of the Infection in the Susceptible Potato Cultivar (Désirée), Resistant Potato Cultivar (Innovator), and *Solanum sisymbriifolium* (litchi tomato)

Introduction

Reactive oxygen species (ROS) are toxic molecules that are produced in a wide variety of processes occurring in a plant, including mitochondrial respiration, photosynthesis in chloroplast, or during a response to biotic/abiotic stresses (Mhamdi & van Breusegem, 2018). Reactive oxygen species occur as a part of the molecular dioxygen (O_2) reduction process and include molecules such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Gechev et al., 2006). Out of these the hydroxyl radical is considered to be the most reactive specie, and has the potential to damage cells through processes such as peroxidation of lipids, protein denaturation, and/or DNA mutation (Bowler et al., 1992). The superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) lead to the formation of the $OH^{\cdot}$ through Haber-Weiss or Fenton reactions (Robertson et al., 2000). Production of ROS during the plant defense response is termed an oxidative burst and is an important part of defenses against an invading pest. The role of ROS in this process is not just to create a cytotoxic environment for the pathogen, but also to signal activation of local and systemic defense responses (Rosso, 2009; Gillet et al., 2017). Oxidative bursts and the generation of ROS occur in two phases in the plant cell. The first phase occurs soon after the presence of the pathogen has been detected; at this stage ROS are a product of a general, weaker reaction, while the second phase oxidative burst is a stronger and more prolonged response to the presence of the pathogen (Lamb & Dixon, 1997). This extended production of ROS is usually one of the early signs of the onset of a process known as the hypersensitive response (HR), which is a highly effective defense response leading to induction of programmed cell death (Bigeard et al., 2015). The HR is an important contributor to plant defense responses against biotrophic pathogens. Biotrophic pathogens are organisms that can infect the plant and feed on it without causing the plant to die (Spanu & Kämper, 2010). A wide variety of plant pathogens are classified as biotrophic, including the potato cyst nematode (PCN), *Globodera pallida*.

The infection process of *G. pallida* involves migration through roots, which occurs intracellularly and involves a great amount of physical damage to plant cells (Rehman et al., 2008). This migration may trigger plant defense responses, including the oxidative burst and production of

ROS, which can damage the nematode. In order to protect itself, the nematodes have evolved a mechanism to scavenge for and remove these toxic compounds. Furthermore, as an aerobic organism *G. pallida* will produce ROS as a byproduct of its own metabolism (Van Voorhies & Ward, 2000), emphasizing even more its requirement for a powerful ROS removal mechanism. Removal of toxins can occur as a part of the antioxidant and detoxifying pathways, which employs a wide variety of ROS scavenging enzymes. Some of the major ROS scavenging enzymes have been found in plant parasitic nematodes, including superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase/glutathione peroxidase system, as well as thioredoxin peroxidase/ thioredoxin reductase system (Robertson et al., 2000; Mittler, 2002).

The main roles of these ROS scavenging enzymes are to reduce the amounts of the intracellular and extracellular ROS, as well as to prevent the formation of the highly reactive hydroxyl radical (Callahan et al., 1988; Rosso, 2009; Gillet et al., 2017). One major antioxidant enzyme that functions to prevent the formation of hydroxyl radical is superoxide dismutase (SOD). Superoxide dismutase (EC 1.15.1.1) is a metalloenzyme that protects the cell by catalyzing the conversion of the superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The presence of all three classes of SOD enzyme has been shown in different organisms, including yeast, fruit flies, mice, and plants (van Loon et al., 1986; Blackney et al., 2014; Carlsson et al., 1995; Gupta et al., 1993). Different classes of superoxide dismutase have been characterized in free-living, plant parasitic, and animal parasitic nematode species (Giglio et al., 1994; Liddell & Knox, 1998; Henkle-Dührsen et al., 1997; Tang et al., 1994; James et al., 1994; Rosso, 2009: Molinari & Miacola, 1997). Nematodes, such as *Onchocerca volvulus, Bursaphelencus xylophilus, Meloidogyne incognita,* and *G. rostochiensis,* have all shown the presence of extracellular SOD or SOD-3 in their secretomes (Henkle et al., 1991; Shinya et al., 2013; Bellafiore et al., 2008; Robertson et al., 1999). Secreted SOD has been implicated to be involved in enhancing the virulence of the pathogen during parasitism of the host, by providing the protection from the host derived ROS (Youseff et al., 2012; Lattemann et al., 1999).

We have characterized (Chapter 2) a putative superoxide dismutase from *G. pallida,* which contains a secretion peptide at the N-terminus suggesting that it may be secreted. The expression of this enzyme during PCN infection in the susceptible potato cultivar Désirée, resistant potato cultivar Innovator, and immune host, *S. sisymbriifolium* was studied here. The immune host, *S. sisymbriifolium*, was chosen because this is an effective trap crop for PCN thought to be resistant to *G. pallida* (Scholte & Vos, 2000; Dandurand & Knudsen, 2016). This plant can cause PCN to hatch at relatively high numbers, yet the nematodes cannot complete their life cycle in the roots of *S.*

sisymbriifolium and will die (Scholte, 2000; Timmermans et al., 2006). Furthermore, the HR seems to be an important contributor to the defense process in the *S. sisymbriifolium* (Timmermans, 2005; Kooliyottil et al., 2016). Since the HR results in extended production of ROS, we wanted to see if this might have any effect on the expression of *SOD-3* in *G. pallida* parasitic J2s. The immune host, *S. sisymbriifolium*, is capable of triggering an HR very early upon nematode infection, where the earliest response was seen around 2 to 4 days after infection (Kooliyottil et al., 2016). This was one of the reasons why we looked at the gene expression of the *Gp-SOD3* at the early time points of infection, more specifically 24, 48, and 72 hours after infection. Another reason why we chose these time points was that mining through the transcriptome of *G. pallida* infecting susceptible potato or *S. sisymbriifolium*, revealed that in a resistant environment antioxidant and detoxifying enzymes, including the SODs, may have been upregulated 24 hours post inoculation (Kooliyottil et al., 2019). In this chapter we present gene expression analysis for the superoxide dismutase from *G. pallida* during the early stages of the infection process.

Methods and Materials

Plant Material

Susceptible potato (*Solanum tuberosum)* cv. Désirée and resistant potato cv. Innovator were propagated vegetatively for two weeks in a sterile plant tissue culture medium containing Murishige and Skoog (MS) salts (Murashige and Skoog, 1962). Seeds of the immune host, *Solanum sisymbriifolium*, were planted in plastic trays and grown for four weeks under standard greenhouse conditions (10^{o}C night-time temperature to 18^{o}C day-time temperature, and a 16:8-h (day:night)). *Solanum sisymbriifolium* seeds were provided by Chuck Brown, USDA-ARS. Next, both plants in tissue culture and *S. sisymbriifolium* plants were transferred into root trainers (Haxnicks™, TDI Brands, USA) and grown under standard greenhouse conditions for an additional four weeks.

Globodera pallida Plant Inoculation

After plants have been growing in the root trainers for four weeks, a single root from each plant was isolated and placed in a glass tube (10 cm L x 0.5 cm W) containing sterile sandy loam soil and sand (2:1) mix. Collected pre-parasitic second stage juveniles (J2s) were sterilized in 100 μg/ml of ampicillin, streptomycin, and 0.125% w/v benzethonium chloride (Kooliyottil et al. 2016). Sterilized J2s were re-suspended in a 0.1% agarose prior to the plant inoculation. 500 J2s per plant

were then delivered onto the isolated root. Roots were covered with soil and left in the greenhouse until extraction.

Collection of G. pallida from the Roots of Infected Plants

Globodera pallida was collected from roots of infected plants 24 h, 48 h, and 72 h after the inoculation. Infected roots were separated from plants and gently washed with DI H_2O. Next, they were cut into small pieces (~2cm in length) and added to cold sterile DI H_2O. This was followed by blending (WARING Commercial®, WARING Products Division, CT) at low speed for 2 seconds (5x). Blended roots were washed through sieves arranged in a following order 2.8 mm/500 µm/250 µm/90 µm/25 µm/20 µm (Humboldt, IL, USA). Parasitic nematodes were collected from the smallest sieve size. Individual nematodes were picked from this sieve under a microscope (Leica, M 80, Germany) and transferred into a 1.5 ml tube (VWR International, Radnor, PA). Once collection was completed, nematodes were washed with sterile DI H_2O; excess supernatant was removed and the sample was frozen in liquid nitrogen for 1 minute and stored at -80^{o}C until used.

Quantitative Real-Time PCR (qRT-PCR)

We examined the expression of the *Gp-SOD3* at the early stages of infection by a quantitative real-time PCR (qRT-PCR) analysis. Total RNA isolated from the collected parasitic second-stage juveniles (J2s) was extracted and reverse-transcribed in order to produce a cDNA library as mentioned above (see General Materials and Methods). On average we used 70 nanograms of total RNA for each sample. The Applied Biosystems ViiA 7 system was used for the qRT-PCR analysis using the Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). Our results were normalized using the EiF-F and EiF-R primer set amplifying *G. pallida EiF4* gene, as an internal control (Table 2.1). SODqF6 and SODqR6 (Table 2.1) were used to check the expression of the *Gp-SOD3* using the qRT-PCR. CT method ($\Delta\Delta$CT) was used to obtain the relative gene expression values for *Gp-SOD3*.

Sequencing Analysis

Since the SODqF6 and SODqR6 primer set was amplifying the fragment of the *Gp-SOD3* that had shared homology with its paralog GPLIN_000150100, the fragment was sequenced to ensure *Gp-SOD3* detection. The fragment amplified from the cDNA library prepared from pre-parasitic

(non-infectious) *G. pallida* J2s, as well as representative parasitic nematode cDNA libraries, was purified using the DNA Clean and Concentrator-25 Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. For each observed time point we have chosen a representative parasitic cDNA libraries prepared from the parasitic nematodes infecting the roots of susceptible potato, resistant potato, and *S. sisymbriifolium*. Purified PCR fragments were sequenced using the GenScript (Piscataway, NJ, USA) sequencing service.

Data Analysis

The data was analyzed using the SAS software, Version 9.4. Copyright © 2016 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. In order to analyze if there is any statistical variance between replicates we used the GLIMMIX Procedure, with the significant value at $p<0.05$. The experiment was designed as a random complete block with three treatments and three replicates. All of the graphs were prepared using the GarphPad Prism (Graphpad Software, La Jolla, California, USA). Chromatogram analysis was conducted using the SnapGene software (from GSL Biotech; available at snapgene.com). Sequence alignments were done using the Clustal Omega (Sievers, 2011) server.

Results

Collection of G. pallida from Roots of Infected Plants

Globodera pallida was collected from roots 24h, 48h, and 72h post infection. Across three replicates for each time point, approximately 200, 100, or 100 nematodes were recovered from susceptible potato cv. Désirée, resistant potato cv. Innovator, or the immune host *S. sisymbriifolium*, respectively.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of *Gp-SOD3* when nematode infect roots of susceptible potato, Désirée, resistant potato, Innovator, and the immune host plant, *S. sisymbriifolium*. The expression of *Gp-SOD3* from the parasitic nematodes isolated from different plant roots was compared to the expression of the gene in the pre-parasitic

(non-infectious) *G. pallida* J2s. The relative expression of *Gp-SOD3* in pre-parasitic *G. pallida* J2s was normalized to 1. The raw amplification curves for the qRT-PCR analysis are shown in Appendix A. For parasitic *G. pallida* J2s infecting roots of all three plants, there was a significant increase in the expression of *Gp-SOD3* compared the expression in pre-parasitic J2s ($P<0.05$; Fig 3.1). This expression remained significantly higher in parasitic J2s compared to the pre-parasitic J2s for all of the observed infection time points (Fig 3.1).

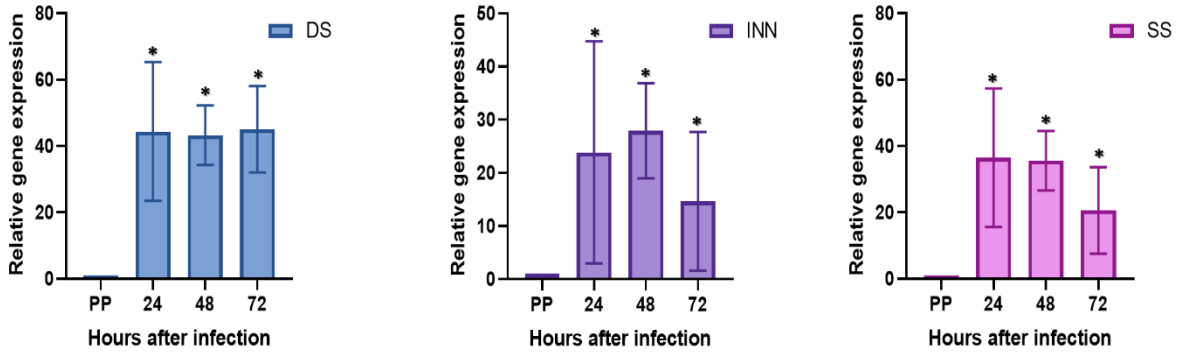

Figure 3.1. Quantitative real-time PCR (qRT-PCR) results from the pre-parasitic and parasitic J2s showing the relative gene expression of the *Gp-SOD3* in pre-parasitic compared to the parasitic J2s. Time point PP refers to the expression of *Gp-SOD3* in the pre-parasitic (non-infectious) *G. pallida* J2s; this expression was normalized to 1. *G. pallida EiF4* gene was set up as an internal reference control. Error bars represent standard error of the mean (S.E.M.), with three biological replicates. Statistical significance was determined using the natural log transformation of the data. *-$P<0.05$; DS-Désirée; INN-Innovator, SS- *S. sisymbriifolium*

Next, we wanted to know if there is any significant difference in the expression of *Gp-SOD3* between the parasitic J2s infecting roots of different plants. We did not observe any significant difference in the expression of this gene when *G. pallida* J2s are infecting roots of susceptible or resistant plant ($P>0.05$). However, although not significant, there is a slight trend towards differences in expression of *Gp-SOD3* early in the infection process. For example, we observed that the highest expression of *Gp-SOD3* was in the parasitic J2s isolated from the roots of the susceptible potato (Fig 3.2). Also, this expression appeared to be constant over time. However, for the expression of *Gp-SOD3* in parasitic nematodes facing the resistant environment there was a slight decrease in the expression of *Gp-SOD3* at 72 hours post infestation compared to the expression of this genes in nematodes infecting susceptible host. This slight drop in the expression was observed in the parasitic J2s collected from roots of either resistant potato cultivar or *S. sisymbriifolium*. Nonetheless, there was no significant difference in the expression of *Gp-SOD3* in the parasitic J2s from Désirée, Innovator, or *S. sisymbriifolium* ($P>0.05$).

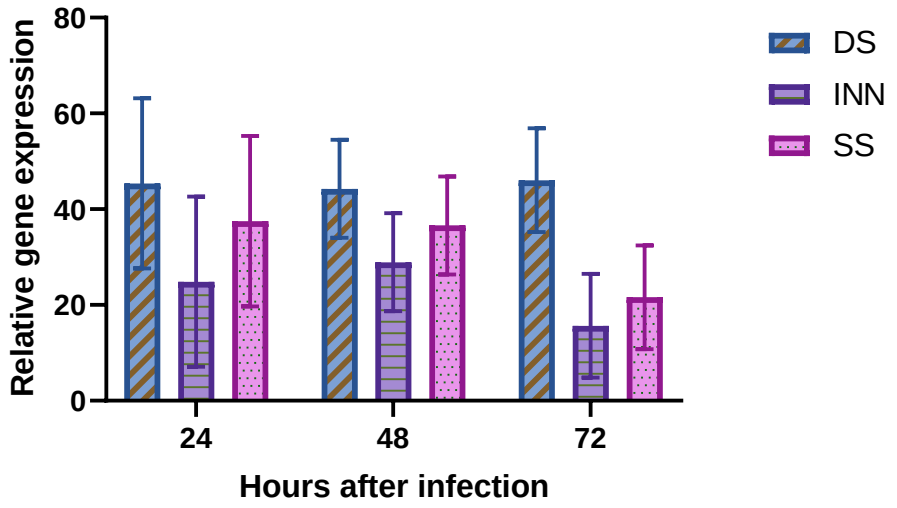

Figure 3.2. Quantitative real-time PCR (qRT-PCR) results from the parasitic *G. pallida* J2s showing the relative gene expression of the *Gp-SOD3* across three different time points (24, 48, and 72 hours after infection). Expression of *Gp-SOD3* in the pre-parasitic (non-infectious) *G. pallida* J2s was normalized to 1. *G. pallida EiF4* gene was set up as an internal reference control. Error bars represent standard error of the mean (S.E.M.), with three biological replicates. DS-Désirée; INN-Innovator, SS- *S. sisymbriifolium*

Table 3.1. Calculated means for the relative gene expression values obtained from the qRT-PCR analysis with the respective standard error of the mean (S.E.M). These values were plotted in Figure 3.1 and Figure 3.2. DS-Désirée; INN-Innovator, SS- *S. sisymbriifolium*

Hours post infection	Plant	Mean ± Standard Error of the Mean
24	DS	45.3923±17.7896
	INN	24.8630±17.7896
	SS	37.4835±17.7896
48	DS	44.2613±10.2172
	INN	28.9183±10.2172
	SS	36.6003±10.2172
72	DS	46.0640±10.8305
	INN	15.6437±10.8305
	SS	21.6020±10.8305

Sequencing analysis

Gp-SOD3 differs from its paralog GPLIN_000150100 (see Cloning and sequencing of the *Gp-SOD3;* Chapter 2*)* at the 5' end, within the first 100 bp of the sequence, however the homology between the two genes increases towards the 3' end. Due to the difficulty to design a primer set that would amplify a region at the 5' end of the *Gp-SOD3* and that was showing a 100% standard curve

efficiency, a primer set amplifying a 124 bp portion beyond the divergent region of the *Gp-SOD3* was used; more specifically between positions 258 and 382 bp. In comparison, this primer set was aligning to a 124bp region in the GPLIN_000150100 nucleotide sequence, between the positions 219 and 343 bp. If aligned to each other, the two genes carried 76% homology in this specific region, as seen in the alignment of their cDNA sequences (Clustal Omega (Sievers, 2011)) (Fig 3.3).

```
GPLIN_000150100      CTAAACGGAACCATCAACGGGCTCAGCGCGGGCGACCACGGCTTCCACTTTCACCAATTT     60
Gp-SOD3              CTGAATGGCAGCATCAACGGCCTCACGCCGGGCGACCATGGCTTTCACTTTCACCAGTTT     60
                     ** ** ** * ********* ****   ********** ***** *********** ***

GPLIN_000150100      GGCGACCTCTCCAAGGCCTGTATGGCCTCGGGTGCGCACTTCAACCGCACGGCAAACGC     120
Gp-SOD3              GGCGACCTTTCGCAGGCGTGCGCAGGGGCGGGTGCGCATTTCAATCCGCACAACAAAAAG     120
                     ******** **  **** **    *   ********** ***** ******  ****

GPLIN_000150100      CACG    124
Gp-SOD3              CACG    124
                     ****
```

Figure 3.3. Alignment of the portion of the *Gp-SOD3* cDNA sequence, amplified using the SODqF6 and SODqR6 primer set, with the cDNA sequence of the paralog GPLIN_000150100. Fully conserved regions are marked with (*), and the points of variation between the two sequences are shaded in yellow. Alignment was conducted using the Clustal Omega (Sievers, 2011) server.

Even though, the SODqF6 and SODqR6 primer set did not align 100% with the cDNA sequence of the paralog GPLIN_000150100, there was still a sufficient amount of similarity to make us concerned. We wanted to ensure that in our gene expression analysis we were only looking at the relative gene expression of *Gp-SOD3*, and not the GPLIN_000150100. The melt curve analysis of the qRT-PCR showed a single peak, suggesting the expression of a single, specific product. Nevertheless, since the fragments that the SODqF6 and SODqR6 primer set aligns to have the same size in both genes, we could not use the size of the amplified fragment to distinguish between *Gp-SOD3* or the paralog. So, we decided to sequence this specific fragment from the pre-parasitic cDNA library, as well as from the representative parasitic nematode cDNA libraries at different time points and from different plants. Subsequent chromatogram analysis of the sequencing results has shown that across all of the sequenced samples for all of the points of variation between the two genes, there are single, sharp sequencing peaks that correspond to the sequence predicted for *Gp-SOD3* (Fig 3.4). One exception was for the nucleotide position 119, that shows a sharp peak for the nucleotide base A (corresponding to the *Gp-SOD3* sequence), but there is also a smaller peak for the nucleotide base G (corresponding to the GPLIN_000150100 sequence). The presence of this smaller peak would indicate a change in the encoded amino acid from lysine to arginine. However, this was the only

exception, and the chromatogram analyses for all of the other point of variance were a strong indicator that *Gp-SOD3* was amplified with the SODqF6/SODqR6 primer set.

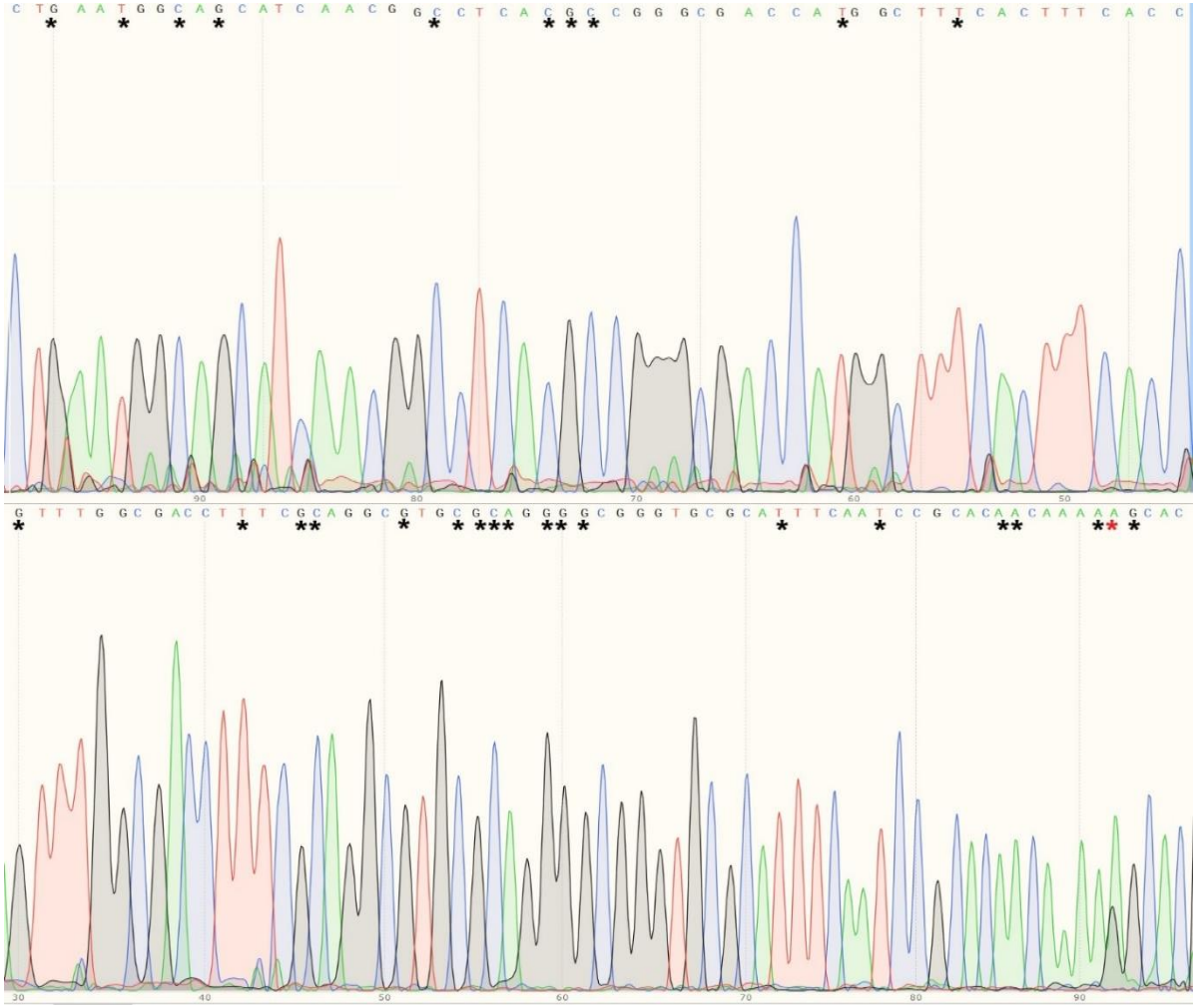

Figure 3.4. Chromatogram analysis for the sequenced fragment amplified from the parasitic cDNA library (corresponding to the parasitic J2s infecting the roots of DS at 72 hours after infection). Nucleotide positions that are the points of variation between the *Gp-SOD3* and GPLIN_000150100 sequences are marked with (*). Note that all of the points of variation have single peaks corresponding to the nucleotide sequence of *Gp-SOD3*. The only exception is for the nucleotide position 119, marked with red (*). Chromatogram analysis was conducted using the SnapGene software (from GSL Biotech; available at snapgene.com).

Discussion

Aerobic organisms, including nematodes, employ antioxidant and detoxifying enzymes to counter not only self-produced reactive oxygen species (ROS), but also any products of the reduced oxygen that might be in its environment (Molinari & Miacola, 1997; Henkle-Dührsen & Kampkötter, 2001). Amongst these enzymes, superoxide dismutase (SOD) is unique in the way that it is the only

antioxidant enzyme that can scavenge for superoxide. Previously we have shown that the potato cyst nematode, *G. pallida*, expresses a putatively secreted superoxide dismutase that we referred to as *Gp-SOD3*. Our *in silico* analysis indicates that *Gp-SOD3* contains conserved sites that are specific for the copper-zinc isoform of SOD, including a total of 7 sites for binding of metal ions and 2 disulphide bond sites (Tian et al., 2011). Expression of *Gp-SOD3* was found to be localized in the hypodermal tissue lining the nematode cuticle which suggests that the enzyme might be secreted to the outer surface of the nematode. In other studies different antioxidant enzymes localized in the hypodermal tissue, were found on the surface of the nematode (Prior et al., 2001; Jones et al., 2004). Further analysis is required in order to confirm if Gp-SOD3 is found on the cuticle of *G. pallida*.

Superoxide dismutase enzymes, especially the Cu-Zn SOD isoform, contribute to the successful infection process of many pathogens and pests (Beaman & Beaman, 1990; Youseff et al., 2012; Lattemann et al., 1999). For example, a mutant of the necrotrophic fungus *Botrytis cinerea,* lacking in expression of cytoplasmic Cu-Zn SOD (*sod*-1) had reduced pathogenicity on the susceptible *Arabidopsis* and tomato plants (Lopez-Cruz et al., 2017). When comparing expression of SOD1 and SOD3 in the animal parasitic nematode *Acanthocheilonema viteae*, it was seen that although both SOD classes were expressed throughout the life cycle of this parasite, SOD3 was upregulated when infective L3 larvae were transferred from the vector to its natural host, while the expression of SOD1 did not change (Lattemann et al., 1999). It was suggested that this increase in the expression of the extracellular Cu-ZnSOD might help *A. viteae* to combat ROS produced by its host (Lattemann et al., 1999). We examined the role *Gp-SOD3* to determine if it may have a similar role in the infection process of *G. pallida*, since host derived ROS will cause stress to the nematode, indicating its requirement for an effective ROS-scavenging mechanism.

As discussed in Chapter 2, *Gp-SOD3* shares the highest level of amino acid sequence similarity with (69%) with one of its paralogs, GPLIN_000150100. We wanted to ensure that in our gene expression analysis we were only looking at the relative gene expression of *Gp-SOD3,* and not the GPLIN_000150100. Since the two paralogs differ mostly at the N-terminus, our aim was to design a primer set that was amplifying a fragment of *Gp-SOD3* corresponding to this region. In spite of several attempts, we were unable to design a primer set specific for this region that was efficient for the subsequent qRT-PCR analysis. We used a primer set that amplified a fragment of the *Gp-SOD3* close to a region that is homologous to its paralog GPLIN_000150100. Sanger sequencing was used to ensure the specificity of the product being amplified. With the exception for one single nucleotide polymorphism (SNP), chromatograms suggest that from both pre-parasitic and parasitic nematode cDNAs, we are amplifying a fragment matching to the *Gp-SOD3* nucleotide sequence. At

one variation point, corresponding to the nucleotide position 119, there seemed to be two peaks corresponding to the nucleotide base predicted by either the *Gp-SOD3* or GPLIN_000150100 sequence, respectively. This would cause a missense mutation, causing a shift from lysine to arginine. However, since both of these amino acids are positively charged, this residue would still retain its basic function (Sokalingam et al., 2012). These sequencing results were a strong indicator that we are observing a relative gene expression of *Gp-SOD3.*

Across all three time points (24, 48, and 72 hours after infection), we saw a significant increase in the expression of *Gp-SOD3* in parasitic J2s compared to the expression in pre-parasitic J2s. This agrees with our hypothesis that *Gp-SOD3* might have a protective role for the nematode during exposure to oxidative stress. During both compatible and incompatible interactions between the nematode and the plant, an extensive amount of damage to the plant cells is occurring due to the intracellular migration of the nematode (Rehman et al., 2008). Nematode migration may be triggering basal defense responses in the plant, including the production of ROS, which could be related to the observed increase in *Gp-SOD3* expression. However, in the susceptible plant, these basal defense responses are predicted to be suppressed once the nematode initiates the sedentary phase of its life cycle and formation of the feeding site (Tran, 2016). Perhaps, one of the future directions for this study could be to look at the relative gene expression of *Gp-SOD3* at the later stages of infection. This would allow us to check if any changes in the expression are occurring with the progress of the infection and nematode development. Nonetheless, we do show that for these early time points of infection, the relative gene expression of *Gp-SOD3* increases significantly when *G. pallida* transitions from the pre-parasitic (non-infectious) to the parasitic (infectious) stages of its life cycle in all three plants.

Furthermore, we wanted to know if parasitic *G. pallida* J2s are showing any significant difference in the expression of *Gp-SOD3* when infecting roots of a susceptible potato, resistant potato, and immune trap crop. In our experiments, there was no significant difference in the expression of this gene when comparing nematodes infecting susceptible, resistant, or immune environments. This may indicate that the expression of *Gp-SOD3* is upregulated when the nematode is exposed to a stressful environment, such as during the infection process, regardless of whether the infection is occurring in a susceptible or resistant plant. Although we did not observe any significant changes in the expression when comparing parasitic *G. pallida* J2s, we could speculate about a slight trend occurring in the expression pattern. We observed that for the susceptible potato plant, the expression of *Gp-SOD3* was slightly higher at all three time points compared to the resistant potato or *S. sisymbriifolium.* This could be due to the response of the nematode to the basal defenses activated

by the plants at these early phases of infestation. Another cultivar we have tested in our study was the resistant potato, Innovator. Innovator is classified as a partially resistant potato variety, that causes a decrease in the reproduction of the nematode and increase in the production of adult males (Trudgill *et al.*, 1987; Phillips & Blok, 2008). The defense process occurring in this plant might be affecting some of the points that are crucial for successful parasitism of the nematode, such as the formation and/or expansion of the feeding site (Goverse & Smant, 2014). Hampering of the normal development of the feeding site may prevent it from providing sufficient nutrients for the nematode, leading to the development of more adult males compared to the adult females (Goverse & Smant, 2014). Although not significantly, for all three time points the overall lowest expression of *Gp-SOD3* was in parasitic nematodes from the resistant potato compared to the other plants. This trend could coincide with the predicted late onset of defense response in Innovator.

On the other hand, for the *G. pallida* J2s infecting the roots of the *S. sisymbriifolium* the expression of *Gp-SOD3* was constant at 24 and 48 hours after infection. However, although not significant when compared to the expression in other two plants, at 72 hours after infection, there was a slight drop in the expression of *Gp-SOD3*. Rapid and potent defense response has been seen in the roots of *S. sisymbriifolium* early upon *G. pallida* infection (Kooliyottil et al., 2016). There are several factors that might be contributing to the potent resistance of *S. sisymbriifolium* to the nematode infection, such as action of certain toxic compounds or expression of defense-related genes (Sasaki-Crawley, 2013). In Sasaki-Crawley, 2013, they saw that upon nematode infection several defense-related genes, seem to be upregulated in the roots of the *S. sisymbriifolium* compared to the susceptible potato plant. One of these genes, was a chitinase gene that was strongly induced in the roots of *S. sisymbriifolium* 3 and 10 days after infection compared to the roots of a susceptible potato (Sasaki-Crawley, 2013). Studies have shown that silencing certain chitinases in cotton has led to an increased susceptibility to the infection by a fungus *Verticillium dahlia* (Xu et al., 2016), as well as that the expression of a specific class IV chitinase enhanced oxidative burst and production of ROS in the *Capsicum annuum* (Kim et al., 2015). It is possible that at this 3 days post infestation time point, increased expression of the chitinase gene in the roots of *S. sisymbriifolium* could also be causing increased generation of ROS. This, in turn, could be overwhelming the antioxidant pathway of the nematode, leading to the death of the nematode represented through the observed slight decrease in the expression of *Gp-SOD3*. Nevertheless, we were unable to observe any statistically significant difference in the expression of *Gp-SOD3* between parasitic J2s infecting the roots of susceptible potato cultivar, resistant potato cultivar, or *S. sisymbriifolium*. There was a great amount of variance

in our biological replicates (discussed below), that could have prevented us from observing any statistically significant difference in the relative gene expression.

Analyzing our gene expression data and extrapolating conclusions about the expression of *Gp-SOD3* in the parasitic J2s infecting roots of different plants was very difficult due to the variability found amongst the biological replicates of our experiment, as well as due to lack of measure of variability in the control (pre-parasitic) J2s sample. Reasons for this variation between the biological replicates may be as follows: 1) collecting all nematodes at specific time points is challenging because the process is time consuming which is especially problematic for studies such as this one, since we are looking at the expression of a gene that is predicted to encode for a stress related enzyme which may lead to differences in expression; 2) due to low numbers of parasitic nematodes isolated from the plant roots. This meant that small amounts of total RNA could be extracted, limiting subsequent qRT-PCR analysis. In the future, this problem could be avoided by using a single nematode RNA extraction in order to obtain greater amounts of the total RNA. For instance, a recent study has used this method to isolate high quality RNA from a single *C. elegans* nematode that was used in a subsequent transcriptome analysis (Serra et al., 2018). Another study has utilized single nematode RNA extraction to look at the transcriptome differences and similarities of an entomopathogenic nematode, *Steinernema feltiae*, during *in vitro* and *in vivo* conditions (Chang et al., 2019).

A further consideration is that focusing on only early time points post infestation contributed to the inconsistency that we have seen between the biological replicates. The difficulty with observing the plant-nematode interactions at early stages of infection is ensuring that all of the collected nematodes are at the same stage of the infection process (Holbein et al., 2016). At 24, 48, and 72 hours post infection, *G. pallida* is still in its early parasitic J2s stage, migrating through the roots and trying to establish the syncytium. Once the nematode begins the sedentary lifestyle and starts feeding on the syncytium, it will begin moulting into adult life stages. Moulting will cause changes in the shape and size of the nematode, making it easier to distinguish nematodes that are at the same point of the infection (Han et al., 2018). However, it is impossible to notice any differences in the shape of early parasitic J2s, making it challenging for us to determine if we are observing synchronized infection. On the other hand, we could have not used later parasitic stages for this study, since in the roots of *S. sisymbriifolium* there is no development past the second stage juvenile (Sasaki-Crawley, 2013; Kooliyottil et al., 2016). Using growth pouches (Mega International, MN, USA) might have helped with achieving synchronized infection (Cotton et al., 2014), however maintaining healthy plants with good root system proved to be difficult using this method (Dr. Joanna Kud, personal

communication). Another possible option for obtaining more synchronized infection could be the use of Pluronic gel. Temperatures of the Pluronic gel can be modified so that only the nematodes that are the same point of infection are collected (Sasaki-Crawley, 2013). Sasaki-Crawley, (2013), proposed that this method can be used to observe all of the stages of the development of parasitic *G. pallida* nematodes. Additionally, using micro-rhizosphere chambers (micro-ROCs) could have been another method for an improved synchronization of the infection process, since the absence of soil around the isolated root could allow for a more precise delivery of pre-parasitic J2s on the root surface (Kooliyottil et al., 2017).

Our study indicated that the expression of *Gp-SOD3* was not significantly different when nematodes are infecting susceptible or resistant environment. This confirms the results of the transcriptome analysis that was the basis for this research. In this transcriptome analysis, at 24 hours post infestation, it was shown that, although not statistically significant, parasitic *G. pallida* J2s from the roots of *S. sisymbriifolium* have slightly higher expression of *Gp-SOD3* compared to the parasitic nematodes infecting roots of the susceptible potato cultivar (Kooliyottil et al., 2019). Although, we did observe a slightly lower expression of *Gp-SOD3* in parasitic J2s from *S. sisymbriifolium* compared to the susceptible host, our data also indicated no significant difference.

However, expression of *Gp-SOD3* significantly increased when the nematode initiates parasitic stages of its lifecycle in all three types of plants. This upregulation was consistent whether we were observing compatible or incompatible interactions, indicating that this enzyme may be important for the regulation of stress encountered when initiating parasitic lifestyle and may be of continued importance during all of its life stages.

Chapter 4: Effects of Allyl Isothiocyanate (AITC) on *Globodera pallida* Detoxification Metabolism and *GST* Expression

Introduction

Besides the antioxidant pathway, which was the focus of the majority of this research project, another defense pathway controlled by the DAF-16 and SKN-1 transcription factors is the nematode detoxification pathway (Gillet et al., 2017). This pathway works towards the detoxification and excretion of internal (endobiotic) or external (xenobiotic) compounds (Gillet et al., 2017; Lindblom and Dodd, 2009), and employs a variety of enzymes that are activated at different phases of the detoxification metabolism. An enzyme family that makes up one of the two enzyme families responsible for the Phase II of the detoxification pathway is glutathione S-transferase (GST; see Chapter 1).

Glutathione S-transferase (GST, EC 2.5.1.18) is predicted to convey a variety of roles for the cell. Some of these include regeneration of the S-thiolated protein (formed as a result of oxidative stress), catalysis of the conjugation of the GSH to the xenobiotic or endobiotic substrate, reduction of lipid hydroperoxides (results in the formation of the oxidized glutathione- GSSG), and catalytic functions in the metabolic pathways that are not involved in the detoxification (Sheehan et al., 2001; Babu et al., 2012). GSTs can also function as ligand-binding proteins, where they are involved in transport and storage of a variety of hydrophobic non-substrate compounds, such as hormones, metabolites, and drugs (Reinemer et al., 1996). In addition, GSTs have been thought to be involved in herbicide resistance in plants (Cummins et al., 2013), in anti-cancer drug resistance (Townsend & Tew, 2003), as well as resistance to insecticides in insects (Enayati et al., 2005). These homo- or heterodimeric mostly cytosolic enzymes, are found in bacteria, fungi, plants, and animals (Campbell et al., 2001; Calmes et al., 2015). Large and multiplex, the GST superfamily is divided into several classes depending on the substrate specificity and localization in the cell (Leiers et al., 2002). Soluble GSTs have been divided into seven species-independent classes including Alpha, Mu, Pi, Theta, Sigma, Zeta, and Omega (Campbell et al., 2001).

This study was based on a recent transcriptome analysis that looked at the transcriptome of *G. pallida* in early parasitic phases (Kooliyottil et al., 2019). Transcriptome analysis identified a gene predicted to encode for a GST as slightly upregulated when nematode was parasitizing the roots of

the trap crop *S. sisymbriifolium*. Since our study focused on gaining insight into different defenses occurring in the nematode, we wanted to know if this gene was responding when *G. pallida* was exposed to certain types of stressful environments, such as nematicidal compounds. A recent study found expression of *GSTs* was increased when the free living nematode, *Caenorhabditis elegans*, was exposed to allyl isothiocyanate (AITC) (Hasegawa et al., 2010). They also showed that nematodes exposed to AITC were able to withstand more additional oxidative stress than nematodes not exposed to AITC, suggesting that the GSTs were not only contributing to the detoxification of AITC, but also increasing the resistance of the nematode to any subsequent stresses (Hasegawa et al., 2010). Allyl isothiocyanate (AITC) is a compound that contains an isothiocyanate group and is derived from sinigrin, a type of glucosinolates that occurs widely in members of the Brassicaceae plant family (Buskov et al., 2002; Fahey et al., 2001). This hydrolysis of sinigrin into isothiocyanates occurs through hydrolysis and has been shown to suppress a variety of pests, including *G. pallida* (Brown and Morra, 1997; Dandurand et al., 2000; Zasada et al., 2008; Zasada et al., 2009; Meyer et al., 2011; Dandurand et al., 2017). The objective of this part of the study was to determine what impact exposure of *G. pallida* to sublethal concentrations of AITC has on defenses occurring in this nematode, in particular on expression of the gene encoding for GST. Here we present the isolation and cloning of a gene that is predicted to encode for one of the GSTs from *G. pallida*, as well as expression analysis for this gene when nematode is exposed to AITC.

Methods and Materials

Amplification of the Coding Sequence, Cloning, and Sequencing Analysis

The *G. pallida* transcriptome (see Chapter 2; Amplification of the Gene, Cloning, and Sequencing Analysis) indicated that several genes encoding for the enzymes in the detoxification pathway were slightly upregulated in early stages of parasitism of the trap crop *S. sisymbriifolium* (Kooliyottil et al., 2019). The corresponding sequences for these genes were obtained from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/). Further homology searches were done using the BLASTn and BLASTp tools. The SignalP 5.0 server was used to check the presence of the signal peptide (Armenteros et al., 2019) and TMHMM algorithm was used to check for any transmembrane domains (Krogh et al., 2001). In our study we focused on *Gp-GST* (GPLIN_001198400). The *Gp-GST* gene sequence was used to design specific primers (Table 2.1). Primer GSTF2 aligned with the 5’ end of the sequence and reverse primers, GSTR2,

aligned with the 3' end of the sequence. In order to obtain the coding sequence, the gene was amplified from the cDNA (see Chapter 1; General Methods and Materials). *Gp-GST* was amplified in a 50µl PCR reaction containing 200ng of cDNA template (see RNA Isolation and Reverse Transcription; Chapter 1), 10X Standard *Taq* Reaction Buffer (NEB, Ipswich, MA), 100µM of each dNTPs, 10µM of each primer, and 0.625 units of Standard *Taq* polymerase (NEB, Ipswich, MA). PCR amplification reaction was carried out in Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA) using the following program set up, initial denaturation at 95ºC for 30 seconds, 30 cycles at 95ºC for 30 seconds, 60ºC for 50 seconds, and 68ºC for 1 minute, followed by a final extension at 68ºC for 5 minutes and a hold at 4ºC. Obtained PCR product was tested for correct size by agarose gel (1%) electrophoresis and purified using QIAquick PCR Purification Kit (Qiagen, Hildren, Germany). Purified PCR product was ligated into the pCR™8 TOPO® TA vector using the pCR™8/GW/TOPO® TA Cloning Kit (Sigma Aldrich, St. Louis, MO, USA). Following ligation, the products were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) using heat-shock transformation by following the protocol provided by the manufacturer. We selected 3 positive colonies and cultured them in order to extract plasmids (QIAprep Spin Miniprep Kit, Qiagen, Hildren, Germany) for sequencing with the universal M13F and M13R primers (Table 2.1). Plasmids were sequenced by the GenScript (Piscataway, NJ, USA) sequencing service in order to ensure the correct sequences of the insert.

Allyl Isothiocyanate (AITC) Exposure and the Visual Assessment Assay (VAA)

A 10 mM stock solution of allyl isothiocyanate (AITC; Sigma Aldrich, St. Louis, MO, USA) was prepared using acetone. Treatment dilutions had final AITC concentration of 50, 100, 200, and 400 µM and were prepared from the stock solution using 0.5% acetone. Exposure to 0.5% acetone was used as a control. Around 10-20 freshly hatched pre-parasitic (non-infectious) J2s of *G. pallida* were placed in a 96-well plate (Corning, Corning, NY, USA) containing 100 µl of the appropriate AITC dilution or control. Three replicates were used for each treatment solution. The activity of the J2s and the effect of AITC were observed at 1, 2, and 3 hours post exposure to AITC using an inverted compound microscope (Leica Microsystems, model DMi1). The impact of AITC was scored based on the visual assessment assay (VAA) described by Zasada et al., (2008), where different treatment wells were scored on a scale of 0 to 3. Score of 3 was given to wells in which nematodes were moving continuously and seemed to be unaffected by the treatment, and score of 0 was given to wells in which most of the nematodes did not show any movement and were straight. This assay was used to determine the sublethal concentration of AITC for the gene expression analysis.

Semi-quantitative Real-Time PCR (Semi qRT-PCR)

We examined the expression of the *Gp-GST* after the exposure to the sublethal concentration of AITC by a semi-quantitative real-time PCR (qRT-PCR) analysis. Approximately 1000 freshly hatched pre-parasitic *G. pallida* J2s were exposed to AITC for 3 hours. Second stage juveniles exposed to 0.5% acetone for three hours were used as a control sample. Following the exposure to either AITC or the control, the exposed J2s were washed three times in sterile DI H_2O and the total RNA was extracted and reverse-transcribed in order to obtain cDNA as mentioned previously (see Chapter 1; General Materials and Methods). On average we used 200 nanograms of total RNA for each sample. Our results were normalized using the EiF-F and EiF-R primer set amplifying *G. pallida EiF4* gene, as an internal control (Table 2.1). GSTF3 and GSTR1 (Table 2.1) primers were used to check the expression of the *Gp-GST* using the semi-qRT-PCR. We have set up two different 50µl PCR reactions (for the control sample and for the treatment sample) containing 200 ng of cDNA template, 10X Standard *Taq* Reaction Buffer (NEB, Ipswich, MA), 100µM of each dNTPs, 10µM of each primer, and 0.625 units of Standard *Taq* polymerase (NEB, Ipswich, MA). PCR amplification reaction was carried out in Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA) using the following program set up, initial denaturation at 95ºC for 30 seconds, 30 cycles at 95ºC for 30 seconds, 60ºC for 30 seconds, and 68ºC for 1 minute, followed by a final extension at 68ºC for 5 minutes and a hold at 4ºC. PCR reaction was stopped after cycles 20, 22, 24, 26, 28, 30, 32, and 34, and 5µl of the mixture was collected. Obtained PCR product was tested for appearance of a band by agarose gel (1%) electrophoresis.

Results

Identification of the Gp-GST from G. pallida

The focus of this study was on identification of genes associated with the antioxidant or the detoxifying pathway of the nematode. Besides *Gp-SOD3* we also identified a gene predicted to encode for an enzyme belonging to the detoxification pathways. We referred to this gene as *Gp-GST,* however in the *G. pallida* genome database it is identified as GPLIN_001198400. Nucleotide and amino acid sequences for this gene was obtained from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/).

Amplification, Cloning, and Sequencing of the Gp-GST from G. pallida

Specific primer set (Table 2.1) was designed based on the *Gp-GST* sequence and was used to amplify the entire coding region of this gene from the cDNA obtained from *G. pallida* pre-parasitic J2s. Amplified product was cloned into a pCR™8 TOPO® TA vector. Subsequent sequencing revealed that the coding sequence of the *Gp-GST* encoded for a product of 621 bp (Fig 4.1), which is the size that was predicted by the *G. pallida* genome database. The nucleotide sequence was translated into an amino acid sequence (Gasteiger et al., 2003) to obtain a putative full length open reading frame that encodes for a protein of 206 amino acids in size. There was no signal peptide predicted at the N-terminus of Gp-GST using the SignalP 5.0 server (Fig B.3) (Armenteros et al., 2019) and no transmembrane domains were detected with the TMHMM algorithm (Krogh et al., 2001). *In silico* analysis indicated that Gp-GST was predicted to encode for a soluble glutathione S-transferase N-terminal domain (PS50404) and a soluble glutathione S-transferase C-terminal domain (PS50405).

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atggtccaatacaaattgtattacttcgatcttcgcggtataggcgagcccattcgcctg
 M  V  Q  Y  K  L  Y  Y  F  D  L  R  G  I  G  E  P  I  R  L
ctcctccattacgttggccaacaatttgaagacgttcgcttcggcaaggaggaatggccg
 L  L  H  Y  V  G  Q  Q  F  E  D  V  R  F  G  K  E  E  W  P
actaaatacaagtcgaaatttttctacgggaaagcgccggtgttggaggttgacgggaag
 T  K  Y  K  S  K  F  F  Y  G  K  A  P  V  L  E  V  D  G  K
cagctgggccaaagcacgacaattttgcgctttttagcagagaaatttgcattggccggt
 Q  L  G  Q  S  T  T  I  L  R  F  L  A  E  K  F  A  L  A  G
aaggatgagtgggagaaggcaaaggccgacgaaatcatcaattttcaaaaggatgctaac
 K  D  E  W  E  K  A  K  A  D  E  I  I  N  F  Q  K  D  A  N
acggagtttgccccgtacatttttgccaaactgggattccgcgaaggcgatttggacaaa
 T  E  F  A  P  Y  I  F  A  K  L  G  F  R  E  G  D  L  D  K
ctccgtacggaagtgcttgagccaggcgtcaagcgcattttcccgttgtttgaggcgctt
 L  R  T  E  V  L  E  P  G  V  K  R  I  F  P  L  F  E  A  L
ttgaaggagtccggttcggactatatgctcccgtccggcttgtccatggtggactttcag
 L  K  E  S  G  S  D  Y  M  L  P  S  G  L  S  M  V  D  F  Q
gtcggcaacttcttgtacaccttcaccaagttggagccggacacgatcaaggcgtacccg
 V  G  N  F  L  Y  T  F  T  K  L  E  P  D  T  I  K  A  Y  P
gagttggtcaaatatgtggagcgtgtccatgcactgcctcagctgcagaagtatttgcag
 E  L  V  K  Y  V  E  R  V  H  A  L  P  Q  L  Q  K  Y  L  Q
cagcgtccgcaagaccgctga
 Q  R  P  Q  D  R  -
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Figure 4.1. The coding and translated amino acid sequence for the *G. pallida Gp-GST* sequence, GPLIN_001198400.

Allyl Isothiocyanate (AITC) Exposure and the Visual Assessment Assay (VAA)

Visual assessment assay (VAA) was conducted as described by Zasada et al., (2008) in order to observe the impact of the AITC on the overall activity of the *G. pallida* J2s. The activity of J2s in

the control treatment (exposure to 0.5% acetone) remained the same over time. Visual assessment assay showed that there was an immediate effect of the AITC on the activity of J2s compared to the control sample, and the activity continued to decrease with increased exposure. Furthermore, a greater decrease in the activity of J2s with the increasing concentration of AITC in the treatment sample was observed. Three hours after exposure, most of the *G. pallida* J2s exposed to the highest concentration of AITC (400 µM) exhibited almost no movement and appeared straight, receiving a score of 0 (Fig 4.2). After the exposure to 100 µM of AITC for three hours, there was a slight decrease in the overall activity of J2s, however most of the nematodes were still showing some degree of motility. Based on the results of the VAA, the concentration of 100 µM of AITC was determined to be sublethal for the *G. pallida* J2s and was used for the subsequent gene expression analysis.

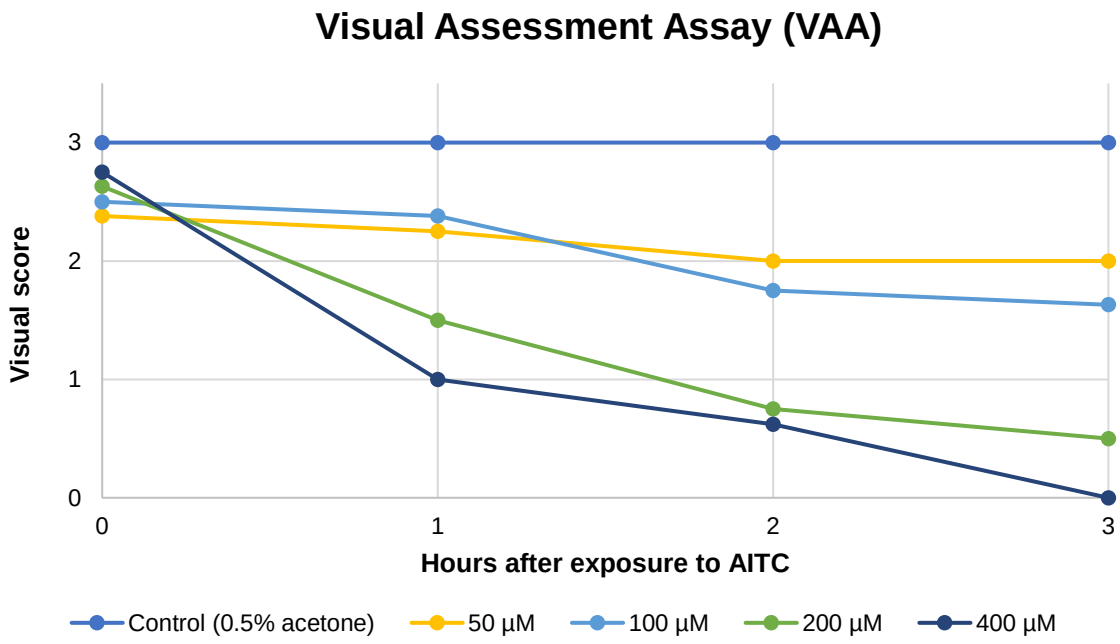

Figure 4.2. Results of the visual assessment assay (VAA) after exposure of *G. pallida* J2s to various concentrations of the allyl isothiocyanate (AITC) and the control (0.5% acetone) over time. The activity of J2s was scored immediately after the exposure, 1, 2, and 3 hours after the exposure to AITC.

Semi-quantitative Real-Time PCR (Semi qRT-PCR)

Semi-quantitative real-time PCR (semi qRT-PCR) was used to analyze the expression of *Gp-GST* when *G. pallida* J2s were exposed to sublethal concentration of AITC. Based on the results of the VAA, freshly hatched J2s were exposed to 100 µM of AITC for three hours. The expression of *Gp-GST* from the nematodes exposed to the AITC was compared to the expression of this gene in the

G. pallida J2s exposed to 0.5% acetone for three hours. This semi qRT-PCR showed a slight increase in the expression of *Gp-GST* following the exposure to 100 μM of AITC for three hours compared to the J2s that were exposed to 0.5% acetone (Fig 4.3).

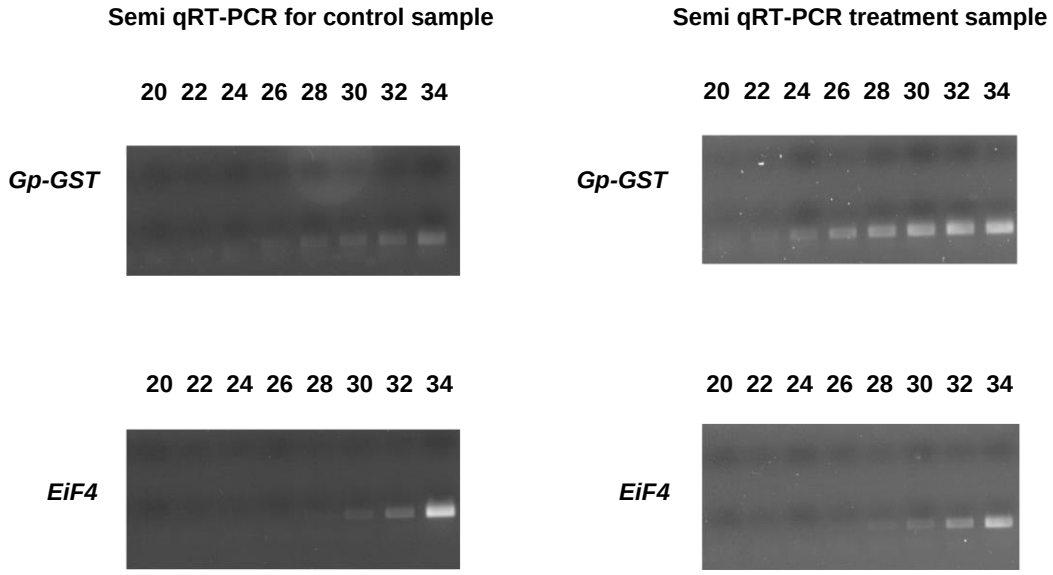

Figure 4.3. Semi-quantitative real-time PCR (semi qRT-PCR) results from the *G. pallida* J2s exposed to allyl isothiocyanate (AITC) showing the gene expression of the *Gp-GST* after the exposure to 100 μM of AITC for three hours. Expression of *Gp-GST* in the *G. pallida* J2s exposed to 0.5% acetone was used as a control. *G. pallida EiF4* gene was set up as an internal reference control.

Discussion

Antioxidant and detoxification pathways are considered to contribute greatly to the defenses activated when nematode is exposed to a stressful environment (Gillet et al., 2017). An important enzyme family contributing to the detoxification metabolism is glutathione S-transferase (GST). A gene that is predicted to encode for one of the GSTs from the potato cyst nematode *G. pallida* was identified in a recent transcriptome study of this nematode (Kooliyottil et al., 2019). In the transcriptome analysis, this gene was shown to have a slightly higher expression when nematode is infecting the resistant plant compared to the susceptible plant (Kooliyottil et al., 2019). For our study, we were interested in the response of antioxidant and detoxification pathways when nematode is exposed to different sources of stress. We wanted to see if this *G. pallida* gene, that we referred to as *Gp-GST* and that was identified in the transcriptome analysis, was expressed when the nematode was exposed to sublethal concentrations of AITC. Alyll isothiocyanate (AITC) is the hydrolysis product

of sinigrin, a glucosinolate found in many brassicaceous plants. Sinigrin is present in high levels in *B. juncea*, and has been shown to be an effective control measure for *G. pallida* (Aires et al., 2009; Lord et al., 2011; Brolsma et al., 2014; Dandurand et al., 2017).

We obtained the sequence for *Gp-GST* from the *G. pallida* genome (https://parasite.wormbase.org/Globodera_pallida_prjeb123/Info/Index/), and amplified it from the *G. pallida* pre-parasitic J2s cDNA. This was followed by cloning and sequencing using the Sanger DNA sequencing platform. Sequencing has revealed that the sequence for the *Gp-SOD3* predicted by the *G. pallida* genome was correctly assembled and it encoded for what appeared to be an ORF without any premature stop codons. Further *in silico* analysis revealed this gene was predicted to encode for a soluble GST N-terminal and C-terminal domain. Glutathione S-transferases make up a large enzyme family, where within classes a lot of focus is placed at the N-terminus (Sheehan et al., 2001). This terminal tends to be more conserved since it involves GSH-binding site, while C-terminus is less conserved within classes and it might contribute to the substrate specificity (Sheehan et al., 2001). Several GSTs have been identified from plant parasitic nematodes, such as *Bursaphelenchus xylophilus, Pratylenchus coffeae, Meloidogyne incognita,* and *G. pallida* (Dubreuil et al., 2007; Bellafiore et al., 2008; Haegeman et al., 2011; Kikuchi et al., 2011; Cotton et al., 2014; Espada et al., 2016). In root knot nematode (RKN) *M. incognita,* one glutathione S-transferase, GST-1, was localized in the subventral esophageal gland (Abad et al., 2008; Dubreuil et al., 2007). Although transcript of this enzyme was localized in the glands of *M. incognita,* there was no signal peptide detected for this GST-1 sequence (Dubreuil et al., 2007). Moreover, an enzymatically functional GST was found in the secretions of the pine wood nematode, *B. xylophilus* (Espada et al., 2016).). *In silico* analysis did not predict any signal peptide for the Gp-GST, indicating that the product of *Gp-GST* may not be secreted by *G. pallida.* However, further analysis is required to determine whether the GST enzyme is secreted by *G. pallida*. Secreted GSTs could be benefiting the nematode through the protection from the ROS or through the modifications of the response of the host plant to the presence of the nematode (Dubreuil et al., 2007).

We saw that there was a slight increase in the expression of *Gp-GST* when *G. pallida* J2s were exposed to sublethal concentration of AITC compared to the exposure to the control (0.5% acetone). Isothiocyanates have been shown to be strong inducers of detoxification enzymes, especially GSTs, both *in vitro* and *in vivo* (Munday & Munday, 2004; Munday, 2002; Hasegawa et al., 2010). GSTs were induced in the fungal pathogen *Sclerotinia sclerotiorum* following an infection of *B. napus* and exposure to isothiocyanates of this plant (Rahmanpour et al., 2009). Isothiocyanates were shown to cause strong induction of GSTs in the bladder of rats, where these enzymes might be

helping to decrease the rate of bladder cancer (Zhang et al., 2006). Induction of this enzyme was also observed in *C. elegans* following an exposure to AITC (Hasegawa et al., 2010). This suggests that the induction of the glutathione S-transferases has an important protective role against harmful effects of isothiocyanates on a wide range of organisms. Moreover, protection provided by these enzymes may be necessary for the infection process and life cycle of pathogens. Mutants of the necrotrophic fungus *Alternaria brassicicola* that lacked the ability to express three different GSTs had significantly reduced abilities to cause successful infection on its host *B. oleracea* (Calmes et al., 2015). Also, these mutants were more susceptible to isothiocyanates produced by the host plant compared to the organisms that had functional GSTs (Calmes et al., 2015). Silencing GST-1 in *M. incognita* led to decreased production of eggs, but it did not impact the ability of the nematode to infect plant roots (Dubreuil et al., 2007). The absence of this GST-1 in *M. incognita* could have had an effect on the completion of its life cycle, by possibly affecting the development of females (Dubreuil et al., 2007). Further understanding of the particular role of the enzyme, GST, in *G. pallida* detoxification could help provide novel control strategies for this pest.

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Appendix A- Raw Amplification Curves for the qRT-PCR Analysis

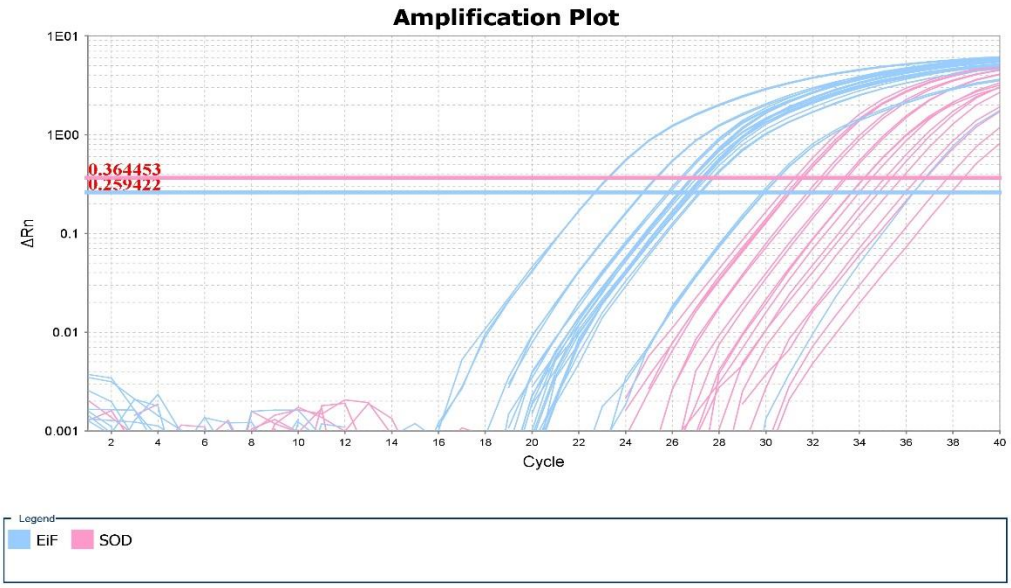

Figure A.1. Raw amplification curve for the qRT-PCR analysis for all three biological replicates of susceptible potato, resistant potato, and *S. sisymbriifolium,* collected 24 hours post infestation. Three technical replicates were used for every sample tested.

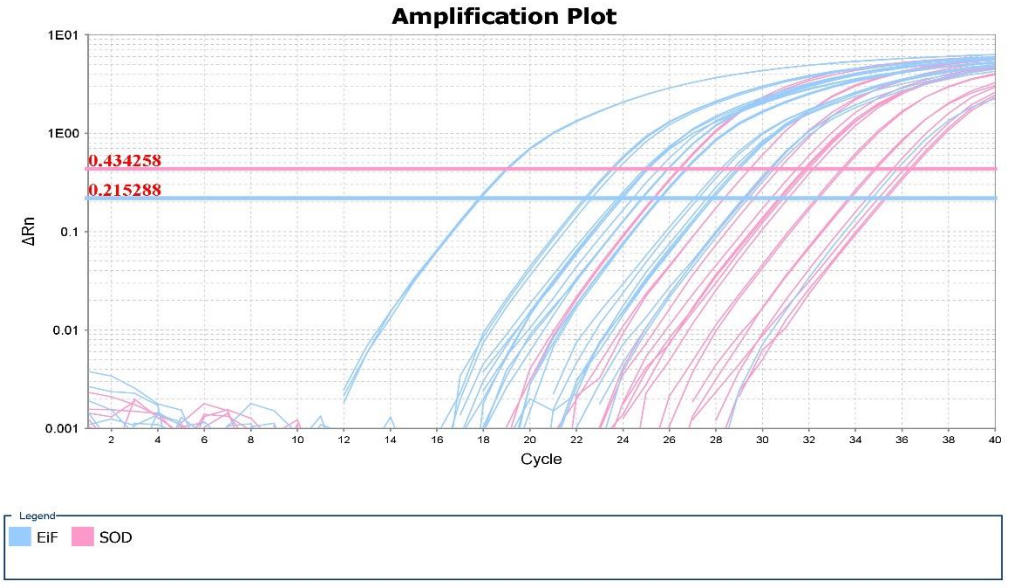

Figure A.2. Raw amplification curve for the qRT-PCR analysis for all three biological replicates of susceptible potato, resistant potato, and *S. sisymbriifolium,* collected 48 hours post infestation. Three technical replicates were used for every sample tested.

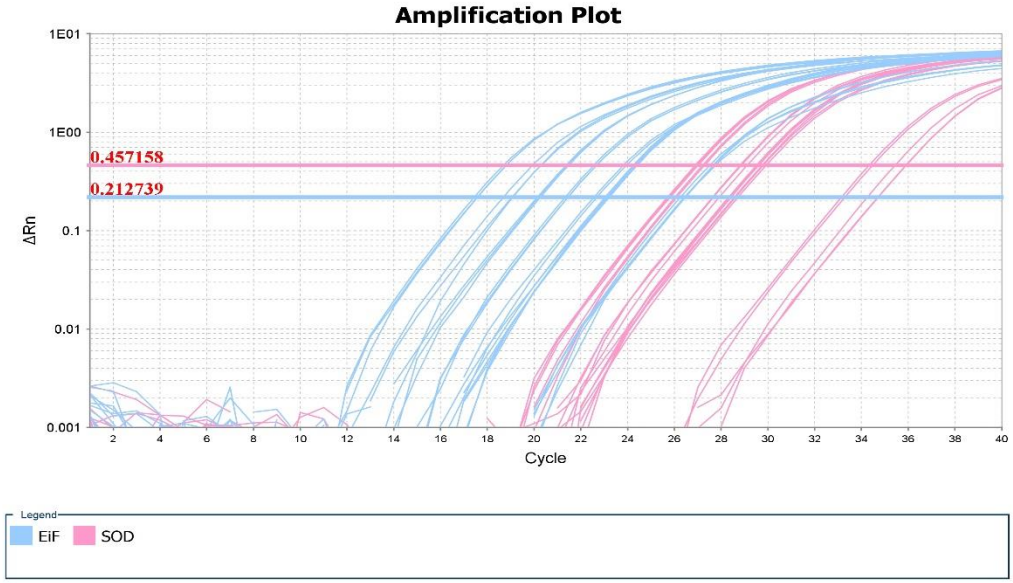

Figure A.3. Raw amplification curve for the qRT-PCR analysis for all three biological replicates of susceptible potato, resistant potato, and *S. sisymbriifolium*, collected 72 hours post infestation. Three technical replicates were used for every sample tested.

Appendix B- Signal Peptide Analysis

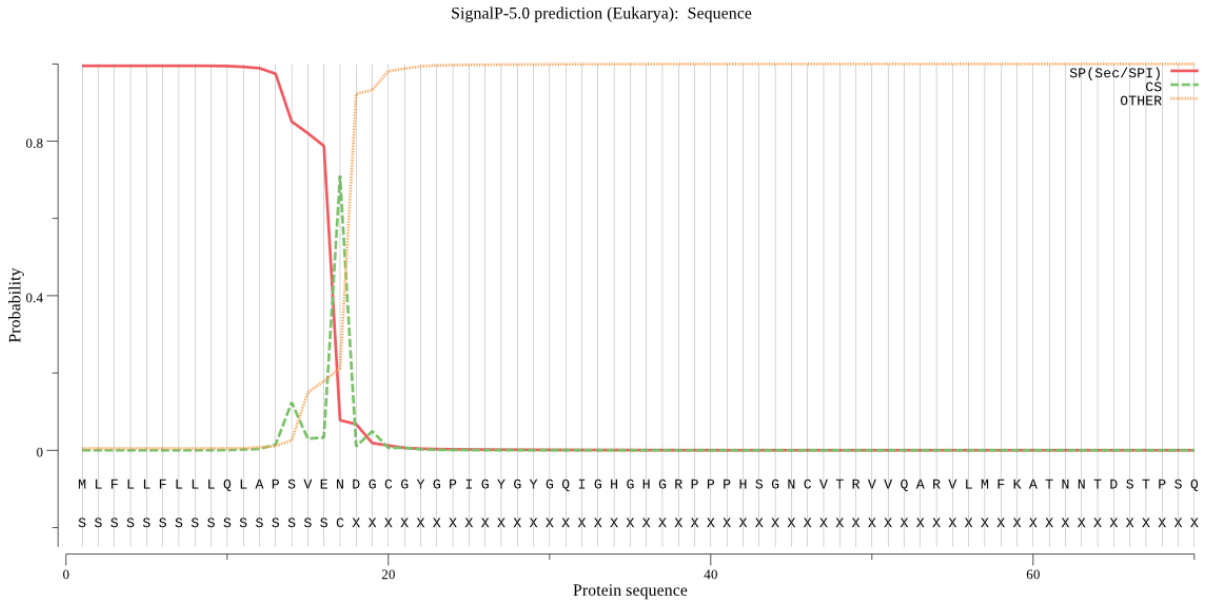

Figure B.1. Signal peptide predicted to be between amino acid residues 17 and 18 at the N-terminus of Gp-SOD3. SignalP 5.0 server (Armenteros et al., 2019) was used for signal prediction.

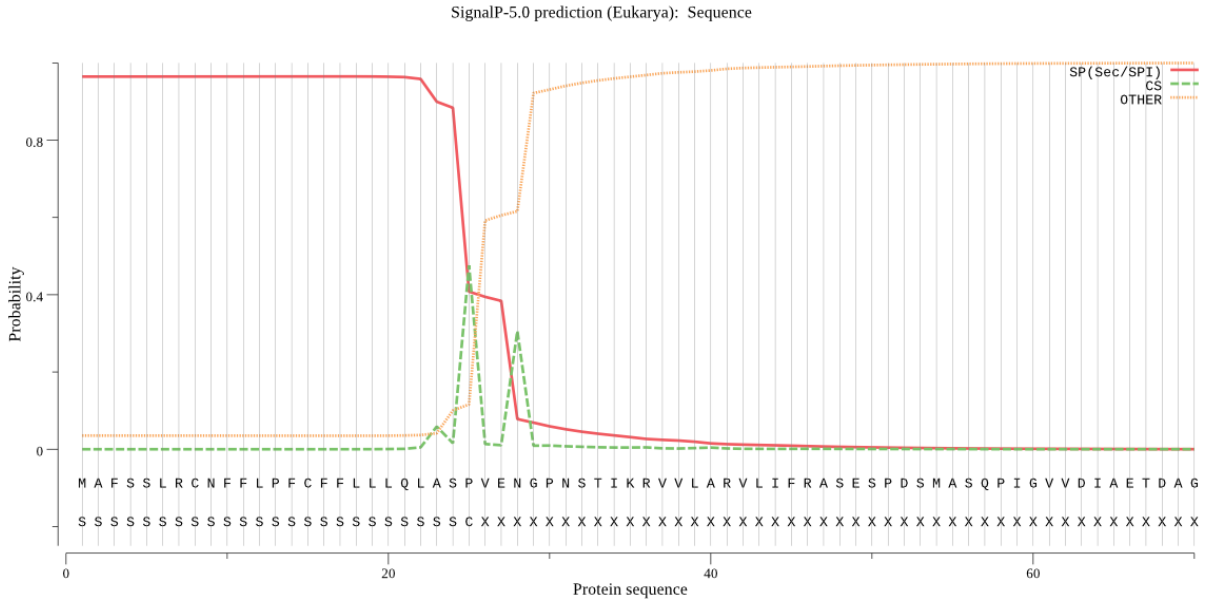

Figure B.2. Signal peptide predicted to be between amino acid residues 25 and 26 at the N-terminus of GPLIN_000150100 (paralog of Gp-SOD3). SignalP 5.0 server (Armenteros et al., 2019) was used for signal prediction.

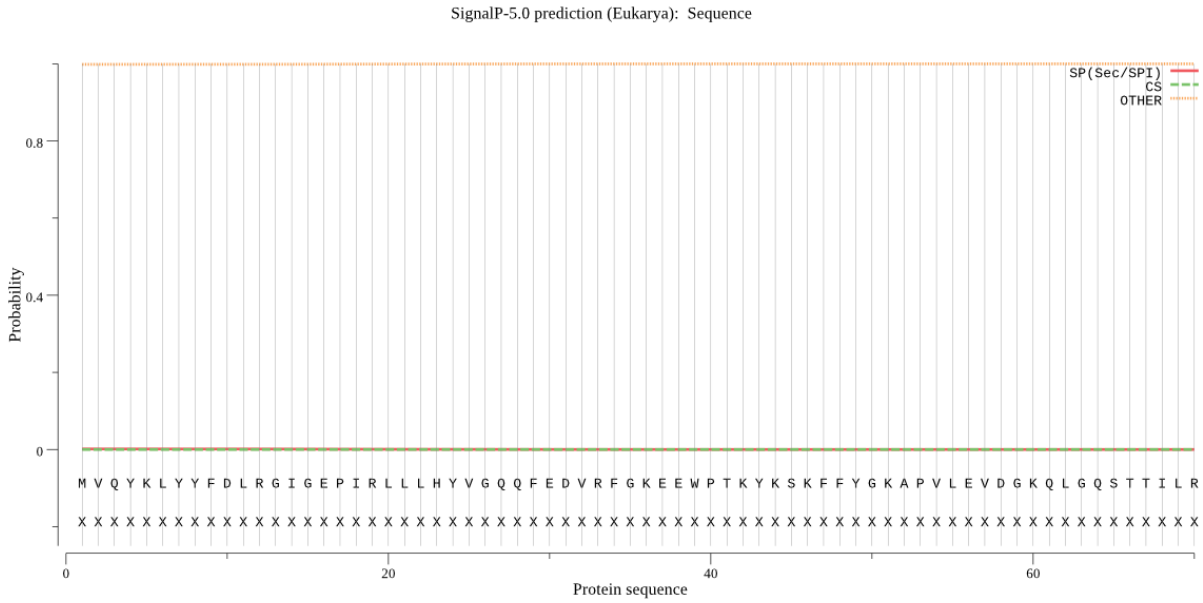

Figure B.3. Absence of the signal peptide predicted for the Gp-GST. SignalP 5.0 server (Armenteros et al., 2019) was used for signal prediction.