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Analysis of the role of polygalacturonase inhibiting proteins in the interaction between plants and nematodes

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Dedication

I dedicated my dissertation to my parents and teachers for their efforts and support through out my academic career

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Abstract

Plant-parasitic nematodes (PPNs) are a great threat to crops, causing billions of dollars of losses worldwide. Cyst nematodes and root-knot nematodes belong to a small group of sedentary endoparasitic PPNs that parasitize the roots of a wide range of crop species. Both of these nematodes invade the roots as infecting juveniles (J2s) and establish a feeding site in the plant root that functions as their nutrient source. An effective plant defence relies on the recognition of pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) by surface-localised pattern-recognition receptors (PRRs). In addition to PAMPs, PRRs can also recognize damage associated molecular patterns (DAMPs), which are molecules produced by plants in response to damage. Nematodes' invasion of plant roots and their subsequent migration inside the roots are likely to damage the plant's root cells, thereby generating cell wall fragments (oligogalacturonides OGs). OGs can act as DAMPs. Recognition of PAMPs or DAMPs by PRRs leads to the activation of defence responses in the host plant, which are designated as PAMP-triggered immunity (PTI). PTI can restrict development and growth of invading pathogens. Though nematode invasion activates PTI in plants during early stages of infection, PTI responses may in turn be suppressed by the invading nematodes during the establishment of their feeding sites. Recent studies have shown that plants use surface-localised receptors to recognise PAMPs released by nematodes, and thereby trigger PTI responses. However, recognition of DAMPs and activation of downstream pathways during plant-nematode interactions remained unexplored. In this study, we characterised the role of polygalacturonase-inhibiting proteins (PGIP*)* in the model plant *Arabidopsis thaliana* during infection with the beet cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. PGIP is encoded in *Arabidopsis* by a small gene family consisting of two genes, *PGIP1* and *PGIP2*. Expression of these genes is induced when the plant is wounded or attacked by pathogens. In order to inhibit pectin degradation by microbial polygalacturonase (PGs), the plant deploys PGIP in the cell wall. This PG-PGIP interaction leads to the production of elicitor-active OGs (oligomers of α-1, 4-linked galacturonic acids), which can be sensed by a plasma membrane localized receptor called *WAK1* (wall-associated kinase). The *WAK1* receptor, on sensing elicitor-activated OGs, sets off a number of defence

responses in the plant such as accumulation of phytoalexins, oxidative burst, callose deposition, and production of glucanase and chitinase.

Quantitative RT-PCR (qRT-PCR) analysis showed that *PGIP* genes are particularly strongly induced upon cyst nematode invasion of roots. To analyse spatio-temporal expression of *PGIP* genes during plant-nematode interaction, we developed promoter::GUS lines and observed strong staining at an early stage (1dpi) of *H. schachtii* infection. However, this staining was not observed when the plants were infected with *M. incognita* at the same stage. Pathogenicity testing with loss-of-function mutants (*pgip1, pgip2*) and overexpression lines (*35S*::*PGIP1, 35S::PGIP2*) revealed that *PGIP1* expression limits the ability of cyst but not root-knot nematodes to parasitize the host roots. A recent transcriptome analysis of host roots during the migratory stages of *H. schachtii* infection revealed that, during this stage, a number of genes involved in secondary metabolism (camalexin and indole glucosinolates production) were strongly and differentially upregulated. Because loss-of-function *PGIP1* mutants (*pgip1*) were hypersusceptible to beet cyst nematode infection, we reasoned that activation of the secondary metabolism genes in these mutants might be impaired. To investigate this hypothesis further, we compared Col-0 and *pgip (pgip1 and pgip2)* roots for the expression of the secondary metabolism genes with or without infection via qRT-PCR. We found that induction of the secondary metabolism genes is impaired in *pgip* mutants, especially in *pgip1* during migratory stage.

A detailed characterization of a putative PG sequence from *H. schachtii* showed a very low sequence similarity to a PG from *M. incognita* and any other known PG from bacteria, fungi, insects, and nematodes. Therefore, we concluded that cyst nematodes do not encode a functional PG and activation of PGIP during cyst nematode infection is independent of a typical PG-PGIP interaction.

In conclusion, our findings provide insights into distinct perception of damage responses by host during cyst and root-knot nematode parasitism at the molecular level. Clarifying further details of these responses may lead to advances in breeding strategies for nematode resistance.

Zusammenfassung

Pflanzenparasitäre Nematoden (PPN) verursachen jährlich weltweit Ernteausfälle in Millionen-Dollar-Höhe. Zysten- und der Wurzelgallennematoden gehören zu einer kleinen Gruppe von sedentären Nematoden, welche an einer Vielzahl an Nutzpflanzen parasitieren. Beide dringen als nicht vollständig entwickelte juvenile (J2s) Nematoden in die Wurzel ein, um dort ein Nährzellensystem zu bilden, welches dann als Nährstoffquelle genutzt wird.

Die Abwehr von diesen endogenen Wurzelschädlingen kann nur durch eine funktionierende Immunabwehr der Pflanze gewährleistet werden. Dabei werden bestimmte molekulare Strukturen, sogenannte Pathogen- oder Mikroben-assoziierte molekulare Muster (PAMPs oder MAMPs), durch spezifische Pathogen-Recognition Rezeptoren (PRRs) an der Zelloberfläche der Pflanze erkannt. Diese Rezeptoren erkennen allerdings nicht nur PAMPs und MAMPs, sondern auch sogenannte DAMPs, Damage-assoziierte molekulare Muster. DAMPs sind Moleküle, welche als Reaktion auf mechanische Schäden gebildet werden. Das Eindringen und Wandern von Nematoden in der Wurzel kann solche Zellschäden verursachen und damit zur Bildung von Zellwandfragmenten, den Oligogalakturoniden (OG's), führen. OG's werden als DAMPs erkannt. Die Aktivierung der PRRs durch DAMPS oder PAMPs lösen in der Pflanze eine Immunreaktion aus, die PAMP-Triggered Immunity genannt wird (PTI). Das Ziel dabei ist, Entwicklung und Wachstum des Eindringlings einzuschränken. Interessanterweise wird anfänglich, wenn der Nematode in die Wurzel eindringt, PTI induziert, im Laufe der Bildung der Nährzellenstruktur aber kann diese PTI-Reaktion wieder unterdrückt werden. Neueste Studien haben gezeigt, dass oberflächenlokalisierte Rezeptoren PAMPs von Nematoden erkennen und PTI aktivieren. Jedoch ist das Erkennen von DAMPs und die folgende Aktivierung von Signalkaskaden während der Pflanzen-Nematoden-Interaktion bisher noch weitestgehend unerforscht.

In dieser Arbeit wurde nach Infektion der Modellpflanze *Arabidopsis thaliana* mit dem Rübenzystennematoden *Heterodera schachtii* sowie dem Wurzelgallennematoden *Meloidogyne incognita* die Funktion eines Zellwand-Proteins, dem Polygalakturonasen-Inhibierenden Protein (*PGIP*) untersucht. *PGIP* wird in *Arabidopsis* von einer kleinen

Genfamilie codiert, welche durch die beiden Gene *PGIP1* und *PGIP2* repräsentiert wird. Die Expression beider Gene wird durch Verwundung der Pflanze bzw. Zerstören der Zellwand durch Schädlinge induziert. *PGIP* inhibiert das Enzym Polygalakturonase (PG), welches von Pathogenen gebildet wird, um Pektine in der Zellwand abbauen zu können. Diese PG-PGIP Interaktion, führt zur Synthese von OG's, die von *WAK1* (Wall Associated Kinase 1), einem Rezeptor mit Kinase Aktivität in der Plasmamembran, erkannt werden. Die Aktivierung von *WAK1* durch OG's führt zu einer Reihe von Abwehrmechanismen in der Pflanze, wie die Synthese von Phytoalexinen, Reaktiver Oxygen Spezies (ROS), Kalloseabscheidung und der Bildung von Glukanasen und Chitinasen.

Quantitative PCR (qPCR) Ergebnisse haben gezeigt, dass die Genexpression von *PGIP* stark hochreguliert ist, wenn der Zystennematode in die Wurzeln eindringt. Um die Expression von *PGIP* zeitlich und gewebespezifisch während der Pflanzen-Nematoden Interaktion analysieren zu können, wurden Promoter::GUS-Linien entwickelt. Das Ergebnis resultierte in einer starken blauen Färbung während des ersten Infektionsstadiums von *H. schachtii* (1 Tag nach Infektion). Allerdings führte die Infektion mit *M. incognita* zu keiner Färbung in dieser ersten Infektionsphase.

Um die Pathogenität beurteilen zu können, wurden loss-of-function Mutanten (*pgip1*, *pgip2*) und Überexpressions Linien (*35S::PGIP1, 35S::PGIP2*) generiert. Diese Mutanten zeigten, dass *PGIP1* die Entwicklung weiblicher Rübennematoden limitiert, hingegen der Wurzelgallenenematode in seiner Entwicklung nicht eingeschränkt ist. Eine neuere Transkriptom-Analyse der Wirtswurzeln während der ersten Infektionsphase ergab, dass in diesem Zeitraum bestimmte Gene, die bei der Synthese sekundärer Pflanzenstoffe (Camalexin und Indol-Glucosinulate) involviert sind, signifikant hochreguliert waren. Eine Beeinträchtigung der sekundären Pflanzenstoffsynthese könnte demnach die beobachtete erhöhte Anfälligkeit der loss-of-function *PGIP1* Mutante (*pgip1*) gegenüber dem Rübenzystennematoden erklären. Um unsere Hypothese stützen zu können, wurde DNA von infizierten und nicht infizierten Col-0 und pgip Wurzeln (*pgip1*, *pgip2*) durch eine qRT-PCR amplifiziert und hinsichtlich darin involvierter Gene verglichen. Tatsächlich konnte festgestellt werden, dass bestimmte Gene, die zur Induktion von sekundären Pflanzenstoffen benötigt werden, in den loss-of-function

Mutanten abreguliert sind, besonders in *pgip1* während der Wanderungsphase. Die Gensequenz von PG ist putativ und wurde, um sie genauer charakterisieren zu können, mit bekannten PG Gensequenzen von *M. incognita*, Bakterien und Pilzen verglichen und resultierte in nur minimalen Ähnlichkeiten. Daraus schließen wir, dass der Rübenzystennematode keine funktionelle PG kodiert und die Hochregulierung von *PGIP* während der Infektion unabhängig von einer typischen PG-PGIP Interaktion ausgelöst wird. Mit dieser Arbeit konnten wir tiefere Einblicke in die Immunabwehr gegenüber Zysten- und Wurzelgallennematoden auf molekularer Ebene gewinnen. Weiterführende Untersuchungen könnten für bestimmte Züchtungstechniken mit Hinblick auf Pathogenresistenzen genutzt werden.

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Acronyms and Abbreviations

AGA	Apiogalacturonan
ATP	Adenosine-5′-triphosphate
BAK1	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
CAMV	Cauli flower mosaic virus
BIK1	BOTRYTIS-INDUCED KINASE 1
cDNA	Complementary DNA
CW	Cell wall
ddH2O	Double distilled water
CRISPR	Clustered regularly interspaced short palindromic repeats
C. elegans	*Caenorhabditis elegans*
CDPKs	Calcium-dependent protein kinases
CWDEs	Cell wall-degrading enzymes
CTAB	Hexadecyltrimethyl-ammonium bromide
DAMPs	Damage associated molecular patterns
DNA	Deoxy ribonucleic acid
DORN	Does not respond to nucleotide
ddH2O	Double distilled water
dpi	Days post infection
EDTA	EthylenediaminetetraAcetic acid Di- sodium salt
EF-Tu	Elongation factor thermo unstable
e.g.,	Exempli gratia (For example)
EGF	Epidermal growth factor
EPNs	Entomopathogenic nematodes
ETI	Effector triggered immunity

FLS2	Flagellin sensing 2
FP	Feeding plug
FT	Feeding tube
GC	Giant Cells
H. schachtii	*Heterodera schachtii*
Hpi	Hour post infection
HG	Homogalacturonan
HgCl2	Mercuric chloride
HMGB	High mobility group box
HR	Hypersensitive responses
IJ	Infective juvenile
ISC	Initial Syncytial Cell
J2	Juvenile stage 2
JA	Jasmonic acid
$K4(Fe(CN)_6$	Potassium Ferrocyanide
KOH	Potassium hydroxide
LB	Lauria-Broth
LRRs	Leucin rich repeats (LRRs)
LysMs	Lysine motifs
MAMPs	Microbial associated molecular patterns
MAPKs	Mitogen-associated protein kinases
µm	Micro meter
$\mu g\ mL^{-1}$	Micro gram per milli liter
mm	Milli meter
MS	Murashige & Skoog

N	Nematode
NB	Nucleotide binding
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaOCl	Sodium hypochloride
OGs	Oligogalacturonides
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
PEPR	Peptide receptors
PGs	Polygalacturonases
PGIP	Polygalacturonase-Inhibiting Protein
PPNs	Plant-Parasitic Nematodes
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PVP	Polyvinyl pyrrolidone
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RBOHD	Respiratory burst oxidase homolg D
RLKs	Receptor like kinases
RLPs	Receptor-like proteins
RNA	Ribonucleic acid
RNAi	RNA interference
RKN	Root-knot Nematode
ROS	Reactive oxygen species
rpm	Revolution per minute
S	Syncytium

SA	Salicylic acid
SAR	Systematic acquired resistance
SE	Standard error
T-DNA	Transfer DNA
UTR	Untranslated region
WAK	Wall-associated kinase
XGA	Xylogalacturonan
YEP	Yeast extract peptone
$ZnCl_2$	Zinc chloride

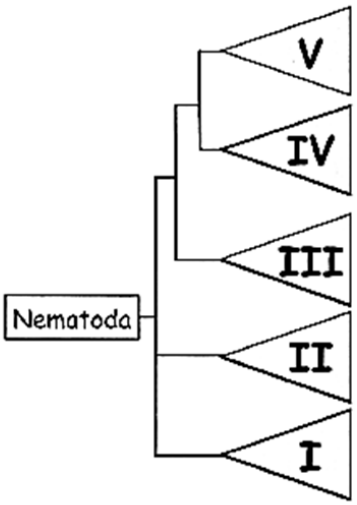
1. Introduction

Nematodes are multicellular organisms belonging to the phylum Nematoda. The word "*nematode*" is derived from two Greek words: *nema* meaning thread, and *toid* meaning form. Nematodes are classified into five clades (Silvestre and Cabaret, 2004), each of which has parasitic species (Figure 1.1). Members of this phylum are the most ancient group of animals on earth, having existed for an estimated one billion years (Wang et al., 1999). They are widely distributed and have adapted to disparate ecosystems such as fresh and salty water, different kinds of soil, various elevations and both trophic and Polar Regions. They are the most abundant animals on earth (Platt, 1994), including about 25,000 known species (Hodda, 2011; Zhang, 2013). However, this figure is increasing continously with the discovery and identification of new species (Elling, 2013).

Nematodes are slender bilaterally symmetrical worms. They are generally about 5–100 μm in diameter and 2.5 mm long (Nyle and Weil, 2009). Most are small, even microscopic, but some can be metres long (Ruppert et al., 2004). The largest nematode, found in the placenta of the sperm whale, is called *Placentonema gigantissima*, which reaches 8-9 metres long (Gubanov, 1951). The nematode body is triploblastic—that is derived from three embryonic cell layers: ectoderm, mesoderm and endoderm. Unlike other bilaterally symmetrical animals having a true coelom, nematodes are pseudocoelomate, having no true body cavity. Instead their body cavity is found between the mesoderm and the endoderm that makes up the walls of the gut.

Nematode anatomy is also very simple, being devoid of respiratory and circulatory system (Figure 1.2). The outer part of the skin, the cuticle, protects the nematode during exposure to environmental stress. The nematode's mouth is located at the anterior end and opens into a buccal cavity, whereas the anus forms the posterior opening for the digestive system that runs inside the nematode from head to tail. The nematode nervous system is composed of circum-pharyngeal nerve ring which is build up from 4 nerve ganglia that runs along the length of the body. From the nerve ganglia arises 6 longitudnal nerves that runs down to various parts of of the digestive and reproductive system. The sophisticated sensory organs help them to sense host and and to mate and reproduce. Two types of chemosensory structures are present in nematodes. Those located at the anterior end (head) are called amphids; they occur in pairs, and each

consists of 12 sensory neurons. The other type of sensory structure, located at the posterior end (tail), is the phasmid. Both amphids and phasmids are similar in structure, but the latter are smaller. In addition to its simple anatomy, the nematode body is transparent, allowing visualization of its internal organs. For these reasons, nematodes are used as models in molecular biology, cell biology and neurobiology for e.g. *Caenorhabditis elegans*. First identified in 1900 by zoologist Emile Maupas in the soil of Algiers, these nematodes are non-parasitic and non-infectious, present in soil, and feed on bacteria. They are microscopic, about 1mm long, and are of no economic importance to human beings. Two sexes exist: a hermaphrodite (self- fertilizing) and a male. After being introduced by Sydney Brenner in 1963 as a model organism, these nematodes have been used mainly in the fields of molecular biology and neuroscience. They are notable for being easy to handle in a laboratory setting and have a rapid life cycle of three days under optimum environmental conditions. Moreover, their entire genome has been sequenced, revealing 60–80% gene homology with humans (Kaletta and Hengartner, 2006; McDonlad et al., 2006). Reverse genetic tools such as RNAi and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 have played a significant role in the study of gene function in *C. elegans* (Fire et al., 1998; Hsu et al., 2014; Dickinson and Goldstein., 2016). Although it offers certain advantages, *C. elegans* also presents a few drawbacks. For example, its body lacks many organs and types of tissue, such as brain, blood, and internal organs, and is evolutionarily divergent from humans. Moreover, its small size presents difficulties to conduct experiments related to biochemistry, molecular biology and histology limiting the degree of understanding tissue-specific signaling.



Order	Examples	Host
Strongylida	Trichostongyles, small strongyles, hookworms	animal
Rhabditida	*Caenorhabditis elegans*	free-living
Strongiloides		animal
Tylenchida	*Meloïdogyne* spp., *Globodera* spp.	plant
Ascaridida	*Ascaris* spp., *Toxocara* spp.	animal
Spirurida	*Brugia* spp., *Onchocerca* spp.	animal
Triplonchida	*Paratrichodorus* spp., *Trichodorus* spp.	plant
Dorylaimida	*Xiphinema* spp.	plant
Trichocephalida	*Trichinella* spp., *Trichuris* spp.	animal

Figure 1.1: Phylogenetic organization of the phylum Nematoda (Silvestre and Cabaret, 2004)

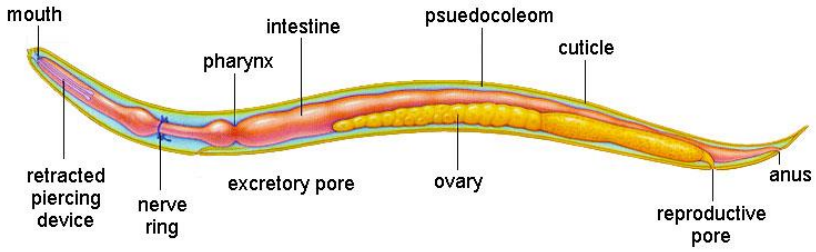


Figure 1.2: Nematode anatomy (Source: University of Illinois)

1.1 Types of Nematodes

Because nematodes are very diversified, their hosts also differ from species to species and can include both plants and animals. However, some of them can utilize microbes as a food source (Manum et al., 1994). Nematodes that infect plants are called plant-parasitic nematodes (PPNs) while the ones infecting animals are called animal-parasitic nematodes. Some insect feeding nematodes are called entomopathogenic nematodes (EPNs) and those feeding on microbes are called microbivores. Both plant and animal parasitic nematodes are damaging to agricultural

crops and livestocks, however, enthomopathogenic nematodes are used in the field of crop protection as a bio-control agent (Lacey and Georgis, 2012).

The different feeding groups are classified on the basis of the structure of their mouthparts (Figure 1.3). Those nematodes that feed on bacteria have a simple tubular mouth with a slightly modified cuticle around the oral opening that ushers food towards the stoma. Those that feed on fungi have a stylet resembling that of plant-parasitic nematodes which helps to puncture the cell wall of fungi in order to suck the internal contents, whereas predator nematodes use teeth to puncture various invertebrates for feeding. Because this work focuses on plant-parasitic nematodes, I have described them in more detail here.

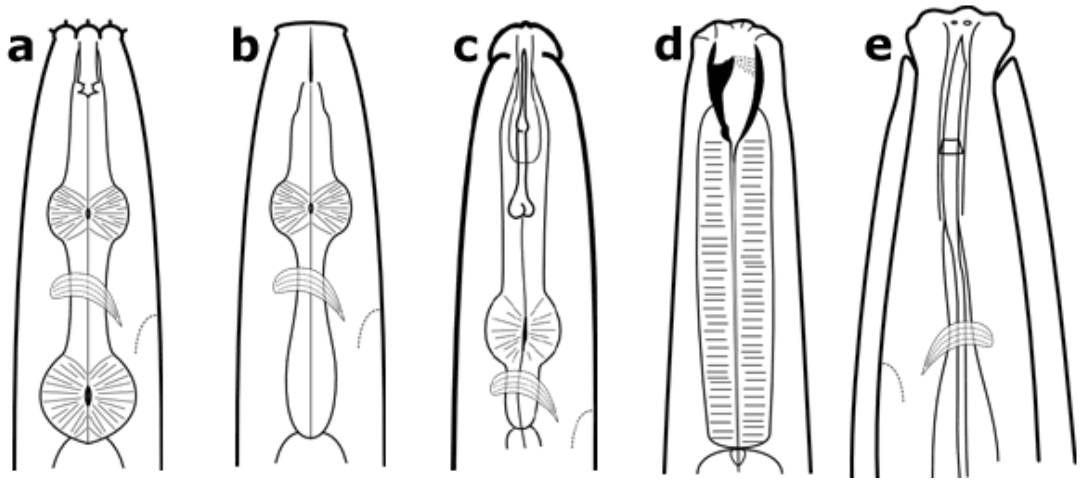


Figure 1.3: Classification of nematodes feeding groups based on the structure of their mouthparts. a) Bacterial feeder have a tubular mouth to suck up bacteria, **b)** Fungivores have a thin stylet but lack stylet knobs, **c)** Plant parasitic feedeing on plants, **d)** Predator nematodes feed on invertebrates animals and has sharp teeth to puncture the prey, **e)** Omnivore. **(Drawing: Ugarte and Zaborski, University of Illinois).**

1.2. Plant parasitic nematodes

Plant parasitic nematodes belong to kingdom Animalia under the phylum nematoda. They were first reported in 1743 by Needham, who observed that nematodes in wheat galls caused severe crop damage. Afterwards, Miles Berkely in 1855 discovered another nematode species, which caused galls on cucumber roots and was termed as the root knot nematodes. In 1859, H. Schacht, a botanist in Bonn discovered tiny worms that parasitized the beet roots and caused so-called

“beet sickness” disease. This observation was confirmed a few years later by A. schmidt (1871), who named this nematode *Heterodera schachtii* in the honour of its discoverer. Since discovery of these plant parasisitc nematodes many decades back, plant nematology has emerged as a very important aspect in the field of plant protection.

About 10% of all nematodes can parasitize plants and comprise more than 4,100 known species (Decraemer and Hunt, 2006; Jones et al., 2013). They include members of the order Tylenchida, Aphelenchida, Dorylaimida and Triplonchida (Table 1). They are small microscopic animal ranges between 0.2 mm (*Paratylenchus* spp.) to about 12 mm (*Paralongidorus* spp.) in length. The body is cylindrical in shape which is tapered at the end. Their body is also surrounded by a transparent surface coat, called cuticle, which protects the nematodes in the soil from various biotic and abiotic stresses. Being transparent, one can easily see their internal organs like the digestive and reproductive systems through a microscope. Unlike higher animals, they lack a respiratory and circulatory system and are depending on diffusion via cuticle. The digestive system is tube like structure which includes stylet, oesophagus, intestine and rectum that open through the anus at the end of the tail. Food is passed through oesophageal lumen that runs between the stylet base and the oesophago-intestinal junctions. Most of the plant parasitic nematodes are included in the taxonomic order Tylenchida and are being characterised by a three-part esophagus: the anterior procorpus, median bulb and basal bulb. There are normally three esophageal glands in this group, along with one dorsal and two sub-ventral glands, but the number increases to six in some nematodes e.g. *Hoplolaimus spp*. At the anterior end (head) there is a needle like structure called stylet which is the piercing apparatus of the plant parasitic nematodes. This stylet is extensible and is used by the nematode to pierce cell wall for sucking the nutrients. This suction is generated through the contraction of the median bulb. In addition to this, nematodes also secrete certain effector proteins into the host tissues through the stylet to facilitate parasitism. In addition to PPNs, certain nematodes that feed on fungi also possess stylet.

Character	Tylenchida	Aphelenchida	Dorylaimida	Triplonchida
Stylet	Stomatostylet	Stomatostylet	Odontostylet	Onchiostylet
Pharynx	Three parts: corpus, isthmus and basal bulb	Three parts: corpus, isthmus and basal bulb	Two parts: distinct corpus and post corpus	Two parts: corpus gradually broadening into posterior part
Pharyngeal gland number	3 glands	3 glands	3–5 glands	5 glands
Position of gland opening	Dorsal gland opens near the spear base	Dorsal gland opens in metacorpus, anterior to valve	All glands open to body of gland	All glands open to body of gland
Metacorpus	Width <75 % of body width	Large, width almost (>75 %) of body width	Absent	Absent
Isthmus	Present	Absent in Aphelenchoididae	Absent	Absent
Pharyngeal gland	In a bulb or overlapping lobe	In a bulb (Paraphelenchidae) or overlapping lobe	In posterior bulboid expansion	In posterior bulboid expansion

Table 1: Different characteristics of four plant parasitic nematodes orders (Adapted from Manzaniilla-Lopez and Hunt, 2009).

PPNs can be separated in to both male and female but some species can also reproduce asexually through parthenogenesis. The male reproductive system consists of one or two testis, seminal vesicle and a vas deferens that ends through a common opening called cloaca. At the tail region is a pair of spicules that facilitate in coupling with the females. In the same region are present caudal bursa that are able to hold the female during copulation. In comparsion to male reproductive system, the female reproductive system is more variable and consists of one or two ovaries, spermatheca, uterus and vagina that open to the outside via the vulva. In some species of PPNs, sperms are stored in the spermatheca which is a specialised structure of the uterus.

Based on their life style, plant parasitic nematodes can be categorised into different types (Figure 1.4). Those that feed on the root from outside are called ectoparasites. Most nematodes in this group are migratory and feed on the outer part of the cortex cells and on epidermal tissue (Schouteden et al., 2015). These nematodes complete their entire life cycle while feeding from the outside and lay their eggs in the soil as *Paratylencus* spp. does. Some ectoparasitic species such as the ring nematode (*Criconemoides* spp.), can feed at one site for a prolonged period and are termed sedentary ectoparasites. These nematodes feed mainly on root hairs superficial

cortical tissues and cause severe damage to plants. Sites of root elongation and cell division, as well as root tips, are the preferred feeding sites for ectoparasitic nematodes.

Nematodes that penetrate the root and feed from inside are called endoparasites (Schouteden et al., 2015). After attachment to the root, the infecting juvenile can remain either migratory, as in the case of lesion nematodes, or become sedentary, as cyst nematodes do, and moult through four life stages to reach its adult form. Migratory endoparasitic nematodes can move through the root tissue and cause serious damage during their migration (Jones et al., 2013). These nematodes have no permanent feeding site and can kill the plant cell by sucking the cytoplasm through stylet before moving on. The severe damage they cause to the root creates opportunities for other microbes, such as bacteria and fungi, to further damage the root system (Zunke, 1991; Jones and Goto, 2011). Sedentary endoparasitic nematodes, by contrast, invade the root after hatching and become sedentary by maintaining a permanent feeding site throughout their life cycle; they are considered to be the most damaging plant-parasitic nematodes (Gheysen and Mitchum, 2011; Jones et al., 2013). The two main nematodes in this group are root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* and *Globodera* spp.).

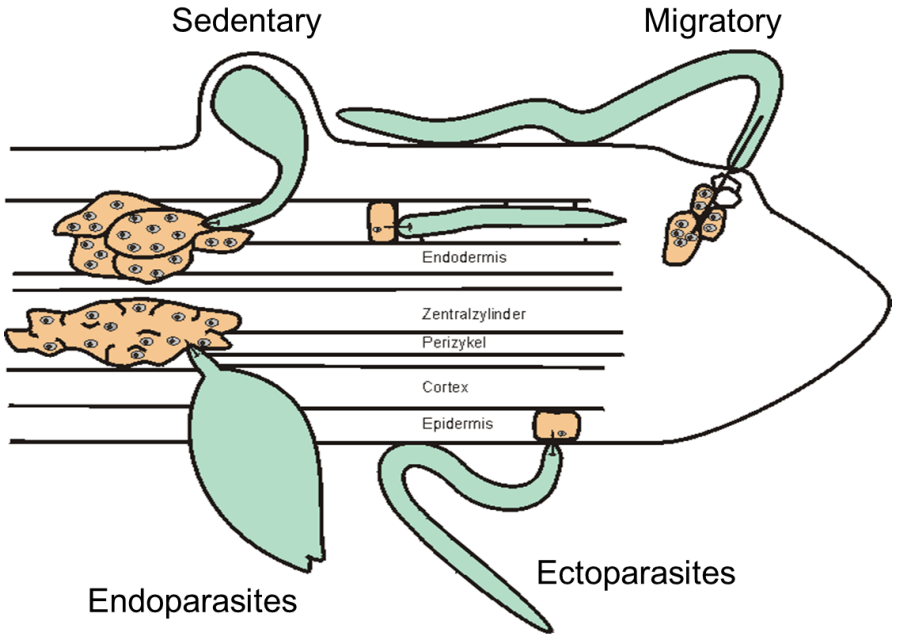


Figure 1.4: Parasitic strategies of plant parasitic nematodes (Drawing: Florian M. W. Grundler).

PPNs can damage all kind of crops, including rice, corn, cotton, legumes, sugar beet, potato, soybean and cereals like wheat. They are an important agricultural pest, causing economic damage of USD 80 billion per year—making them a serious threat to the agriculture industry (Nicol et al., 2011). For farmers, they are a kind of hidden enemy being not visible with naked eye, live in the soil and causes non specific symptoms. They attack the roots and decreases plants ability to absorb water and nutrients from the soil. This results in stunted growth, chlorosis, wilting and ultimately yield loss of the infested crop.

1.2.1 Root-knot nematodes (*Meloidogyne* spp.)

Root-knot nematodes (RKNs) are sedentary endoparasites of many plant species and are widely distributed around the globe. They were first discovered by Berkeley in 1855 on the roots of cucumber and since then have been recognized as an important pathogen of more than 3,000 plant species (Ehwaeti et al., 1999; Niu et al., 2016) with 100 species of RKN reported so far (Karssen et al., 2013).

The life cycle of RNK last 20-40 days and begins with hatching of an infective juvenile (J2) upon sensing of root exudates. The J2s are vermiform and move freely in the soil in search of the host root. After finding the root, they migrate intercellularly towards the root tip, make a U-turn towards the vascular cylinder (Wyss et al., 1992; Mende, 1997) and use its needle -like stylet to puncture 5–10 procambial cells. These initial cells enlarge to form giant cells (GCs) first named by Treub in 1886, and serve as a feeding site for nematode growth and development. This feeding site differs from procambial cells both morphologically and physiologically. The GCs undergo successive nuclear division without cytokinesis, resulting in many nuclei within each cell leading to a significant increase in their size. The central vacuole breaks down into many small vacuoles, the cell wall becomes thickened with many ingrowths to increase solute uptake from the vascular cylinder (Jones and Payne 1978; Huang, 1985; Wiggers, 1990; Almeida-Engler et al., 2011; Ji et al., 2013; Rodiuc et al., 2014; Niu et al., 2016). The cortex and the pericycle cells surrounding the GCs divide and proliferate uncontrolled to disrupt xylem leading to the formation of a pseudo-organ called a gall that contains 5-7 GCs (Figure 1.5; Escobar et al., 2015).

In addition to these morphological changes, certain physiological changes also occur within the GCs including a marked amylase and invertase activity, starch accumulation, and suppression of different defence-related genes (Ibrahim, Hosseini et al., 2011). A microarray study of excised galls was conducted in *Arabidopsis* and tomato upon infection with *Meloidogyne incognita* and *Meloidogyne javanica* during different developmental stages (Jammes et al., 2005; Portillo et al., 2013). Most of the differential expressed genes were involved in metabolism showing that GCs act as strong sinks. Moreover, genes related to cell cycle, protein synthesis and DNA processing were highly up regulated in galls in comparison to uninfected roots (see Siddique and Grundler, 2015 for review).

The nematode feeds on this metabolically active site, and moults into a third-or fourth-stage juvenile to become an adult male or female. The female takes a pear shape, and the male becomes vermiform, leaving the root to mate sexually with a female in species such as *M. hapla*; however, most *Meloidogyne* species can reproduce asexually. After becoming sexually mature, the female secretes a gelatinous matrix from its vulva (Maggenti and Allen, 1960) that can make a pore through the surrounding gall tissue (Orion, 1987; Orion and Franck, 1990).The female can deposit her eggs in this gelatinous matrix, forming an egg mass that flows out through the pore into the rhizosphere. The gelatinous matrix also plays a role in protecting the egg mass from soil microbes because of its antimicrobial activity.

The most important and widespread species of economic importance are *M. incognita*, *M. hapla*, *M. javanica*, *M. arenaria* and *M. chitwoodii*. RKNs cause galls on damaged roots that are the primary symptoms of RKN infection below ground. The formation of such galls on roots can block the transport of water and nutrients to the rest of the plant, stunting growth, leading to chlorosis and reducing yield. As a result, it is estimated that majority of the PPN-related crop losses are alone caused by RKN (Abad et al., 2008).

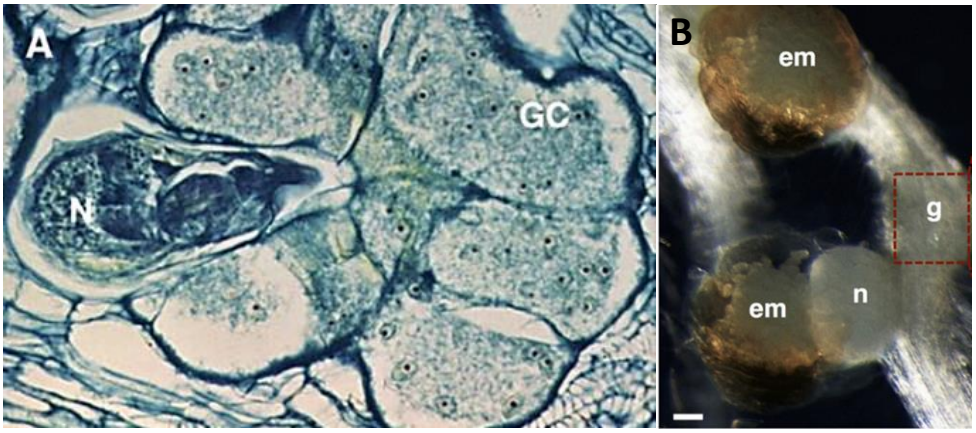


Figure 1.5: Morphology of giant cells and gall. A) Cross-section of the giant cell (GC) system initiated by a root-knot nematode, *M. incognita* (Davis and Mitchum, 2005). **B)** A gall induced in *Arabidopsis* root containing a mature female nematode, and associated gelatinous matrix filled with egg masses. em, egg masses; g, gall; n, nematode; Bars= 50µm (**Picture: Rodiuc et al., 2014**).

1.2.2 Cyst nematodes (*Heterodera and Globodera* spp.)

Cyst nematodes are sedentary biotrophic endoparasitic nematodes that cause extensive damage to many crops plants worldwide. They attack a wide range of crops in tropical, subtropical and temperate regions. There are around 60 known species of the genus *Heterodera* alone (Wouts and Baldwin, 1998). Cyst nematodes infesting potato (*Globodera pallida*), sugar beet (*Heterodera schachtii*), cereals (*Heterodera avenae* and *Heterodera filipjevi*) and soybean (*Heterodera glycines*) are of particular economic importance (Jones et al., 2013). An important charatersitic of this pest is able to survive in soil even in harsh environmental conditions when there is no suitable host around. The reason is that the eggs remain dormant and protected inside the hard body wall of the dead female called cyst for many years. This makes it difficult in eradicating this pathogen by implementing control strategies such as crop rotation (Fleming and Power, 1998).

Like RKNs, cyst nematodes also start their life cycle from an egg. When no host is available, the eggs contained inside the cyst remain dormant until the infective juveniles are stimulated to hatch in the presence of host-derived root exudates (Perry, 2002). The infective juvenile (J2) invades the root and migrates destructively and intracellularly through the living root tissue until it reaches the inner cortex to the vascular cylinder (Niblack et al., 2006). Upon reaching the

vascular cylinder, it carefully pierces its stylet into the host cell, and then waits for the cell response. If the protoplast collapses or the stylet becomes covered with a layer of callose like material, the stylet is retracted unless it finds a cell that does not respond adversely to J2 probing (Golinowski et al., 1997). Such a cell, usually a procambial or pericycle cell of the vascular cylinder becomes the initial syncytial cell (ISC) (Golinowski et al., 1997). The nematode then secretes certain effector proteins produced in the subventral glands (Wyss, 1992; Goellner et al., 2001) including enzymes to hydrolyse the polysaccharide components of the cell wall such as cellulose, hemi-cellulose and pectin producing local cell wall dissolution and fusion of adjacent plant cells (Bohlmann and Sobczak, 2014). This degradation of the plant cell wall during migration and its dissolution within syncytium is also achieved by plant's own cell wall degrading enzymes (CWDEs) such as expansins and cellulases within the feeding site (Baum et al., 2007; Goellner et al., 2001; Wieczoreck et al., 2006, 2008). These events produce a highly metabolically active syncytium (Hussey and Grundler, 1998) enriched with cellular organelles such as of mitochondria, ribosome, and smooth endoplasmic reticulum. The vacuole is broken in to small vacuoles (Golinowski, Grundler et al., 1996; Golinowski et al., 1997), and the cell wall around syncytium thickens with similar ingrowths as observed in GCs (Figure 1.7; Jones and Northcote, 1972).

Prompted by the occurrence of such dramatic morphological changes, some studies have examined the physiological changes within the syncytium. Microarray data of syncytia induced by *H. schachtii* in *Arabidopsis* and soybean roots by *H. glycines* showed differential expression of genes in comparison to uninfected roots, with many of these genes not even expressed in uninfected roots (Klink et al., 2007; Szakasits et al., 2009). The function of most of these differentially expressed genes relates to metabolic activities (Szakasits et al., 2009). A series of studies found an enrichment of sugars and amino acids in the syncytium as compared to uninfected root, indicating the importance of carbohydrates and amino acids for the development of *H. schachtii* (Hofmann et al., 2007; Siddique and Grundler, 2015). Moreover, certain studies have shown that the amount of starch content is much higher in the syncytium, something likely to support nematode development. This is confirmed by an evident decrease in susceptibility in plants whose starch biosynthesis has been impaired after infection with *H. schachtii* (Hoffmann et al., 2008)

After establishing the feeding site, the nematode remains associated to it for several weeks. During this time it molts into a third- and a fourth-stage juvenile and then it becomes an adult male or female (Figure 1.6). Females remain sessile and assume a lemon shape, whereas males become vermiform and then leave the root in search a female for mating. After mating, the female produces 200-300 eggs and then dies. Its outer body toughens and turn into a cyst containing the eggs in which the juveniles develop to the second, pre-infective stage. The eggs inside are protected by the tough protective sheet of the cyst against any environmental barriers in the soil. Root exudates of suitable host will trigger the juveniles to hatch; in their absence the juveniles can remain dormant as long as 20 years (Grainger, 1964).

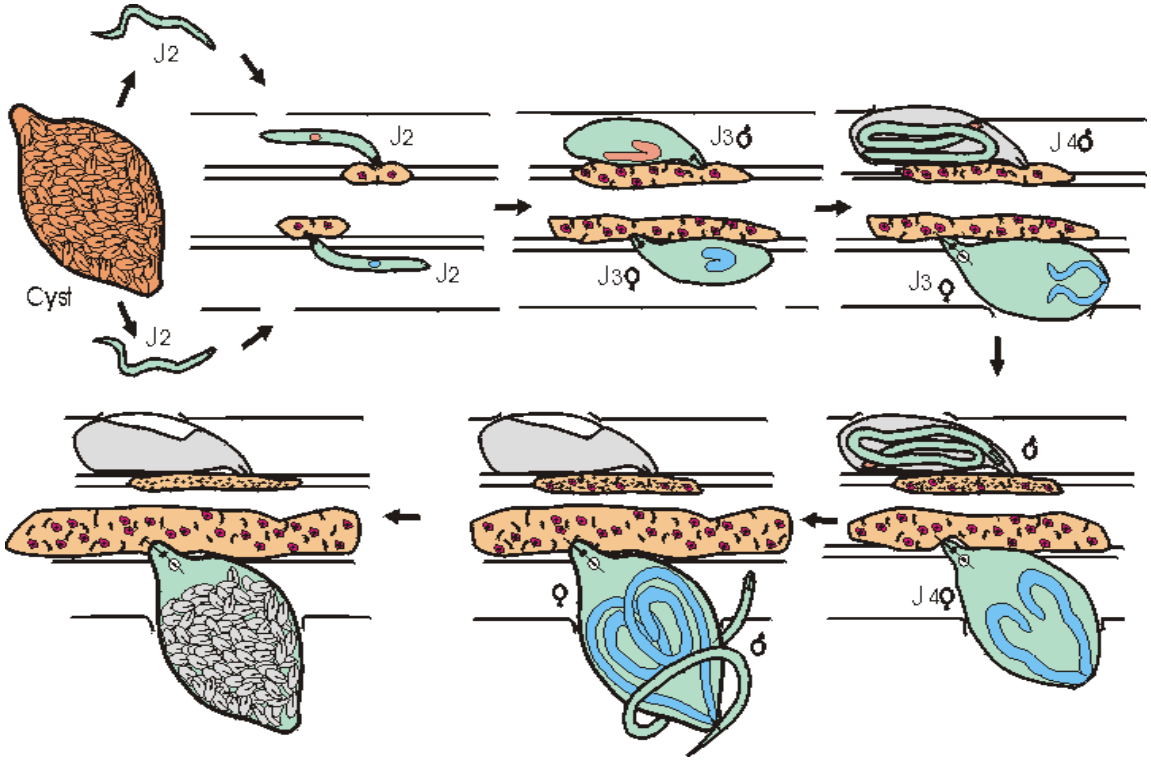


Figure 1.6: Life cycle of cyst nematode *Heterodera schachtii.* The infective juveniles (J2s) invade the roots and migrate destructively towards the vascular cylinder and establish initial syncytial cell. The nematode attached to the feeding site for several weeks, become sedentary and molts into a third- and a fourth-stage juvenile to become an adult male (vermiform) and female (pear shaped). The male leaves the root and search a female for mating. After fertilsation, the female dies and her body turns brown to become cyst with 200-300 eggs. The eggs hatch again in the presence of suitable host plant **(Drawing: Florian M. W. Grundler).**

In terms of damage to agriculture crops, cyst nematodes come second after root-knot nematodes. Yield losses caused by cyst nematodes are difficult to estimate because symptoms do not arise

until the infestation is severe (Atkinson, 1996). The annual crop losses worth USD 1.5 billion are alone caused by soybean cyst nematode in the United States (Chen et al., 2001). Potato cyst nematodes are responsible for 9% of the total potato production globally (Turner and Rowe, 2006).

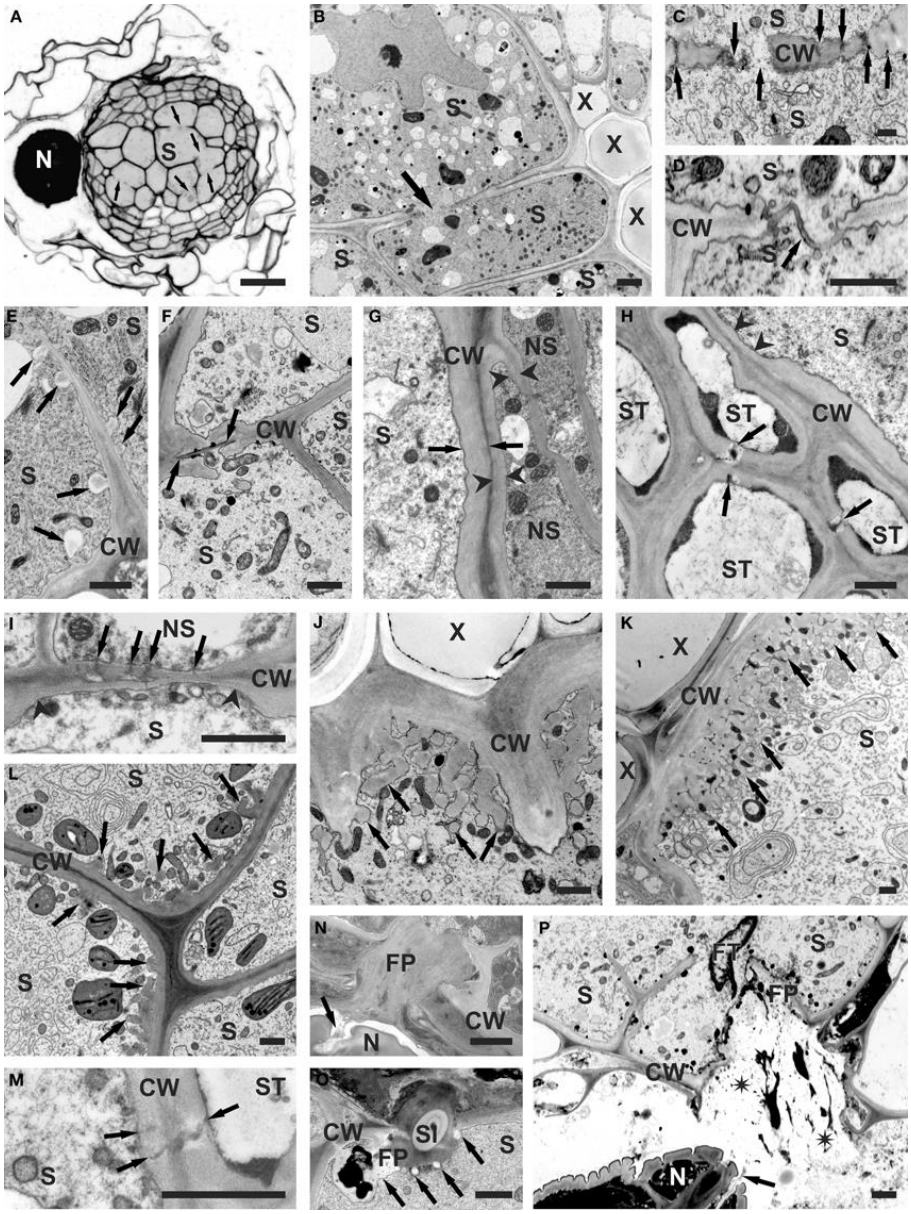


Fig 1.7: Morphological changes in *Heterodera schachtii* induced syncytia in *A. thaliana* roots. **A)** Anatomical structure of syncytium with arrows pointing to cell wall openings. **B)** Ultrastructure of syncytium. Arrow indicates cell wall opening. **C)** Openings of cell wall formed by widening of plasmodesmata (arrows). **D)** Cell wall dissolution leading to cell wall openings formed without involvement of plasmodesmata. Arrow indicates middle lamella

covered with plasmalemma. **E)** Paramural bodies (arrows) formed at extensively digested part of internal cell wall. **F)** Casparian stripe (between arrows) covered with newly deposited cell wall in untypical syncytium induced in the endodermis. **G)** Comparison of thickness of outer syncytial cell wall (between arrows) and cell wall of non-syncytial cells (between arrowheads). **H**) Thin part of outer syncytial cell wall (arrowheads) facing sieve tube. Arrows indicate plasmodesmata between sieve tubes. **I)** A group of plasmodesmata (arrows) at thin part of outer syncytial cell wall (between arrow heads) facing non-syncytial parenchymatous cell. **J)** Single cell wall ingrowths (arrows) formed at syncytial wall facing vessels. **K)** Well-developed system of cell wall ingrowths (arrows) formed at syncytial wall facing vessels. **L)** Unusual localization of poorly developed cell wall ingrowths (arrows) on wall between syncytial elements. **M)** Plasmodesmata (arrows) between syncytial element and sieve tube. **N)** Feeding plug in syncytial cell wall. Secretions emanating from nematode amphids are marked with arrow. **O)** Feeding plug with inserted cross-sectioned nematode stylet. Callose depositions are indicated with arrows. **P)** Broken feeding plug in syncytial wall. Spilled syncytial cytoplasm is marked with asterisks. Arrow points to amphidal secretions. Light microscopy **A)** and transmission electron microscopy micrograms **(B–P)** of syncytia at 2 **(E, O),** 5 **(A, B, D, F, N, P)**, 10 **(G, H, I, J, M)**, and 13 **(C, K, L)** days post inoculation. CW, cell wall; FP, feeding plug; FT, feeding tube; N, nematode; NS, non-syncytial cell; S, syncytium; Sl, stylet; ST, sieve tube; X, xylem vessel. Bars = 20 μm **(A**) and 1 μm **(B–P)** (Pictures: Bohlmann and Sobczak., 2014).

From the above discussion regarding RKN and cyst nematode, it is evident that both of these phytopathogens have a wide range of host and are not only different on the basis of their phylogeny but also with their infection strategies.

1.3 Plant defence responses

Because many pathogens, including nematodes, pose a serious threat, plants defend themselves from such biotic stress by activating a number of defence responses in case of infection. The pathogens, in their turn, try to manipulate the biology of such responses to achieve successful parasitism. However, even before activation of such defence responses, the pathogen must have overcome certain constitutive barriers such as wax layers, the cell wall and secondary metabolites (Reina-Pinto and Yephremov 2009; Ahuja et al., 2012; Bednarek, 2012). The cell wall, the first barrier to be overcome by plant pathogens, comprises of three layers: the middle lamella, primary layer and secondary layer. The middle lamella, the top most layer of the cell wall, connects the cells to form a strong structure. This layer is mostly composed of pectins, which give strength and flexibility to the plant cell. Pectins are polysaccharides whose structural

classes include homogalacturonan (HG) and rhamnogalacturonan I and II; xylogalacturonan (XGA) and apiogalacturonan (AGA) have also been found (Deng et al., 2006). The primary and secondary cell wall are composed of cellulose and hemicellulose (usually xyloglucan or arabinoxylan) embedded in a matrix of pectins. The secondary cell wall is similar in composition to primary cell wall, having an additional component, lignin, that is a complex network of phenolic compounds and that gives strength to the cell wall. The secondary wall can also protect plants from invading pathogens and microbes. To overcome these structural barriers for successful invasion, pathogens produce certain cell wall-degrading enzymes (CWDEs), including pectate lyases, endo-β-1, 4-glucanase, cellulases and polygalacturonase (PGs) (Vanholme et al., 2007; Goellner et al., 2001; Smant et al., 1998) for the degradation of its main components. PGs are well characterised in fungi and are released to break the alpha 1-4 linkage between the D- galacturonic acid residues of homogalacturonan (Bussink et al., 1992; Wubben et al., 1999; Kalunke et al., 2015). As a result of this activity, cell separation and maceration of host tissue take place, eliciting a number of inducible defence responses by the plant.

Plants are equipped with different kinds of constitutive and physical barriers to counteract a wide range of pathogens such as bacteria, fungus, viruses, insects and nematodes. Because pathogens sometimes overcome such constitutive barriers, plants have developed a system of inducible defence responses that vary from plant to plant. Some plants have strong recognition abilities against certain pathogens and activate immediate defence responses that prevent this pathogen from successfully colonising—these are termed resistance plants. Other plants, having weaker recognition abilities against certain pathogens, lack a timely response to pathogens that overcome such defence responses, which then colonise and damage the host plant. Such plants are called susceptible plants.

Plant-pathogen interactions is a two way communication such that plants must protect themselves from different kinds of pathogen attack by activating their defence systems, and the pathogens in return must overcome such defence responses by manipulating their cellular biology to carry out the successful parasitism that allow their growth and development. Such inducible defence responses are activated by sensing certain molecules from pathogens called elicitors first defined in the early 1970s (Keen, 1975). Elicitors were initially thought to be molecules responsible for the induction of phytoalexins, a secondary metabolite responsible for

defence. However, this term is now used for any molecule responsible for stimulating any kind of plant defence (Ebel and Cosio, 1994). Elicitors are categorised in two groups; general elicitors that are sensed as danger signals in both host and non-host plants to activate plant innate immunity (Nürnberger, 1999; Nürnberger and Brunner, 2002), whereas specific elicitors (effectors) produced and function in those plant species that carry the analogous resistant genes and absence of which can lead to disease (Hammond-Kosack and Jones, 1997; Luderer and Joosten, 2001; Nimchuk et al., 2001).

1.3.1 PTI and ETI responses in plants

General elicitors are either non-self from pathogens/microbes called Pathogen or Microbial associated molecular patterns (MAMPs/PAMPs) and are highly conserved across several pathogens species (Felix et al., 1993) or self-endogenous molecules from the host produced as a result of damage or wounding called damage associated molecular patterns (DAMPs) (Boller and Felix, 2009).

Both PAMPs and DAMPs are recognized by plasma membrane based-receptors first processed in endoplasmic reticulum before being transported to plasma membrane (Frescatada-Rosa et al., 2015). These receptors are also called Pattern recognition receptors (PRRs). These PRRs act as danger signals and activate the downstream defence systems of host plants (Boller and Felix, 2009; Zipfel, 2014) including activation of oxidative burst and mitogen-associated and calcium-dependent protein kinases (MAPKs and CDPKs), ethylene production, and modifications of the host cell wall leading to the restriction in growth and development of the invading pathogen (Figure 1.8; Asai et al., 2002; Boller and Felix, 2009; Holbein et al., 2016). PRRs are either receptor like kinases (RLKs) having an extracellular ligand binding domain, a single trans-membrane domain and an intracellular kinase domain or receptor-like proteins (RLPs), which lack an intracellular kinase domain (Boller and Felix, 2009; Schwessinger and Ronald, 2012; Wang et al., 2010) and require adopter molecules (Zipfel, 2008) for signal transduction. The extracellular ligand binding domain of PRRs contain either leucin rich repeats (LRRs) that are involved in the recognition of bacterial peptides such as flagellin and EF-Tu (Chinchilla et al., 2006, Zipfel et al., 2006), or lysine motifs (LysMs), lectin motifs, or epidermal growth factor (EGF) that specifically binds to carbohydrate-containing molecules, such as fungal chitin, bacterial peptidoglycans, extracellular ATP and oligogalacturonides (Kaku et al., 2006; Miya

et al., 2007; Brutus et al., 2010; Choi et al., 2014), the result of which is the activation of PAMP-triggered immunity (PTI). In the model plant *Arabidopsis thaliana*, well-studied PAMPs/MAMPs::PRRs recognitions include FLAGELLIN SENSING2 (*FLS2*), which specifically perceives bacterial PAMP flg22 and EF-Tu receptor (EFR), which binds directly to elf18 peptide (Zipfel et al., 2004; Chinchilla et al., 2006; Zipfel et al., 2006). Both these PRRs rapidly associate with another LRR-RLK called BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (*BAK1*) upon ligand perception (Roux et al., 2011; Sun et al., 2013). *BAK1* than phosphorylate the cytoplasmic BOTRYTIS-INDUCED KINASE 1 (*BIK1*) which in turn phosphorylate the RESPIRATORY BURST OXIDASE HOMOLG D (RBOHD) that results in downstream defence signalling pathways such as MAP kinase activation and gene expression (Asai et al., 2002; Li et al., 2002; Lu et al., 2010; Li et al., 2014). Loss of either receptor suppresses basal immunity to bacterial infection (Zipfel et al., 2004, 2006; Nekrasov et al., 2009; Saijo et al., 2009).

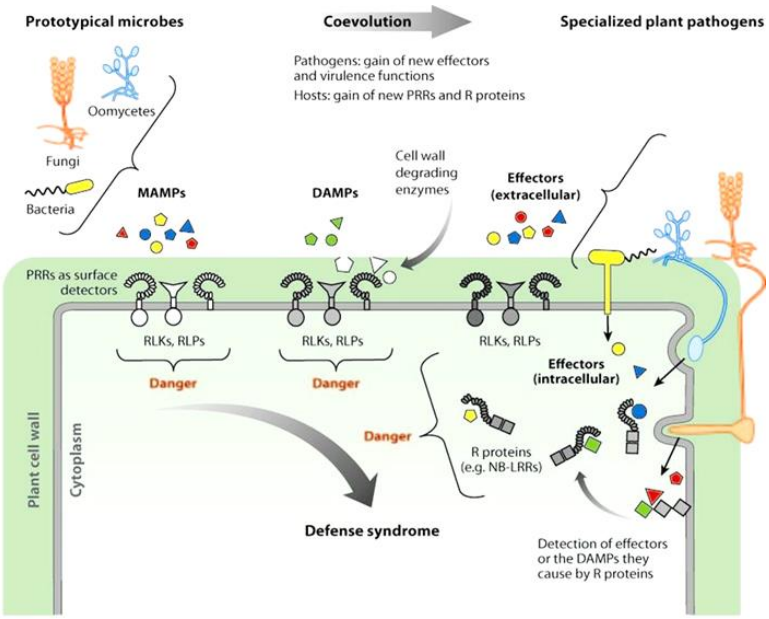


Fig. 1.8: General overview of PTI and ETI responses. Plants recognizing PAMPs, DAMPs and effectors as danger signal to activate innate immunity. These molecules are recognized through receptor like kinase (RLKs) or receptor like proteins (RLPs) in case of PAMPs/DAMPs or through R proteins in case of effectors. After recognition, stereotypical defence syndrome is activated by the plants (Boller and Felix, 2009).

Although PAMP- and DAMP-triggered immune responses are activated in response to different pathogens, details of such responses to nematode infection are largely unknown (Holbein et al., 2016). However, recent work by Manosalva et al. (2015) has revealed that plants may be able to recognise small conserved molecules called ascarosides from nematodes. Nevertheless, the PRR mediating the response to ascaroside remained unknown (Manosalva et al., 2015).

To suppress PTI responses, virulent pathogens secrete certain proteinaceous secretions (effectors) into the host plant cytoplasm to interfere with cellular functions, making the host more virulent (Presti et al., 2015). A number of nematode effectors have been reported to suppress PTI responses of their host plants. In addition to suppression of defence response, effectors play different roles in facilitating parasitism such as chemotaxis mediation, degradation of plant cell wall to facilitate penetration and migration of the invading nematode, and maintaining feeding site functions (Niu et al., 2016). The examples of effector proteins suppressing activation of defence include GrVAP1 from *Globodera rostochiensis* (Lozano-Torres et al., 2014), Ha-annexin from *Heterodera avenae* (Chen et al., 2015) and Hs30C02 from *Heterodera schachtii* (Hamamouch et al., 2012). Plants in turn have evolved a second line of defence that recognized specific effectors through plant receptor R proteins encoded by the R gene (Boller and Felix, 2009; Du et al., 2015; Jones and Dangl, 2006; Sarris et al., 2015). These R proteins are a member of the intracellular nucleotide binding (NB) leucine-rich repeat (LRR) protein family (Jones and Dangl 2006; Monaghan and Zipfel, 2012), sharing structural similarity with animal NOD-like receptor (Inohara and Nunez, 2003; Rairdan and Moffett, 2007). After recognition of specific effectors through the NB-LRR, plants activate a stronger defence responses termed as effector triggered immunity (ETI) (Dodds and Rathjen, 2012) that was formerly called gene-for-gene resistance (Flor, 1971).The activation of ETI may lead to a specific type of programmed cell death known as hypersensitive responses (HR) (Spoel and Dong, 2012). In well-known examples of ETI responses, two effectors, AvrB and AvrRpm1 from *Pseudomonas syringae* are perceived by plant immune receptor *RPM1*, prompting activation of ETI responses, including HR (Mackey et al., 2003). Plant-parasitic nematodes also secrete certain effectors that directly or indirectly interact with plant immune receptors R proteins to induce ETI. Several plant NB-LRR proteins have been identified in recent times that confer resistance to nematodes. For example, the potato cyst nematode *G. pallida* secretes effector protein RBP-1, which interacts with plant NB-LRR protein Gpa2 to elicit defence

responses including HR (Sacco et al., 2009). Similarly, *G. rostochiensis* effector SPRYSEC-19 has been shown to bind directly to SW5, a CC-NB-LRR disease resistance protein, and suppress activation of plant ETI responses (Postma et al., 2012). Although both PTI and ETI responses use their own distinct receptors, they share similar downstream defence signaling pathways (Figure 1.9; Tsuda et al., 2009). Because the present study focuses on PTI responses via DAMP perception in *Arabidopsis* against cyst and root-knot nematodes, I have discussed these in detail here.

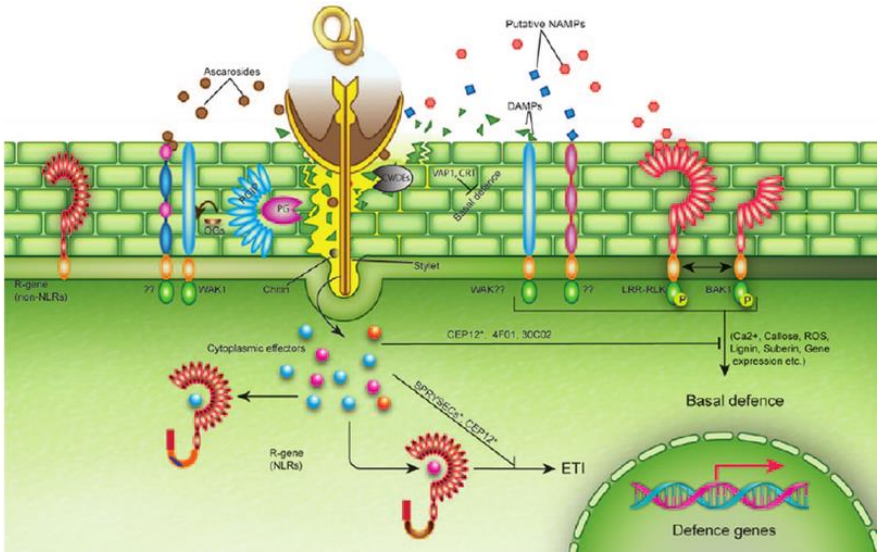


Fig 1.9: An overview of plant immune responses to nematodes. Nematode invasion damages plant cell wall by secreting certain CWDEs such as polygalaturonases (PGs). The plant inhibit the activity of PGs by PG inhibiting proteins (*PGIP*) that result in the production of oligogalacturonides (OGs) that are sensed by *WAK1* receptor to activate immune responses. Plants are also able to recognize nematodes through an unknown NAMPs receptor to activate PTI responses. In order to suppress PTI, nematodes secrete apoplastic (VAP1, Lozano-Torres et al., 2014; CRT, Jaouannet et al., 2013) and cytoplasmic (CEP12, Chronis et al., 2013; 4F01, Patel et al., 2010; 30C02; SPRYSECs, Rehman et al. , 2009) effectors that are counteract by several R-genes in the plant (Holbein et al., 2016).

1.3.2 DAMPs responses in plants

Multicellular organisms including plants some time suffer infection or wounding that must be healed to prevent further damage. Although plants are equipped with pre-existing physical barriers such as cell wall, cuticle, wax layer, and thick woody cover, attack by pests may cause substantial structural damage. Accordingly, plants have evolved sophisticated mechanisms that

enable them to recognise and respond to such damage or danger signals. Consequently, the wounding site is protected to block further entry of opportunistic pathogens. Such kind of responses may occur within very short time frames—within a few minutes to several hours of damage— and involve releasing, perceiving and transducing specific signals that ultimately activates wound related defence genes. To recognize such danger signals (DAMPs), plants may use surface-localised receptor like kinases (Shiu and Bleecker, 2001), which can activate downstream signaling pathways responsible for immunity. For PAMPs, the recognition events and downstream signalling are well studied and characterised (Zipfel, 2014) but not for DAMPs. Although a number of DAMPs and their receptors have been identified, the details of their signaling mechanisms have yet to be thoroughly investigated (Figure 2.1).

The first well-studied DAMP in *Arabidopsis* is the 23-amino acid peptide *AtPEP1* and its homologues *AtPEP2*-8 The *AtPEPs* are derived from their 92-amino acid cytosolic precursor proteins PROPEP1-8, respectively (Huffaker et al., 2007). These *AtPeps* have been shown to be induced upon wounding and are recognized by the LRR-RLKs Pep receptors (*PEPR1* and *PEPR2*) to activate robust PTI responses (Yamaguchi et al., 2006; Yamaguchi et al., 2010; Huffaker and Ryan, 2007; Bartels et al., 2013). In addition to *Arabidopsis*, *AtPEP* homologues have also been identified in crop species. One example, ZmPep1, is found in maize and is known to regulate defence responses against fungal infection, whereas ZmPep3 is known to trigger Jasmonic acid and ethylene production to induce gene expression involved in herbivore defence (Huffaker et al., 2011).

Extracellular ATP (eATP) is also a well-characterised DAMP signal in both plants and animals (Tanaka et al., 2014). Adenosine-5′-triphosphate (ATP) is a rich source of energy that aids cellular metabolism in all organisms and plays an important role in signal transduction (Tanaka et al., 2010). In animals, eATP plays a vital role in a number of cellular processes such as neurotransmission, cell growth, cell death, immune response and inflammation. Two plasma membrane purinoceptors have been identified to transduce these cellular processes: One is the P2X ligand-gated ion channels while the other one is P2Y G-protein-coupled receptor (Ralevic and Burnstock, 1998; Lustig et al., 1993; Abbracchio et al., 2006).

In plants, most of the ATP is maintained inside the cell (Beis and Newsholme, 1975) and can be released into the extracellular matrix on pathogen attack or wounding, either of which serves as a

danger signal (Song et al., 2006; Tanaka et al., 2014). Earlier studies in plants have shown that extracellular ATP contributes to plant growth and development (Tanaka et al., 2010), but a recent discovery that identified *DORN1* as a lectin receptor kinase of eATP (Choi et al., 2014) has confirmed that eATP also acts as a DAMPs signals in plants to induce PTI responses. In this study, loss-of- function *dorn1* plants showed reduced expression of defence genes in response to both ATP treatment and wounding. These results were reversed in lines overexpressing *DORN1* confirming that eATP plays a part in DAMP signaling upon wounding by binding to the extracellular lectin domain of *DORN1* receptor.

Other well-characterized DAMPs in Arabidopsis are oligogalacturonides (OGs) (Ferrari, 2013). OGs are produced as a result of fragmentation of homogalacturon (HGA) a main component of pectins degraded by microbial polygalacturonase enzyme (PGs) or endogenous PG of the plant upon mechanical wounding (Cervone et al., 1989; Bellincampi et al., 2014; Orozco-Cardenas and Ryan, 1999). To inhibit microbial PG activity, the plant cell wall deploys a defence protein called PG-inhibiting protein (*PGIP*), which prevents further degradation of pectins. This leads to the production of elicitor-active OGs that are oligomers of α-1, 4-linked galacturonic acid and that act as danger signals activating a number of defence responses (Benedetti et al., 2015) such as phytoalexins accumulation (Davis et al., 1986), callose deposition, ROS burst, (Bellincampi et al., 2000; Galletti et al., 2008), glucanase and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988) and nitric oxide (Rasul et al., 2012) to induce PTI responses. Recently wall-associated kinase (*WAK1*), an epidermal growth factor (EGF) similar to receptor kinase, has also been identified as a receptor for OGs (Brutus et al., 2010). Among five WAK genes, *WAK1* is the only one induced upon OG treatment and wounding (Denoux et al., 2008; Wagner and Kohorn, 2001). Moreover, when overexpressed in *Arabidopsis, WAK1* increases resistance to pathogens (Brutus et al., 2010). This shows that the trio of PG, PGIP and OG plays a significant role in plant innate immunity.

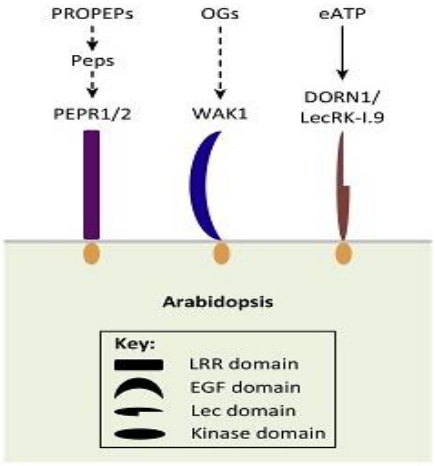


Fig. 2.1: DAMPs::PRRs pairs in *Arabidopsis*. Endogenous peptides (DAMPs) are produced as a result of cleavage of pro-peptides (PROPEPs) and are sensed by *Arabidopsis* leucine-rich repeat receptor kinases (LRR-RKs) *PEPR1* and *PEPR2*. Degradation of cell wall produces oligogalacturonides (OGs) that are sensed by epidermal growth factor (EGF)-like RK *WAK1* while extracellular ATPs are recognized by lectin-domain (Lec) RK DORN1/LecRK-I.9 to induce PTI responses. Arrows in block and dashes indicate direct and lack of direct binding proofs, respectively (Zipfel, 2014).

A fourth class of DAMPs has recently been identified in *Arabidopsis* (Choi et al., 2016). The High Mobility Group Box 1 (*HMGB1*) protein in mammals was the first DAMP to be identified and characterized followed by its related protein in *Arabidopsis AtHMGB3.* There are about 15 genes in *Arabidopsis* that encode HMG box domain-containing proteins and they are subdivided in to four groups. (i) HMGB-type proteins, (ii) A/T-rich interaction domain (ARID)-HMG proteins, (iii) 3xHMG proteins that contain three HMG boxes (iv) The structure-specific recognition protein 1 (SSRP1) (Merkle and Grasser, 2011). In a recent study, Choi et al. (2016) found that extracellular *AtHMGB3* also act as DAMP in plant and can induce similar defence responses as Pep1. However, plasma membrane based receptors that detect and transduce responses to *AtHMGB3* are yet to be discovered.

1.3.3 Role of PG, PGIP and OG in plant-pathogen interactions

Plant pathogens are generally classified in two classes according to their lifestyle: necrotrophs and biotrophs. Necrotrophic pathogens can infect plants by using certain CWDEs and toxins that kill the host and feed on the remains of dead tissue (Stone, 2001). On the other hand, biotrophic pathogens do not kill the host, but rather penetrate the host cell wall to get nutrients from the

living tissue using a specialised feeding structure and manipulating host defence responses without disturbing the cell membrane (Schulze-Lefert and Panstruga, 2003; Mendgen and Hahn, 2004).

Because cell wall is the first barrier encountered by pathogens during the early phases of infection, they secrete certain CWDEs such as pectinases to degrade cell wall components (Jones et al., 1972; Mankarios and Friend, 1980). Among pectinases, PGs are the first enzymes to be secreted by certain biotrophic and necrotrophic pathogens such fungi, bacteria, and insects as a way of hydrolysing homogalacturonan in the plant cell wall to facilitate parasitism (De Lorenzo and Ferrari, 2002; Girard and Jouanin, 1999; Holbein et al., 2016). Amongst the fungi that are known to secrete PGs are *Aspergillus flavus* (Whitehead et al., 1995; Shieh et al., 1997), *Botrytis cinerea* (Favaron et al., 1992; ten Have et al., 1998), *Aspergillus niger* (Maldonado and de Saad, 1998; van Santen et al., 1999) *Alternaria citri* (Isshiki et al., 2001) *Claviceps purpurea* (Oeser et al., 2002) and *Sclerotinia sclerotiorum* (Favaron and Marciano 1992; Li et al., 2004). A number of bacteria also produce PG including *Agrobacterium tumefacians* (Rodriguez-Palenzuela et al., 1991), *Ralstonia solanacearum* (Huang and Allen, 2000) and *Bacillus polymyxa* (Nagel and Vaughn, 1961). Similarly, some species of insects that feed on plants produce PGs in their salivary glands, thereby causing considerable damage to plants (Laurema et al., 1985; Strong and Kruitwagen, 1968; Girard and Jouanin, 1999; Boyd et al., 2002; Frati et al., 2006; Celorio-Mancera et al., 2008).

Recent studies on the role of PGs as a virulence factor have been concluded through gene knockout and cloning strategies. Elimination of the *Bcpg1* gene from *Botrytis cinerea* via partial gene replacement reduced virulence on tomato leaves and apple (ten have et al., 1998). Another study revealed that bacterial wilt pathogen *Ralstonia solanacearum* also needs PGs for successful pathogenesis. Mutant varieties lacking PehA (an endo-PG) show a slow colonisation on egg plants, and bacterial populations observed in the stems were much lower in comparison to the parent strain (Hung and Allen 2000).

To tackle the cell wall degrading activity of microbial PGs, plant cell wall features LRR among which *PGIP* is deployed in the cell wall during early infection for successful inhibition of secreted PGs (De Lorenzo, 2001; Kalunke et al., 2015). *PGIP* is an important family of defence proteins whose members are present in variable numbers, from a small family of two genes in

Arabidopsis (Ferrari et al., 2003) to 16 in *Brassica napus* (Hegedus et al., 2008). In the model plant *Arabidopsis thaliana, AtPGIP1* (At5g06860) and *AtPGIP2* (At5g06870) are positioned on chromosome 5 with a similarity of 76.1% at the amino acid-level and separated by a short stretch of introns of 69 and 83 bp, respectively (Ferrari et al., 2003). Transgenic plants totally lacking *PGIP* activity has not been characterized so far (Kalunke et al., 2015). The crystal structure of *PGIPs* shows a central LRR with 10 imperfect repeating units, each derived from 24 amino acid residues. Most LRR proteins have one β-sheet connected with a helix on the convex side or β-turns, but in *PGIP*, it is organised to form two β-sheets, one of which (sheet B1) occupies the concave inner side of the molecule and contains amino acid residues critical for the interactions with PGs (Di Matteo et al., 2003). This PG-PGIP interaction not only inhibits the cell wall-degrading ability of PGs but also produces elicitor-active oligogalacturonides (OGs) (Figure 2.2; Dixon and Lamb, 1990) with a degree of polymerisation ranging 10-15 (Côté and Hahn, 1994) to activate defence responses.

PGIP genes are not only induced upon infection with pathogens but also with mechanical wounding (Bergmann et al., 1994; Li and Smigocki, 2016). Many studies have examined the role of PGIPs in plant defence against different pathogens. Transgenic *Arabidopsis* plants overexpressing *AtPGIP1* and *AtPGIP2* increase resistance against necrotrophic fungi and decrease disease symptoms. Similarly, the expression of *AtPGIP2* is regulated through jasmonate and needs both *COI1* and *JAR1*. Conversely, the transcript level of *AtPGIP1* is strongly induced by exogenous application of oligogalacturonides but is independent of salicylic acid, jasmonate, or ethylene signalling pathways (Ferrari et al., 2003). Also, transgenic plants expressing an antisense of *PGIP1* in *Arabidopsis* show enhanced susceptibility to *Botrytis cinerea* (Ferrari et al., 2006). The significance of the *PGIP* gene in plant defence has also been reported in transgenic crops. The first successful study reported that a transgenic tomato plant overexpressing pear PGIP (*PcPGIP*) increases the inhibitory activity against *Botrytis cinerea* PG, reducing lesion development (Powel et al., 2000). Similarly, transgenic tobacco and *Arabidopsis* plants overexpressing bean PGIP2 (*PvPGIP2*) have shown similar results against *Botrytis cinerea* infection (Manfredini et al., 2005). A recent study showed that virus induced gene silencing of *GhPGIP1* in cotton enhances susceptibility when challenged with two fungal strains, *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. Similarly overexpressing *GhPGIP1* in transgenic *Arabidopsis* plants conferred resistance to both the

pathogens (Liu et al., 2017). In addition to response to fungi, *PGIP* also play a role in defence against bacteria. A recent study shows the role of *OsPGIP4* playing a role in conferring resistance to bacterial leaf streak in rice (Feng et al., 2016). These studies show that *PGIP* plays a significant role in plant innate immunity. However, information regarding the role of PG, PGIP and OG in plant-nematode interactions is still limited.

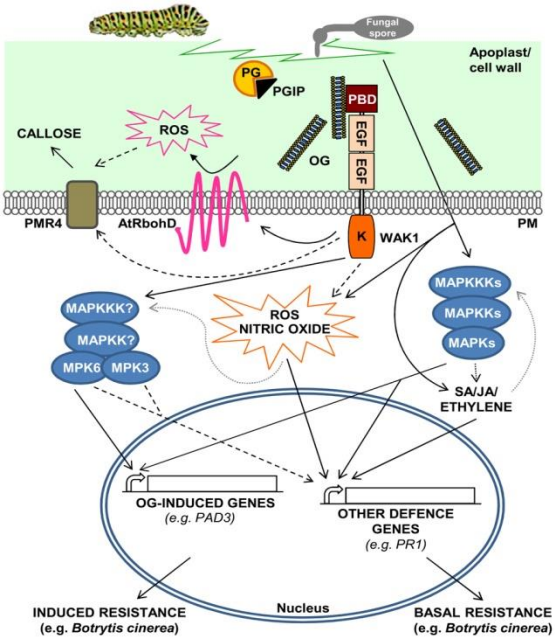


Fig. 2.2: A model of PG, PGIP and OG in plant-pathogen interactions. Fungus secretes cell wall degrading enzymes such as polygalacturonases (PGs) to hydrolyse cell wall components. The plant in return inhibit the activity of PGs by PG inhibiting proteins (*PGIP*) that leads to the accumulation of elicitor active oligogalacturonides (OGs) which are sensed by *WAK 1* receptor to activate immune responses such as ROS burst, MAP kinase activation, callose deposition and nitric oxide etc. (Ferrari et al., 2013).

1.4 Role of PG, PGIP and OG in plant-nematode interactions

In addition to fungi, bacteria and insects, PGs are also secreted by nematodes. The first PG of animal origin isolated from *Meloidogyne incognita*, has been suggested to be secreted into the host plant implicating a role in parasitism (Jaubert et al., 2002). A recent transcriptomic data of pre-infective juvenile of the cyst nematode *Heterodera schachtii* also identified a PG (Fosu-Nyarko et al., 2016). Apart from the role of *PGIP* and its inhibitory activity of PGs, there are also some studies about the role of *PGIP* in defence other than the classical PG-PGIP interactions. In one study Veronica et al (2011) concluded differential expression pattern of

PsPGIP1 between susceptible and resistant pea genotypes against cyst nematode *Heterodera goettingiana* infection. In addition to this, *in situ* hybridization confirms that *PsPGIP1* was localized specifically in syncytia in the resistant genotype suggesting the role of this gene in counteracting the successful establishment of syncytium in host root. Also loss-of-function mutant of *Atpgip1* has prolonged seed germination by prompting pectin breakdown in the seed coat indicating a different role of PGIP genes in plants other than its inhibitory activity (Kanai et al., 2010).

Due to lack of available information about the role of PG, PGIP and OG in plant-nematode interactions and whether OGs are produced as a result of nematode infection to activate defence responses are indeed interesting questions to be answered. In order to address such questions, I focussed my research on the following objectives using *Arabidopsis thaliana* as model plant.

As *PGIP* plays a role against different pathogens and has a well-defined function against a number of fungal strains; however there are not many detail studies about the role of PGIPs against nematodes. My first objective was to characterize the role of *PGIPs* in plant defence against root-knot nematode *Meloidogyne incognita* and cyst nematode *Heterodera schachtii* using *Arabidopsis* as a model organism. In order to achieve this objective I used different molecular tools including screening loss-of-function *Arabidopsis pgip* mutants against the infection of plant parasitic nematodes *Heterodera schachtii* and *Meloidogyne incognita*. I also produced transgenic plants expressing *promoterPGIP::GUS* to investigate the spatio-temporal expression of *PGIPs* during nematode infection.

OGs are well characterized DAMPs in plant innate immunity. However, it is not known whether OGs play a role in DAMP triggered immunity against nematodes and whether they are produced upon nematode infection acting as danger signals and activate basal defence responses like other plant pathogenic-interactions. Therefore, my second objective was to investigate the role of OGs upon nematode infection.

PGs are secreted by a number of pathogens including nematodes to degrade cell wall and facilitate successful infection. However, the role of cyst nematode-derived PGs as pathogenicity factors are not clear. My third objective was to characterize the role of PGs from cyst nematode *Heterodera schachtii*.

2. Material and Methods

2.1 Plant material

Arabidopsis thaliana T-DNA insertion mutants in Col-0 background were ordered from Nottingham Arabidopsis stock center (NASC). The details about the seeds stock are given in Table 6.

2.2 Preparation of medium

The chemical composition and protocols used for the preparation of different media in this study are described as follows:

2.2.1 Preparation of Knop medium

Plants were grown in Knop medium for all experiments except for *M. incognita* infection assays as described previously (Sijmons et al., 1991). The chemicals and composition of the Knop medium is described in Table 2 and Table 3.

Table 2: Chemicals used for the preparation of Knop medium

Stock solution	Chemical	[g L^{-1}]
Stock solution I	KNO_3	121.32 g L^{-1}
	$MgSO_4$ $7H_2O$	19.71 g L^{-1}
Stock solution II	$Ca(NO_3)_2$ 4 H_2O	120 g L^{-1}
Stock solution III	KH_2PO_4	27.22 g L^{-1}
Stock solution IV	FeNaEDTA	7.34 g L^{-1}
Stock solution V	H_3BO_3	2.86 g L^{-1}
	$MnCl_2$	1.81 g L^{-1}
	$CuSO_4$ $5H_2O$	0.073 g L^{-1}
	$ZnSO_4$ $7H_2O$	0.36 g L^{-1}
	$CaCl_2$ $6H_2O$	0.03 g L^{-1}
	H_2MoO_4	0.052 g L^{-1}
	NaCl	2 gL^{-1}

Table 3: Composition of Knop medium litre^{-1}

Composition	Quantity
Sucrose	20g L^{-1}
Daichin Agar	8g L^{-1}
B5 Vitamins (added after autoclaved)	1 mL L^{-1}
Stock solution I	2 mL L^{-1}
Stock solution II	2 mL L^{-1}
Stock solution III	2 mL L^{-1}
Stock solution IV	0.4 mL L^{-1}
Stock solution V	0.2 mL L^{-1}

Double distilled water (ddH_2O) was used to adjust the volume to 1 litre while the pH was adjusted to 6.4 with KOH. The medium was autoclaved to avoid any contamination. After autoclaving, the medium was cooled down to 60^0C. Vitamin B5 was added to the medium, which was poured into the Petri dish plates. For selection of transformed plants, 25mg mL^{-1} of hygromycin was added to the media before pouring.

2.2.2 Preparation of MS medium

Murashige and Skoog medium was used for the infections assays of *M. incognita* (Murashige & Skoog, 1962). The chemical composition for MS medium is shown in Table 4.

Table 4: Preparation of Murashige & Skoog medium

Chemicals	Quantity (g L^{-1})
MS salts including Vitamins and MES buffer	4.7 g L^{-1}
Sucrose	20 g L^{-1}
Gelrite agar	5 g L^{-1}
ddH_2O	1L

Gelrite agar was added after adjusting the pH to 5.7. The media was then autoclaved and poured into the petri dish plates.

2.2.3 Preparation of LB medium

Lauria-Broth (LB) (Bertani, 1951) liquid or solid medium was prepared for growing *E. coli* at 37 ^{0}C. Specific antibiotics with appropriate concentration (50µg mL^{-1} Kanamycin, 10µg mL^{-1} Gentamycin and 100µg mL^{-1} Rifampicin) were added to the media for the selection of transformed bacteria. The composition for the preparation of LB medium is shown in Table 5.

Table 5: Preparation Lauria-Broth (LB) medium (Solid)

Ingredients	Quantity (g L^{-1})
Trypton	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	10 g L^{-1}
Agar	15 g L^{-1}

After mixing the ingredients, 1N NaOH was used to adjust the pH to 7.0. After autoclaving, appropriate antibiotics were added to the LB medium, which was then stored at 4^0 C till further use.

2.2.4 Preparation of YEB medium

Agrobacterium tumefaciens of the strain GV3101 were grown in YEB liquid or solid media supplemented with appropriate antibiotics (Rifampacin and Gentamycin) for 2 days at 28^0 C. The ingredients used for the preparation of YEP media is shown in Table 6.

Table 6: Preparation of YEP medium

Ingredients	Quantity (g L^{-1})
Peptone	10 g L^{-1}
Yeast extract	10 g L^{-1}
NaCl	5 g L^{-1}
Agar	15 g L^{-1}

After mixing the above ingredients, pH was adjusted to 7.0 with 1N NaOH. After autoclaving, appropriate antibiotics were added to the YEB media and stored at 4^0 C.

2.3 Sterilization of Seeds

To avoid any contamination, all transgenic lines were sterilized using 0.7 % sodium hypochloride (NaOCl) for 3 minutes followed by 3-times washing with autoclaved ddH_2O. After washing, seeds were spread on a sterile filter paper in a Petri dish and dried in the sterile bench. After drying the seeds, they were stored at 4^0C for further use.

2.4 Genotyping and Expression check

To confirm wild-type or mutant allele, Salk mutant lines were genotyped by designing genotyping primers through Salk T-DNA primer express (http://signal.salk.edu/tdnaprimers.2.htmL). For GABI-KAT lines, sterilized seeds were grown in Knop media supplemented with 50 mg mL^{-1}antibiotic Sulfadiazine. The lines that survived and grew on this selection media with 100% germination were considered homozygous. Homozygous lines were grown to collect seeds for further experiments while the heterozygous lines with less than 100% survival were discarded. A list of mutant lines with details is given in Table 7.

To check the expression of the target gene in the mutant lines, RNA was extracted from 12 days old mutant and Col-0 plants grown on Knop medium according to the protocol given in below section (c.f. 2.5 RNA extraction). RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosynthesis, Darmstadt, Germany) following manufacturer´ s protocol given below (c.f. 2.6. cDNA preparation).

Table 7: *Arabidopsis* insertion lines details.

Stock Name	Stock centre code	Locus	Insertion site	Gene/mutant name
SALK_001662.33.10.x	N683772	At5g06860	3’ UTR	PGIP1 (*pgip1-1*)
GK-092G09-012001	N665467	At5g06860	Exon	PGIP1 (*pgip1-2*)
GK-717A02-025309	N468738	At5g06870	Exon	PGIP2 (*pgip2-1*)

Arabidopsis Information Resources (TAIR) was used to obtain the sequences of the *PGIP* genes used in this study. Forward and reverse primers for each gene were designed using PRIMER3 software and were ordered from Invitrogen (Life technologies ^{TM}) which are listed in Table 11, 12, 13, 14. After confirming the absence of the transcript (c.f. 2.7 RT-PCR), mutant lines were subjected to *Heterodera schachtii* and *Meloidogyne incognita* infection to have an insight about the role of these genes against these pathogens.

2.5 RNA extraction

RNA extraction for each experiment was performed using RNeasy Plant Mini Kit (Qiagen), following manufacturer´ s protocol. RNA was further digested with DNase1 using DNA-*free*™ DNA Removal Kit (Ambion) following manufacturer´ s protocol. RNA sample was eluted in 30µL of RNase free H_2O. NanoDrop2000 Spectrophotometer (Thermo Scientific Inc., USA) was used to measure RNA concentration and stored at -80^oC.

2.6 cDNA preparation

For cDNA synthesis, High Capacity cDNA Reverse Transcription Kit (Applied Biosynthesis, Darmstadt, Germany) was used following manufacturer´ s protocol as shown in Table 8. The cDNA synthesis was carried out on a C1000 PCR thermocycler (Bio-Rad, Munich, Germany) using the protocol shown on Table 9. Bio-Rad CFX Manager 2.1 Software was used for constructing and running the protocol.

Table 8: Reverse transcription mix for generating cDNA template

Chemicals	**Quantity**
Nuclease free H2O	3.2 µL
10X Reverse Transcription Buffer	2 µL
10X Random Primer	2 µL
25X dNTPs (100mM)	0.8 µL
MultiScribeTM Reverse Transcriptase	1 µL
RNasein	1 µL
RNA	10 µL
TOTAL	**20µL**

Table 9: Thermo cycler conditions for cDNA synthesis

	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Temperature	25 ºC	37 ºC	85 ºC	4 ºC
Duration (min)	10	120	5	∞

2.7 Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to visualize the expression of genes for various experiments. Primers for target gene were designed manually or through software Primer 3 (http://primer3.ut.ee/) and ordered from Invitrogen™ Life technologies. A PCR mix of 25µL for each reaction was prepared by mixing buffer, dNTPs, Taq Polymerase, RNase free water, cDNA and specific primer for each gene (Table 10). The PCR reaction was set up depending on the required experiment: An initial denaturation for 5 minutes, followed by 40 cycles of denaturation at 95^{o}C for 30 seconds. Primer annealing lasted for 30 seconds at 52^{o}C and elongation/extension at 72^{o}C and it was terminated by a longer extension step at 72^{o}C for 5 minutes.

Table 10: RT-PCR reaction mixture for expression analysis

Chemicals	Quantity
Nuclease free H2O	17.4 µL
25X dNTPs (100mM)	0.5 µL
Taq Polymerase	0.1µL
Forward Primer	0.5 µL
Reverse primer	0.5 µL
cDNA	1 µL
TOTAL	25µL

After completion of the PCR run, DNA electrophoresis was used to separate DNA fragments by size. To achieve this, 1% agarose gel was prepared by mixing 1g of Agarose with 100mL 1X TAE buffer. After heating for 2-3 minutes in an oven, the mixture was allowed to cool down and 4µL of PeqGreen was added to it before pouring it slowly in a gel tray. After polymerization, the

comb was removed and the gel tray was placed in a gel tank supplemented with 1X TAE buffer. Samples were loaded along with DNA ladder and were allowed to run at 80 V for 60 minutes. Afterwards, the DNA bands were visualized with UV light in a gel documentation chamber.

2.8 Nematode infection assays

To screen loss-of-function mutants and transgenic plants, infection assays were carried on with both cyst nematode (*Heterodera schachtii*), and RKN (*Meloidogyne incognita*). The infection assays procedure with both the nematodes is described as follow:

2.8.1 Infection assay procedure with *Heterodera schachtii*

For inoculation with *Heterodera schachtii*, cysts were collected from mustard stock culture grown *in vitro* on Knop medium under sterile bench. Around 200-300 cysts were picked with a forcep and were collected in a Baermann funnel containing 3mM sterile $ZnCl_2$ that helps to stimulate the hatching of the pre-infective juveniles (J2). After 5-7 days, the J2s passed through the 100µm sieve and collected in a pipe closed with a clip. After collecting enough J2s, sterilization procedure was carried out to avoid any contamination during the infection process. For this purpose, an 11-µm sieve was used in which the J2s were collected under the sterile bench. 0.05% $HgCl_2$ was then used to surface sterile the nematodes for 3 minutes followed by 3X washing with autoclaved distal water for 3 minutes each. After sterilization, 70-80 J2s plant^{-1} were used to inoculate 12-days-old plants grown in Knop medium. After 12-14 days post inoculation (dpi), the following parameters were recorded.

2.8.1.1 Average number of nematodes plant^{-1}

Average number of males and females nematodes plant^{-1} were counted under the binocular. The females were marked as dots while the male were marked as cross on the Petri dish. Two plants per Petri dish and 20 plants per ecotype were used in each experiment. At least 5-15 female plant^{-1}were observed under optimal inoculation.

2.8.1.2 Average syncytium and female sizes

At 14 dpi, at least 30 infection sites containing syncytia and associated female sizes were photographed using a stereo microscope (LEICA M165 C; Leica Microsystems Ltd,

Germany).The area of female and syncytium was measured using outline tool of LAS software (Leica Microsystems).

2.9 Infection assay procedure with *Meloidogyne incognita*

2.9.1 Harvesting of eggs

To carry out infection assay with *Meloidogyne incognita*, we first harvested their eggs from 3-months-old tomato plants grown in soil. The roots containing galls were washed with water and cut in to smaller pieces with the help of a scissor. Afterwards, the root pieces were grinded for 30 s in 1 litre blender jar containing 1.5 % Sodium hypochlorite followed by washing with water. To collect eggs, four sieves with different pore sizes were used starting from 250 µm on the top followed by sieves of 150 µm, 50 µm, and 25 µm respectively. The water containing eggs were poured on the top sieve and washed several times. The dirt and root pieces remained in the bigger size sieves while clean eggs were collected in the bottom sieve of 25 µm. These eggs were collected in a 50mL tube for further purification. The collected eggs were centrifuged at 3000 rpm for 1 minute. The supernatant was removed and the egg-pellet was washed with 35% sucrose solution followed by a careful suspension in 1.0-1.5 cm tap H_2O. The suspension was again spinned down at 2000 rpm for 3 minutes leading to floating of the eggs at the interface between sucrose and water. The eggs were collected with a glass Pasteur pipette and were transferred to a 25 µm sieve. Washings through water removed the sugar immediately as sugar can damage the eggs (Schaik, 2011).

Purification of eggs was followed by their surface sterilization. For this, the eggs were incubated in 10% sodium hypochlorite for 3 minutes followed by 3X washings with autoclaved water to remove any residual sodium hypochlorite from the eggs. The eggs were then collected in a 50mL falcon tube and 2mL of 22.5mg mL^{-1} gentamycin sulfate and 150 µL of nystatin was added to it. The volume was adjusted to 30mL with sterile water and poured into an autoclaved-hatching chamber sealed with parafilm and stored at 27 ^{0}C in the dark.

After 3-5 days of incubation, the hatched J2s were collected in a 2mL tube and spinned down at 8000 rpm for 45 seconds. The supernatant was removed and 1 mL of 0.5% (w/v) streptomycin-penicillin solution was added. After re-suspension for 20 minutes, the J2s were spinned down at 8000 rpm for 45 seconds. The supernatant was carefully removed and nematodes were

incubated in 0.1% (w/v) ampicillin-gentamycin solution for 20 min. After incubation, nematodes were spinned down at 8000 rpm for 45 sec. Afterwards, they were washed with sterile tap water for 5 minutes followed by centrifugation at 8000 rpm for 45 seconds. A final incubation was performed with 0.1% (v/v) chlorhexidine solution for 3 minutes followed by 3X washing with autoclaved tap water. These surface-sterile nematodes were then used for inoculating the12-days-old *Arabidopsis* plants grown in MS medium with gelrite and put it in dark.

2.9.2 Average gall number and average gall size measurements

After 21 dpi, average numbers of galls were counted under a dissecting microscope binocular. After recording gall number, galls were photographed using a stereomicroscope (LEICA M165 C; Leica Microsystems Ltd, Germany). Average gall sizes were measured by using outline tool of LAS software (Leica Microsystems). Approximately 30 galls were outlined and measured for each experiment.

2.10 Nematode-infected root collection

Both Col-0 and loss-of -function mutants were grown in Knop medium with a 16h light and 8h dark cycle at 25°C. 12-days old plants were infected with 90-100 sterilized J2s of *H. schachtii*. At 10 hours post inoculation (hpi), small root segments surrounding nematode head (0.5 cm) were cut with a forcep and shock frozen in liquid nitrogen. Similarly for sample collection at 10dpi, syncytia associated with female nematodes were cut with a forcep and shock frozen in liquid nitrogen. RNA extraction and cDNA synthesis was performed as described above.

2.11 Quantitative real-time (qRT) PCR

For quantification of gene expression analysis, qPCR was performed using a MicroAmp® fast optical 96 well reaction plate (Applied Biosystems), with an ABI StepOnePlus^{TM}Real Time PCR System (Applied Biosystems, Germany). A total master mix of 20µL was used in each well containing 10 µL SYBR Green (Invitrogen), 0.5 µL 10 nM primers, 8.5µL ddH_2O and 1 µL of cDNA template. Following PCR conditions were used in 40 cycles: First cycle of 95°C for 10 min, followed by 40 cycles with each cycle 95°C for 15 s, 60°C for 60 s. *UBQ5* and *β-tublin* were used as an internal control for each experiment. Difference in gene expression was calculated using Pfaffl's method (Pfaffl, 2001).

2.12 Extraction of Genomic DNA from *Arabidopsis*

For genomic DNA extraction, 12-days-old *Arabidopsis* plants grown on Knop medium were harvested and frozen in liquid nitrogen. The leaf samples were grounded to a fine powder with the help of Precellys homogenizer. To the powder, 0.5 mL of cetyltrimethylammonium bromide (CTAB) buffer was added and incubated for 15 minutes at 55°C. After incubation, samples were spinned down at 12000 rpm for 5min. The supernatant was shifted to a fresh tube followed by the adding 250 µL of Chloroform: Iso Amyl Alcohol (24:1) and mixed by inversion. After this step, the tube was spinned down at 13000 rpm for 1 min to get two phases in the tube. The upper aqueous phase was transferred to a clean tube to which 50 µL of 7.5 M Ammonium Acetate was added followed by 500 µL of ice cold absolute ethanol. For DNA precipitation, samples were incubated for 1 hr at -20 ^{0}C. After precipitation, the samples were centrifuged to get the pellet DNA. Remove the supernatant and washed the DNA pellet twice with 500µL of 70 % ethanol. After washing, spin down the samples at 13000 rpm for 1 min. After removing the supernatant, DNA was allowed to dry for 15 minutes. Finally, the DNA was dissolved in 40-50 µL sterile DNase free water.

CTAB Buffer for Isolation of Genomic DNA

2.0 g CTAB (Hexadecyltrimethyl-ammonium bromide)

10.0 mL 1 M Tris pH 8.0

4.0 mL 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di- sodium salt)

28.0 mL 5 M NaCl 40.0 mL H2O

1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidinehomopolymer) Mw 40,000)

Adjust all to pH 5.0 with HCL and make up to 100 mL with H_2O.

2.13 Gateway cloning

A Gateway cloning system was used to generate all transgenic plants used in this study. The cloning procedure was carried out with a Gateway Cloning Kit from Thermo Fisher Scientific (Inc., Massachusetts (US). This technique comprised of performing a BP reaction and an LR

reaction using BP clonase and LR clonase enzymes respectively. In BP reaction, an entry clone was developed between an attB-flanked target DNA fragment and an attP-containing donor vector. The donor vector used to clone the amplified PCR product was pDONR207 mixed with a BP clonase enzyme according to manufacturer's protocol. The reaction mixture was incubated for 3-4 hours at 25^0C followed by the addition of 1µL of proteinase K and incubation for 10 min at 37 ^{0}C. The mixture was then used to transform competent cells of an *E. coli* strain DH5α. Heat shock for 50s at 42^0C was used for transformation. Transformed cells were grown in a YEB medium at 37^0C for 1.5 hours. Afterwards, they were streaked on LB plates containing 10µg mL^{-1} gentamycin and were let grew overnight at 37^0C. The positive colonies were screened via colony PCR. Afterwards, positive colonies were grown overnight in an LB medium with gentamycin from which plasmid was extracted following day with a Plasmid Extraction Kit (Macherey-Nagel, Düren, Germany). Positive colonies were confirmed through sequencing (GATC Biotech AG, Konstanz, Germany). In addition, a stock culture was prepared by dissolving the transformed cells in LB media in 50 % glycerol and stored at -80°C. This plasmid was then preceded to an LR reaction by mixing it with a destination vector and LR clonase enzyme according to manufacturer's protocol. The gene of interest will be exchanged via homologues recombination from entry vector into destination vector (pMDC162, pMDC32; Curtis and Grossniklaus, 2003). Similar procedure was carried out for transformation as described above except the selection media was replaced with 50µg mL^{-1} Kanamycin. After confirmation of the sequence, the plasmid was directly transformed into competent *Agrobacterium tumefaciens* cells (strain GV3101) and shock frozen in liquid N2 for 5s followed by heat shock at 37^0C for 5 minutes. The transformed agrobacterium was allowed to grow in YEB medium for 2 hours at 28^0C. Afterwards, the bacteria was streaked on LB plates supplemented with appropriate concentration of Rifampicin (35µg mL^{-1}), Kanamycin (50µg mL^{-1}) and Gentamycin (10µg mL^{-1}) and allowed to grow at 28 ^{0}C for 2 days. After confirming colonies with a colony PCR, the positive colonies were grown in the same selection media without agar overnight and stored in 50% glycerol at -80 ^{0}C. Later on this construct is used for transformation of Arabidopsis plants or mutants.

2.14 Cloning and transformation of Promoter::GUS lines

Promoter regions upstream of start codon of *PGIP1* (1214bp) and *PGIP2* (483bp) as previously done by Ferrari et al., 2003 were amplified from genomic DNA using promoters given in Table 13 and cloned in a gateway cloning vector pDONR 207 (Invitrogen) according to manufacturer's instructions. The verified fragments were cloned in an expression vector pMDC162 fused with beta-glucuronidase (GUS) gene (Curtis and Grossniklaus, 2003). These promoter::GUS constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 for the transformation of 4-6 weeks old *Arabidopsis* plants by floral dip method (Clough and Bent, 1998). After drying of plants, seeds (T0) were harvested and sterilized before growing on Knop medium supplemented with 25µg mL^{-1} Hygromycin. Only the transformed plants will survive on the selection medium.Three independent homozygous plants (T2) were selected for further analysis.

2.15 Generation of overexpression and complementation lines

To overexpress *AtPGIP1* and *AtPGIP2*, full-length coding sequence of both genes was amplified from cDNA synthesized from RNA isolated from 12-days-old *Arabidopsis* plants. The primer pairs used to amplify the coding sequences from both genes are listed in Table 14. The amplified PCR product was cloned in to Gateway cloning vector pDONR207 (Invitrogen). The clone fragments were verified through sequencing and introduced again in the gateway system pMDC32 vector under the control of double Cauliflower Mosaic virus (CaMV) 35S promoter to engineer *AtPGIP1* and *AtPGIP2* overexpression. The verified constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was used for the transformation of 4-6 weeks old Col-0 plants by floral dip method (Clough and Bent, 1998). After drying of plants, seeds (T0) were harvested and sterilized before growing on Knop medium supplemented with 25µg mL^{-1} hygromycin. The plants resistant to hygromycin were considered to be transformants and were grown to produce homozygous plants. At least two to three independent homozygous lines with highest up regulation were selected for further studies. Complemented lines of *pgip1* mutants were obtained by cloning a wild-type copy of *PGIP1* gene under the control of CaMV 35S promoter using Gateway Cloning system as described above. Two homozygous complemented lines of the wild-type gene were used in this study.

2.16 Histochemical GUS analysis in syncytium

Homozygous lines were grown in Knop medium and infected with nematodes to analyse the GUS expression in a time-course analysis. The infected roots were incubated with X-gluc solution staining (Biomol, Hamburg, Germany) dissolved in 0.1 M sodium phosphate buffer pH 7.0 containing 0.1% Triton-X 100, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$ and 10 mM Na_2EDTA for 12-14 hours at 37 ^{0}C. After overnight staining, the reaction was stopped and samples were washed with 70% ethanol. Staining was carried out at different time points for *Heterodera schachtii* (1, 3, 5 and 10 dpi) and *Meloidogyne incognita* (1, 3, 7, and 15 dpi). The stained syncytia were photographed with a Leica DM4000 inverted Microscope having LAS software (Leica Microsystems) fitted with an Olympus C-5050 digital camera.

2.17 Measurement of ROS

Apoplastic measurement of hydrogen peroxide in leaf disc was carried out by luminal based detection method as previously described (Roux et al., 2011). *Arabidopsis* plants were grown in Knop medium for two weeks after which leaf discs measuring 3 mm^2 were cut with round cork borer and placed in a 96 well plate (Grenier Bio-One) containing 50-100µL sterile water for 12 hours to reduce wounding response. After overnight incubation, the water was removed and replaced with an elicitation solution comprising of 35µL of 1µM luminol derivative 8-amino-5-chloro-7 phenylpyrido[3,4-d]pyridazine-1,4(2H,3H) dione (L-012) (Nishinaka et al., 1993; Wako, chemicals), 15 µL of 20µg mL^{-1} horseradish peroxidase (Sigma-Aldrich, Germany) and 50 µL of 1µM flg22 peptide. Light emission was measured as relative light units (RLU) in a 96-well Lumino meter (Mithras LB 940; Berthold Technologies). Each ROS experiment was repeated 3 times with similar results.

2.18 Plants treatment with OGs

For OG treatment, *Arabidopsis* seeds were sterilized and grown in 6-well plates containing 5 mL liquid KNOP medium. After 9 days of germination, the media was removed and a 3mL of fresh media was added to the wells before adding 30µL of OGs with a final concentration of 50µg mL^{-1}.

After 24 hours of treatment, the plants were gently placed in a semi solid Knop media and allowed to settle down from any stress for few hours. Water treated plants were used as a control and handled in the same manner. Afterwards, the OG- and water-treated plants were inoculated with 70-80 sterile J2s per plant and evaluate for the infection after 12-14 dpi as described above.

Table 11: Primer sequences used in gene expression analysis

Gene	Locus	Forward Primer	Reverse Primer
JAZ8	At1g30135	TGTGTTTTTCTTCAGATGTTACCC	TCTCTGCTTGCGATCGATATT
PROPEP1	At5g64900	ACGAAGCGAAGAAAGTCACC	TTCGGCTGTTTCGAAGTTCC
NPR2	At4g26120	AAACCGAGTTGCACTTGCTC	AGTGATGTCCGCTTTTCACC
PAD3	At3g26830	TTAAGCTCGTGGTCAAGGAGAC	GACCCATCGCATAAACGTTGAC
CYP81F2	At5g57220	ATCGTGCTAGTGAACGCTTG	TTCGTCCGTTACCAAACACC
CYP71A12	At2g30750	GCTTCTTGAGATCCCTTGCG	GTGATGTGGTGTTTGGTCCC
PGIP1	At5g06860	AGTCCCTGACCTTCGCCTAT	AGCATCACCTTGGAGCTTGT
PGIP2	At5g06870	AACAAGCTTCAAGGCGATGC	AACCTTGGAGAGATCGAACTGG
β-tublin	At5g44340	TTTCCGTACCCTCAAGCTCG	GTGAAGCCTTGCGAATGGGA
UBQ5	At3g62250	GTTAAGCTCGCTGTTCTTCAGT	TCAAGCTTCAACTCCTTCTTTC

Table 12: Primer sequences used in expression check for the mutants

Gene	AGI code	Forward Primer	Reverse Primer
PGIP1	At5g06860	CTGACAGGTCCAATTCCTGAC	AATCCATCAAATAAAACATTTTGAA
PGIP2	At5g06870	TCTTGTCCACTCTCCTCCTCA	CCGGAATACTCCCTGTGATG

Table 13: Primer sequences used in developing promoter::GUS lines

Gene	AGI code	Forward Primer	Reverse Primer
PGIP1	At5g06860	AAAAGGGCAGGCTAGGCTAA	CTGAGGCAATGTCTTCACCA
PGIP2	At5g06870	ACCAAGCTTATCTCTAGGAT	GAGTTTTTATGGAAACTATGATTG

Table 14: Primer sequences used in developing overexpression lines

Gene	AGI code	Forward Primer	Reverse Primer
PGIP1	At5g06860	ATGGATAAGACAGCGACATTGTGTC	TTACTTGCAAATTTCAAGAGGAGCAC
PGIP2	At5g06870	ATGGATAAGACAATGACACTGTTC	TCACTTGCAACTAGGAAGAGG

3. Results

Although PAMP/DAMP-triggered immune responses (PTI) are activated in response to different pathogens, details of such responses to nematode infection are largely unknown (Holbein et al., 2016). But some recent work shows the relevance of PTI in context of plant-nematode interaction. For example, several PTI-deficient mutants were shown to be hypersusceptible to infection by RKN (Peng and Kaloshian, 2014; Teixeira et al., 2016; Mendy et al., 2017). A growing number of PTI-suppressing effectors have also been characterized during last many years (Lozano-Torres et al., 2014; Mantelin et al., 2015). Even so, the role of OGs, PGs, and PGIPs in plant-nematode interactions remained mostly obscured. In this study, we characterized the role of *Arabidopsis PGIP1* and *PGIP2* genes against plant parasitic cyst nematodes *Heterodera schachtii* and root-knot *Meloidogyne incognita.* The results of the experiments are discussed here.

3. 1 *PGIP* genes are induced upon nematodes infection

3. 1. 1 Microarrays data and qPCR validation

To characterise the role of *PGIPs* against nematodes, we first analysed the expression of these genes in our published transcriptomic data. The data revealed that both *PGIP1* and *PGIP2* genes are significantly upregulated in response to infection by *Heterodera schachtii* when compared to control roots at different developmental stages. The expression of both *PGIP1* and *PGIP2* were upregulated at infection site at 10 hours post inoculation (hpi) to a fold change of 3.98 and 1.07 respectively when nematodes were still in their migratory stage (Mendy et al., 2017). Similarly, the published data from Szakasits et al (2009) at 5 and 15 dpi (days post inoculation) also showed an upregulation of fold change 3.70 for *PGIP1*and 0.70 for *PGIP2* (**Table15**).

Locus	Gene Name	Root vs Sync (5 + 15dpi) (Szakasits et al., 2009)	Root vs migratory stage (10 hpi) (Mendy et al., 2017)
At5g06860	*PGIP1*	3.40 *	3.98 *
At5g06870	*PGIP2*	0.70	1.07

Table 15: Expression of *PGIP1* and *PGIP2* in microarrays data upon *H. schachtii* infection. Values are relative fold change. Asterisks indicate significant difference to control.

To validate the chip data results, we cut root segments containing the infection site at different stages of *Heterodera schachtii* infection and did a qRT-PCR (**Figure 1**). The results showed an upregulation to a fold change of 3.11 for *PGIP1* and 1.91 to *PGIP2* at 10 hpi in comparison to uninfected roots. The transcript level was also increased for *PGIP1* at 10 dpi in syncytium associated with females (1.77 fold-change), which was slightly lower for *PGIP2* (0.85 fold change) in same samples.

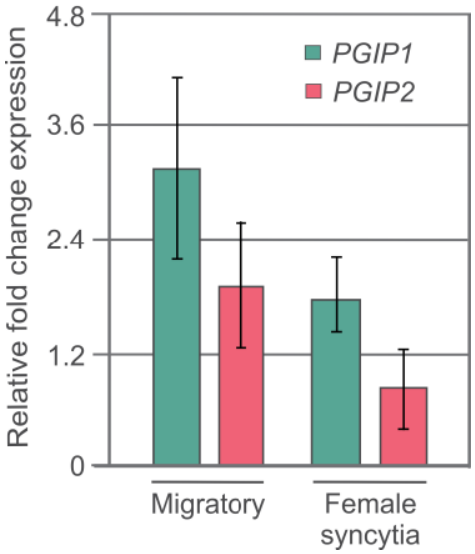


Figure 1: *PGIPs* genes are activated in *Arabidopsis* upon cyst nematode infection. Validation of variations in the expression of *PGIPs* upon nematode infection via qRT-PCR. The values shows a relative fold change upon nematode infection as compared with control roots. *UBQ5* and *β-tublin* was used as housekeeping genes to normalize the data. Bars represent mean ± SE for three independent biological replicates.

As there was no significant up regulation of *PGIP1* and *PGIP2* in different chip data conducted with *Meloidogyne incognita* at 3 dpi, 7dpi,14 dpi and 21 dpi galls, so validation for this data was not done in this study.

3. 1. 2 Activation of *PGIP::GUS* expression in Arabidopsis roots upon cyst and RKN infection

To further understand spatio-temporal expression of *PGIPs* during plant-nematode interaction, we transformed Arabidopsis with *PGIP1*::GUS or *PGIP2::GUS* construct and generated three to five independent homozygous lines. First, we performed a time-course promoter::GUS analysis subsequent to infection by BCN. Although we saw no staining in uninfected root segments, the majority of root infection zones exhibited a strong GUS staining at 1 dpi, 3 dpi and 5 dpi. However, the intensity of GUS staining was reduced considerably at 10 dpi (**Figure 2A**). Next, we analysed the *PGIP1::GUS* and *PGIP2::GUS* upon infection with RKN *M. incognita*. We found no staining at 1 dpi for both *PGIP1* and *PGIP2* but a specific staining was present at 3dpi onwards in giant cells by RKN *M. incognita* (**Figure 2B**).

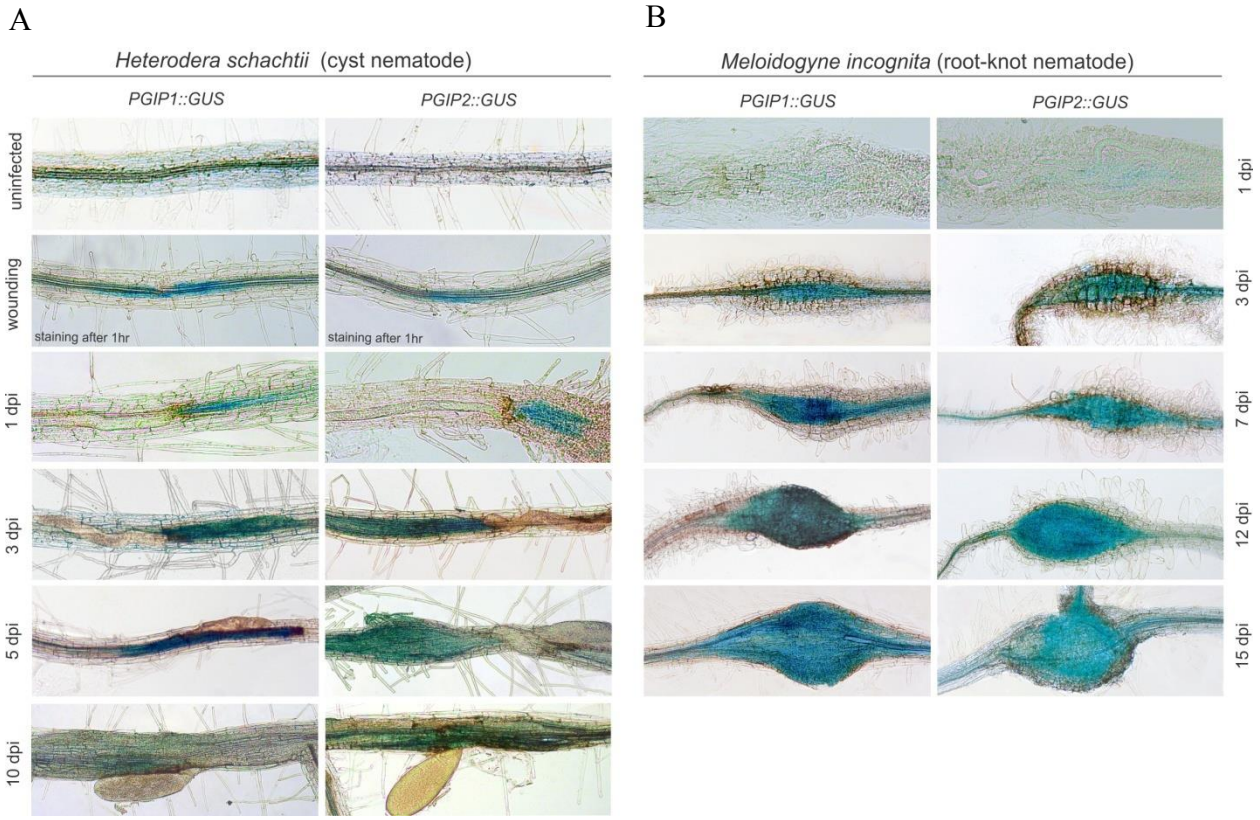


Figure 2: Activation of *PGIP::GUS* expression in Arabidopsis roots upon cyst and RKN infection. (A) Expression of *PGIP1::GUS* and *PGIP2::GUS* in *Arabidopsis* roots upon wounding or *H. schachtii* infection at 1, 3, 5 and 14 dpi, respectively. Scale bar =200 µm (B) Expression of

PGIP1::GUS and *PGIP2::GUS* in *Arabidopsis* roots upon *M. incognita* infection at 1, 3, 7, 12 and 15 dpi, respectively. Scale bar =200 µm.

3. 2 *PGIP*- and OG-mediated signaling is involved in cyst nematode infection

To define a role for *PGIP1* and *PGIP2* against nematode infection, we ordered T-DNA insertion mutants from Arabidopsis stock centre. *pgip1* mutant lines having two independent insertions, one in an exon (*pgip1-1*; **Figure 3A**) and another in 3′ UTR region (*pgip1-2;* **Figure 3B**) were selected for this study. However, a single *pgip2* mutant with a T-DNA insertion in an exon region was selected (*pgip1-2*; **Figure 3C**).

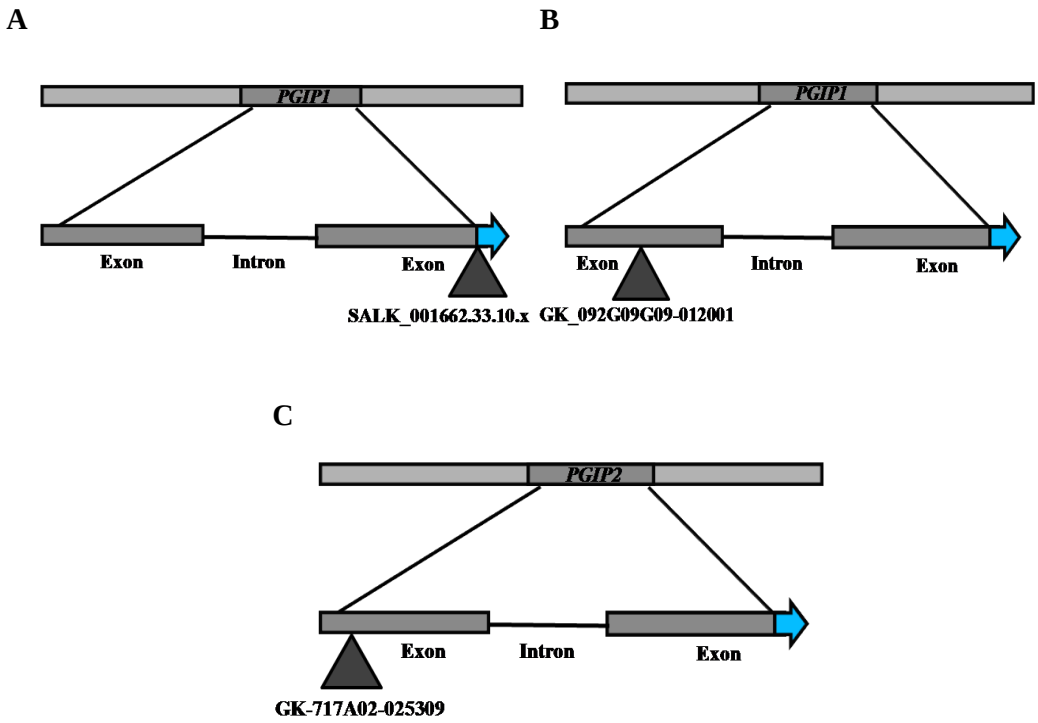


Figure 3: T-DNA insertion map for *pgip1-1*, *pgip1-2* and *pgip2-1*. (**A, B, C**) *Arabidopsis* genes *pgip1-1*, *pgip1-2* and *pgip2* showing the position of T-DNA insertion as indicated by the triangles.

To confirm whether knocking out target gene led to an absence of in selected lines, we extracted RNA from 12-days-old mutant plants and used RNA from Col-0 as control. Primers were designed on either side of T-DNA insertion (Table 11). No expression of either gene was detected through RT-PCR using *β-tubulin* as a positive control and water as a negative control,

which confirmed that the mutants are homozygous (**Figure 4**). Also the homozygous mutants did not show any phenotypic aberrations and could be used for the experiments.

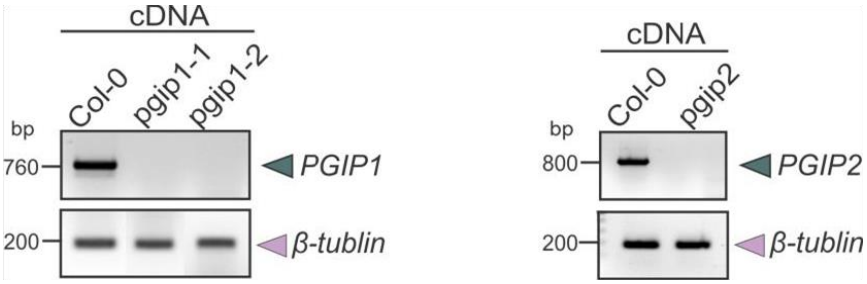


Figure 4: Expression check of *pgip1-1*, *pgip1-2* and *pgip2-1*.Gel picture showing expression check (RT-PCR) from the cDNA of all the tested mutants compared to Col-0. *β-tubulin* was used as a positive control, while water was used as a negative control.

To characterize the role of *PGIPs* in nematode infection, we grew loss-of-function T-DNA insertion mutants for *PGIP1* and *PGIP2* (for *PGIP1*, *pgip1-1* and *pgip1-2*; for *PGIP2*, *pgip2-1*) *in vitro* for 12 days and infected them with J2s of either BCN or RKN, as described in the Methods section. For BCN, we counted the number of females, number of males, average size of syncytium and average size of females at 14 dpi. We found a significant increase in the average number of females in both mutant lines for *PGIP1* (*pgip1-1* and *pgip1-2*) when compared with Col-0 (**Figure 5A and Supplementary Figure 1A**). Similarly, we observed a significant increase in average syncytium size in *pgip1* (*pgip1-1* and *pgip1-2*) but noted no differences in average female size (**Figure 5B, C and Supplementary Figure 1B, C**). Conversely, *pgip2-1* mutants showed no significant differences in average female numbers, average female size and average syncytium size, but a significant decrease in average number of males was observed when compared with Col-0 (**Figure 5D-F**). In comparison to BCN, there was no change in average number or in average size of galls, induced by RKN among all tested lines (**Figure 5G–J**). Altogether, we conclude that knocking out *PGIP1* leads to hypersusceptibility of plants to cyst nematodes but not to root-knot nematodes. *PGIP* has been shown to promote the formation of OGs, which in turn activate host defence responses to restrict pathogen development. To evaluate whether OGs plays a similar role in plant-nematode interaction, we treated the *Arabidopsis* plants with OGs and infected them with BCN (see Methods for details). We found that number of females as well as size of syncytium and females was significantly reduced in plants treated with OGs as compared to water-treated plants (**Figure 5K-M**).

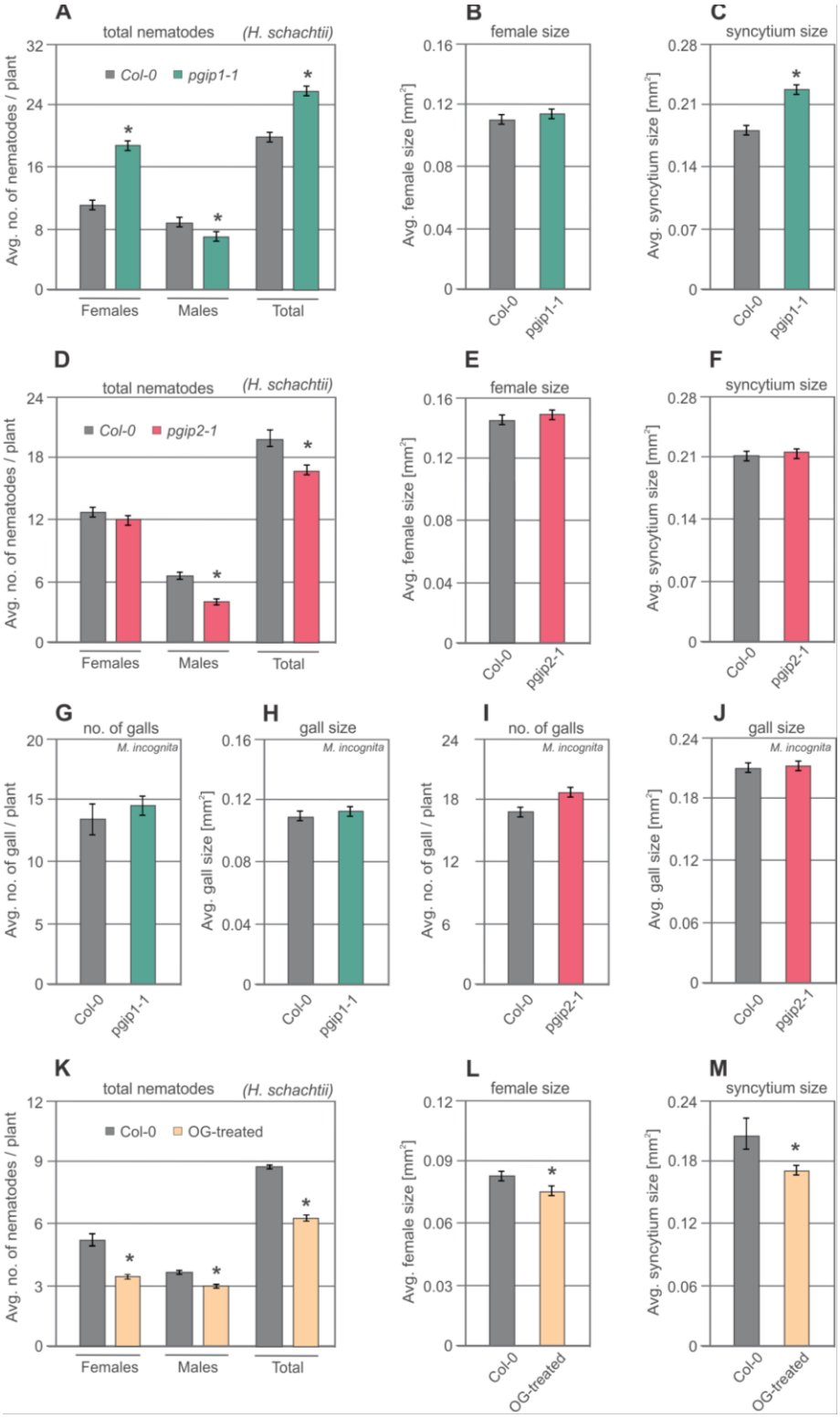


Figure 5: Cyst Nematode infection assays in *pgip1*, *pgip2* and OG-treated plants. (A) Average number of females and males per plant present in Col-0 and *pgip1-1* mutant lines at 14 dai. (B, C) Average female sizes (B) and plant syncytia (C) in Col-0 and *pgip1-1* mutant lines.

(D) Average number of females and males per plant present in Col-0 and *pgip2-1* lines at 14 dai. (E, F) Average female sizes (E) and plant syncytia (F) in Col-0 and *pgip2-1* mutant lines. (G, I) Average number of galls per plant present in Col-0, *pgip1-1* (G) and *pgip2-1* (I) mutant lines at 21 dai. (H, J) Average size of galls per plant present in Col-0, *pgip1-1* (H) and *pgip2-1* (J) mutant lines at 21 dai. (K) Average number of females and males per plant present in water- or OG-treated Col-0 plants at 14 dai. (L, M) Average female sizes (L) and plant syncytia (M) in water- or OG-treated Col-0 plants at 14 dai. Bars represent mean ± SE for three independent biological replicates. Data were analysed using student's T-test ($p< 0.05$). Asterisks represent statistically significant difference to corresponding Col-0.

As *pgip1* mutants are susceptible to CN, so to confirm further whether this suceptiblity is due to absence of *PGIP1*, we transformed *pgip1-1* mutants with *35S::PGIP1* construct and analysed the homozygous plants via nematode infection assays. Indeed, we found that transgenic plants showed no changes in susceptibility to BCN as compared to Col-0 **(Figure 6**).

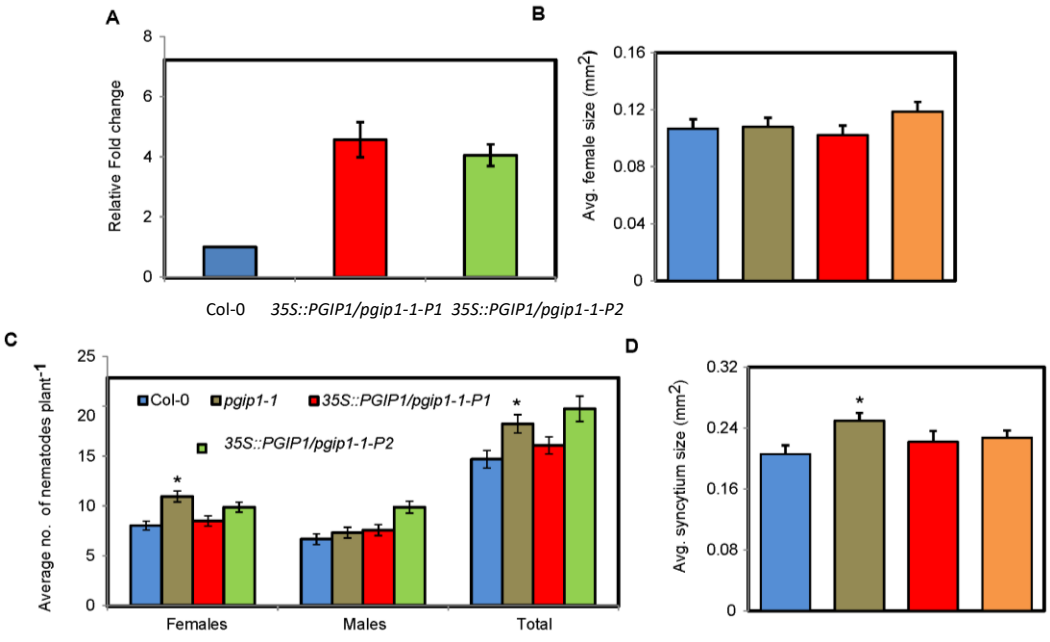


Figure 6: Nematode infection assays in complementation lines for *pgip1* (*35S::PGIP1/pgip1-1*) mutant lines. (A) Two independent homozygous lines overexpressing *PGIP1* in *pgip1-1* background (*35S::PGIP1/pgip1-1-P1, 35S::PGIP1/pgip1-1-P2*) were selected and analysed for changes in transcript abundance of *PGIP1*. Bars are the mean ± SE for three independent biological replicates. (B, D) Average sizes of female nematodes (B) and plant syncytia (D) in Col-0 and *35S::PGIP1/pgip1-1*complemention lines for *pgip1* at 14 dai. (C) Average number of females and males per plant present in Col-0 and *35S::PGIP1/pgip1-1* complementation lines for *pgip1* at 14 dai.

3. 3 Overexpression of *PGIP1* decreases susceptibility to cyst nematode but not to root-knot nematodes

Because loss-of-function *pgip1* mutants were hypersusceptible to *H. schachtii* infection, we hypothesized that overexpression of this gene might reduce susceptibility to nematode infection. To test this hypothesis, we produced transgenic plants expressing *PGIP1* or *PGIP2* under the control of 2xCaMV 35S promoter (35S::*PGIP1*; 35S::*PGIP2*). Three homozygous lines for 35S::*PGIP1* (P2, P9, and P10) that showed the highest upregulation through qRT-PCR were selected for further experiments (**Figure 7A**). No obvious phenotypic changes were observed in the transgenic lines as compared to wild type. We inoculated 12-day-old plants of the transgenic lines along with Col-0 with infective juveniles of *H. schachtii* and collected data after 14 dpi. Although we saw a significant reduction in female numbers as well as in total number of nematodes per plant for P9 and P10, there was no change in both the parameters for P2 (**Figure 7B**). In addition to number of nematodes, we also measured average size of females and syncytium; we found that average size of syncytium was decreased significantly in all three tested lines (**Figure 7C, D**).

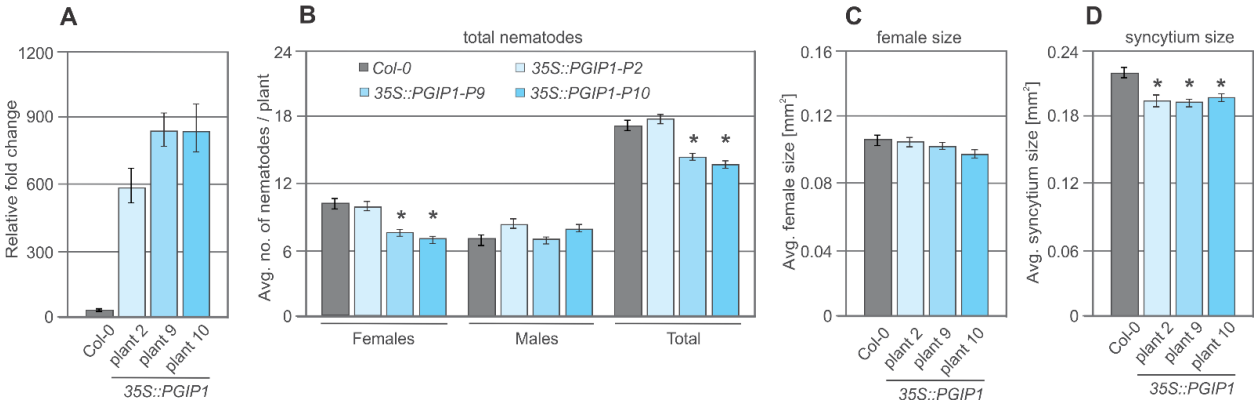


Figure 7: Nematode infection assays in *PGIP1* overexpression lines. (A) Three independent homozygous lines (P2, P9, P10) overexpressing *PGIP1* (*35S::PGIP1*) were selected and analysed for changes in transcript abundance of *PGIP1*. Bars represent mean ± SE for three independent biological replicates. (B) Average number of females and males per plant present in Col-0 and *PGIP1* overexpression lines at 14 dai. (C, D) Average sizes of female nematodes (C) and plant syncytia (D) in Col-0 and *PGIP1* overexpression lines at 14 dai. Bars represent mean ± SE for three independent biological replicates. Data were analysed using student's T-test ($p < 0.05$). Asterisks represent statistically significant difference to corresponding Col-0.

We also tested transgenic plants overexpressing *PGIP2* against infection with *Heterodera schachtii*. Unlike *35S*::*PGIP1*, no significant change was observed in any parameter of any line overexpressing *PGIP2* (**Figure 8A–D**).

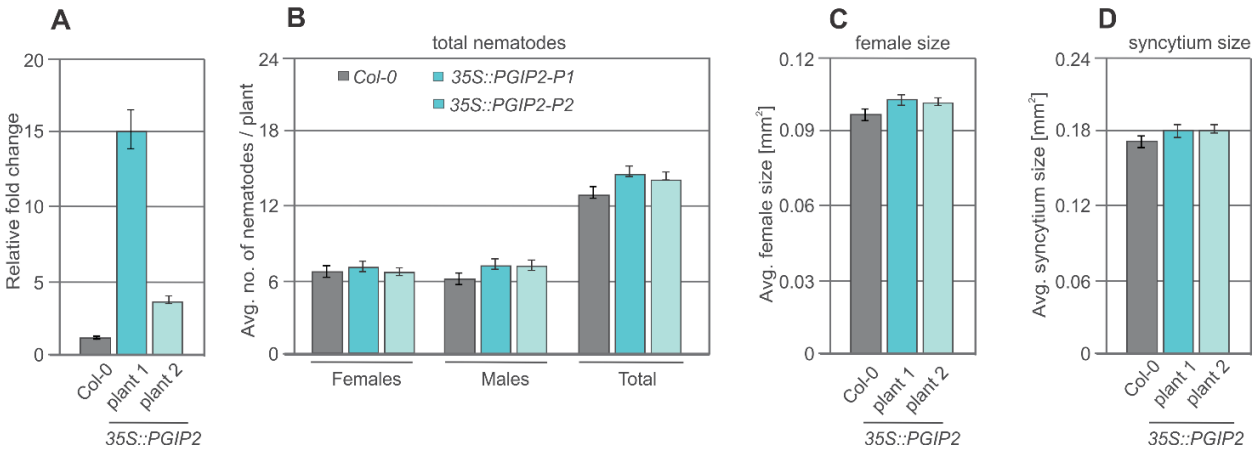


Figure 8: Nematode infection assays in *PGIP2* overexpression lines. (A) Two independent homozygous lines (P1, P2) overexpressing *PGIP2* (*35S::PGIP2*) were selected and analysed for changes in transcript abundance of *PGIP2*. Bars are the mean ± SE for three independent biological replicates. (B) Average number of females and males per plant present in Col-0 and *PGIP2* overexpression lines at 14 dai. (C, D) Average sizes of female (C) and plant syncytia (D) in Col-0 and *PGIP2* overexpression lines at 14 dai. (B-D) Bars represent mean ± SE for three independent biological replicates. Student's T-test was used for data analysis ($p < 0.05$).

We also tested *PGIP1* or *PGIP2* overexpression lines against *Meloidogyne incognita* and found no effect on average number as well as average size of galls (**Figure 9**).

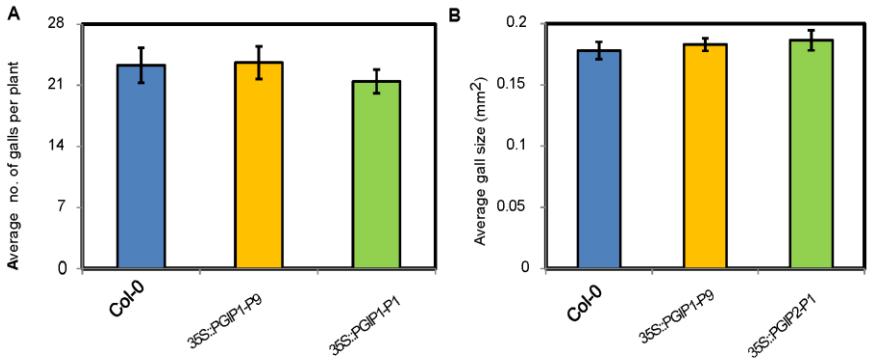


Figure 9: Root-knot nematode infection assays in *PGIP1* and *PGIP2* overexpression lines. **(A)** Average number of galls per plant present in Col-0, and *PGIP1*and *PGIP2* overexpression lines at 21 dai. **(B)** Average size of galls per plant present in Col-0, *PGIP1*and *PGIP2*

overexpression lines at 21 dai. Error bars indicate mean ± SE for three independent biological replicates. Data were analysed using student's T-test ($P< 0.05$).

3.4 *PGIP*-mediated defence responses activate glucosinolate and camalexin responses

Apoplastic production of ROS is one of the hallmarks of PTI responses, which are activated after pathogen attack or elicitor treatment (O'Brien et al., 2012). To investigate whether these PTI responses are dependent on the function of PGIPs and whether the hypersusceptibility of *pgip1* mutants results from impaired production of ROS, we carried out luminal-based detection method of quantitatively evaluating PTI responses. Leaf discs from 2-week-old *pgip1* and *pgip2* plants did not show any significant difference when compared to wild type in response to flg22 (**Figure 10A**). This experiment showed that elicitor-induced ROS production is independent of both *PGIP1* and *PGIP2*, indicating that it plays no role in PGIP-mediated defence responses. Next, we hypothesized that the hypersusceptibility of *pgip1* mutants might be due to impaired expression of defence-related pathway genes. To test this hypothesis, we assessed the expression of a few marker genes that are highly upregulated during the migratory stages of infection in our published transcriptome data (**Supplementary Table 1**). These genes were *JAZ8* (Chini et al., 2007) involved in Jasmonic acid induction and signalling, *NPR2*, a salicylic acid marker gene (Canet et al., 2010) and *PROPEP1*, a member of the PROPEP family that is induced upon wounding (Huffaker et al., 2006). We also tested three genes involved in the synthesis of camalexin and indole-glucosinolate that were also highly upregulated in the chip data. We choose *CYP81F2*, which encodes a cytochrome P450 enzyme and which is involved in indol-3-yl-methyl glucosinolate catabolism (Clay et al., 2009); *CYP71B15* (PAD3), which catalyses the final step in camalexin biosynthesis (Schuhegger et al., 2006; Zhou et al., 1999); and *CYP71A12*, which catalyses the conversion of IAOx to IAN (Millet et al., 2010). After specificity check, the efficiency of the primers was checked through qPCR (**Supplementary Table 2**).

At first, we wanted to know about the expression of these selected marker genes in un-infected roots of *pgip1* and *pgip2* using Col-0 as control. Our data showed no significant change in expression of all tested genes between Col-0 and *pgip* mutants in uninfected roots. These results showed that the tested genes were not impaired in their expression at the basal level **(Figure 10B-G**). Next we wanted to check the expression of these marker genes upon infection with

Heterodera schachtii. We cut root segments at 10 hpi, representing the migratory stage of nematode infection, and used these samples for qRT-PCR analysis. We found no change in expression of *JAZ8, PROPEP1* or *NPR2* in *pgip1 or pgip2* compared with Col-0 (**Figure 10 B-D**). However, upregulation of camalexin and indole-3-glucosinolate genes (*PAD3*, *CYP81F2* and *CYP71A12*) was significantly impaired in *pgip1* compared with Col-0 (**Figure 10 E-G**).

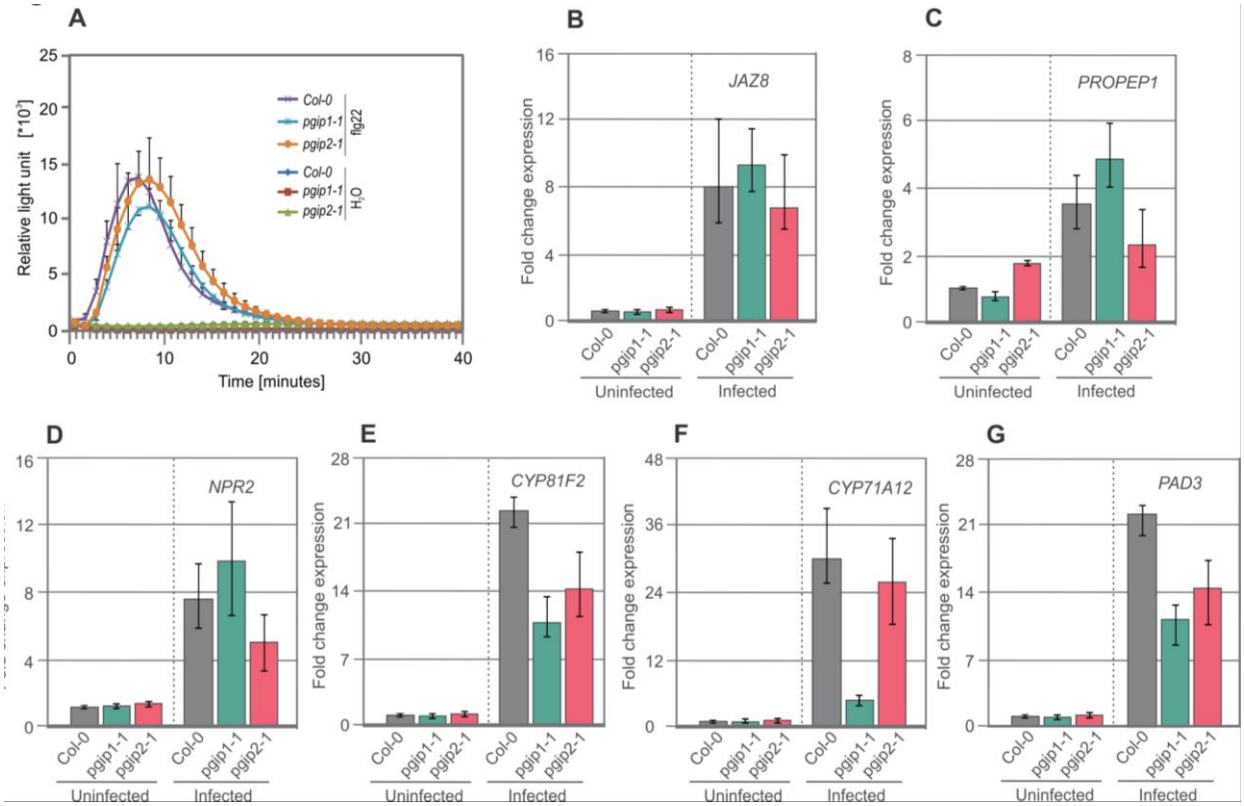


Figure 10: ROS production and gene expression analysis on root segments with migratory stages cyst nematodes. (A) Root segments from Col-0 , *pgip1-1* and *pgip2-1* plants were treated with water, or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. (B-G) Infected and uninfected root segments from Col-0, *pgip1-1* and *pgip2-1* plants were cut and gene expression was measured. Data represent relative expression of the indicated genes with the value in Col-0 plants set to one. Bars represent mean ± SE for three independent biological replicates.

Our results showed that the susceptibility in *pgip1* results from impaired induction of camalexin and indole-glucosinolate biosynthesis pathways. To have a further insight to this hypothesis, we used a double mutant *cyp79b2/b3,* which is strongly impaired in accumulation of glucosinolate and camalexin (Zhao et al., 2002; Kliebenstein et al., 2005). The plants were grown *in vitro* and inoculated with cyst nematodes and number of males and females were counted. The data

showed that numbers of females were increased significantly in *cyp79b2/b3* as compared to Col-0 (**Figure 11a)**. However, we did not observe any significant change in average female and syncytium size between Col-0 and *cyp79b2/b3* **(Figure 11b-c).**

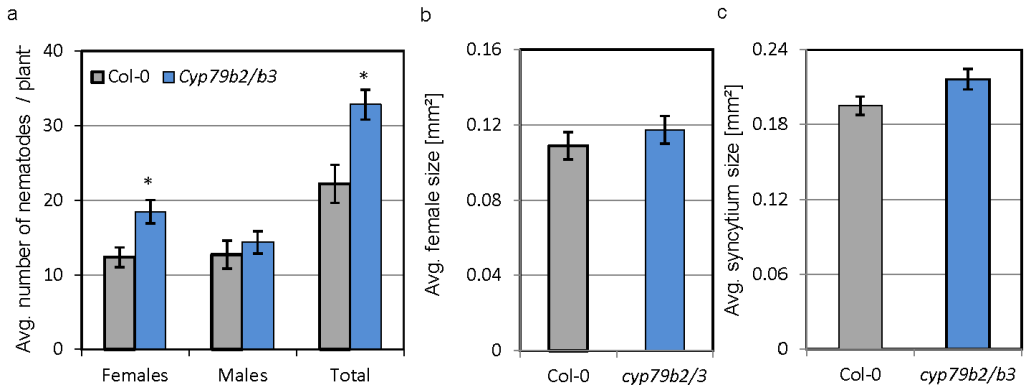


Figure 11: Nematode infection assays in *cyp79b2/b3* lines. (a) Average number of females and males per plant present in Col-0 and *cyp79b2/b3* lines at 12 dai. Average sizes of female nematodes (b) and plant syncytia (c) in Col-0 and *cyp79b2/b3* lines at 14 dai. Bars represent mean ± SE for four independent biological replicates. Data were analysed using student's T-test ($p < 0.05$). Asterisks represent statistically significant difference to corresponding Col-0.

3. 5 Do cyst nematodes encode a PG?

A recent transcriptome analysis of *H. schachtii* (Fosu-Nyarko et al., 2016) identified a contig with a relatively low sequence similarity to PG from RKN *M. incognita* (**Figure 12**; Jaubert et al., 2002). Based on sequence information from Fosu-Nyarko et al., 2016 and our own unpublished draft transcriptome, we amplified a full length PG sequence from *H. schachtii* (see methods for details). This sequence was designated as *HsPG* and encodes for 71 amino acids protein (**Figure 13**).

>BCN_raw_Reads_unmatched_contig_4799 (Fosu-Nyarko et al., 2016)
GTCTGACCATTCTTCACTATATGCACTCTATGCCTCACCCATTTTCTCTGACCATTCTTCACTATATG
CACTCTATGCCTCACCCATTTTCTCTGCACTTCCCACACTATGCATTCTATGCCTCCTCCCCTTTTCC
TGCCCTACCCAGTTTTTCTATGCACTTTACACAAACTGTGGCTCATTTTCTGTTCTCCTCTTTTTCTC
TGTTCTCCTTTCTACTCCTCCAATTGTCCTCCTCCTCCGTTTCCTACCTCCGTTTCAGCTCTTCCTCC
GTTGCCCCAT

Figure 12: Nucleotide sequence of putative PG (Fosu-Nyarko et al., 2016).

>HsPG

MPHPFSLHFPHYAFYASSPFLCPTQFFYALYTNCGSFSVLLFFSVLLSTHAIFFPFRFLSLSALFLYHPR

Figure 13: Amino acid sequence of *HsPG*.

An alignment between *HsPG* and *MI-PG-1* identified a very low percentage similarity between the sequences (**Figure 14**). A detailed search to identify homologues for *HsPG* from genome and transcriptome of two other closely related cyst nematodes (*Globodera pallida* and *Globodera rostoschiensis*) did not yield any *HsPG* homologue (Cotton et al., 2014; Eves-van den Akker et al., 2016). Next we used amino acid sequence of few selected PGs from bacteria, fungi, insects, and nematodes (*HsPG* and *MI-PG-1*) to built up a phylogenetic tree using the Neighbor-Joining method, which was drawn as phylogram. This analysis indicates that *HsPG* form a separate cluster away from all other PGs (**Figure 15A**) and showed no significant similarity to any known PG. A further analysis predicting Pfam domain revealed that *HsPG* does not contain a polygalacturonase domain (pfam12708). To further characterize the putative *HsPG*, we analysed its expression during different stages of nematode development (eggs, pre-infective J2s, J3s, females). We found that *HsPG* is expressed at extremely low level during all tested developmental stages of BCN with the highest expression present in eggs (**Figure 15B**). Based on these data, we conclude that *HsPG* is unlikely to encode a PG and its further characterization was not performed.

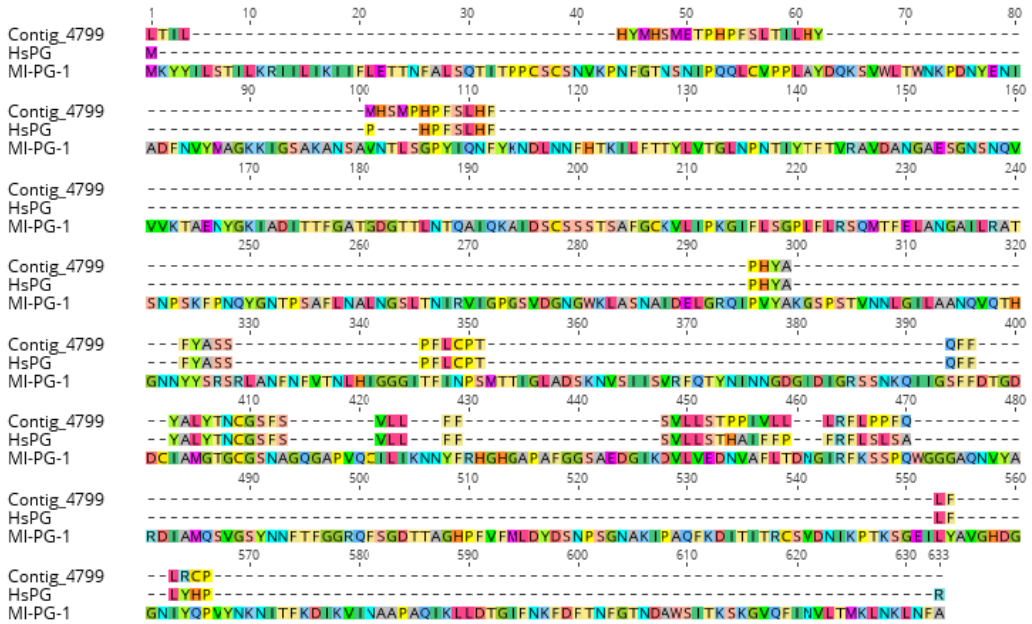


Figure 14: Alignment between contig_4799, *HsPG* and *MI-PG-1*.

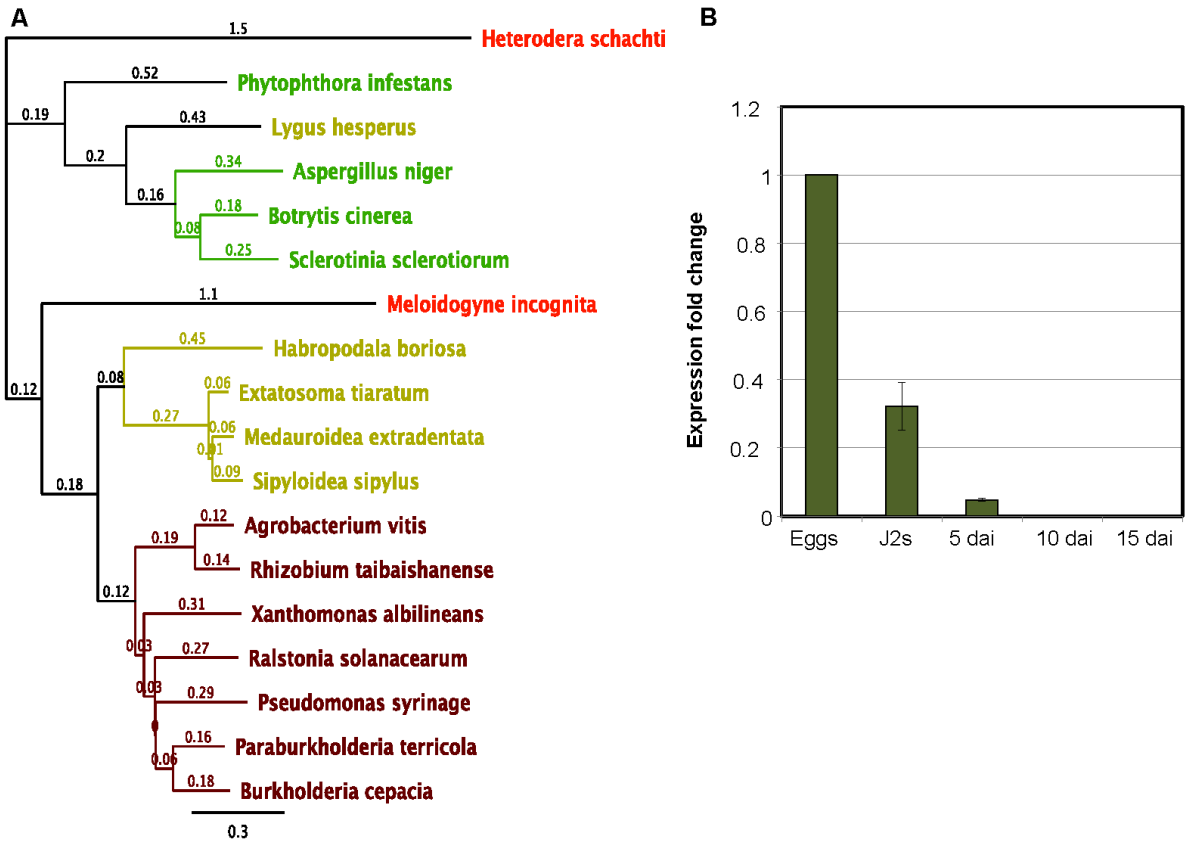


Figure 15: Characterization of polygalacturonase from *H. schachtii*. (A) A phylogenetic tree generated from Neigbour-joining construction method based on alignment of PGs sequence from

bacteria, fungi, insects and nematodes. The number next to each branch indicates a measure of support (B) Changes in expression of *HsPG* during different developmental stages of cyst nematode *H. schachtii*. Actin was used as housekeeping genes to normalize the data. Bars represent mean ± SD for three independent biological replicates.

4. Discussion

Plants are exposed to many different kinds of biotic interference and they must cope with these stresses in order to maintain continued growth and development. For this reason plants have evolved a sophisticated mechanism to sense such dangers through receptors in their cell membranes called pattern-recognition receptors (PRRs). The PRRs can sense conserved molecules from pathogens called pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) or damage-associated self-molecules called damage-associated molecular patterns (DAMPs). Both PAMP and DAMP- can trigger immune responses known as pattern-triggered immunity (PTI). There are numerous studies regarding the role of PTI in different types of plant-pathogen interactions (Boller and Felix, 2009; Nicaise et al., 2009; Tena et al., 2011; Zipfel, 2014); however, details of such responses to nematode infection are largely unknown (Holbein et al., 2016). Nevertheless, recent work shows the relevance of PTI in the context of plant-nematode interaction. For example, several PTI-deficient mutants were shown to be hypersusceptible to infection by RKN (Peng and Kaloshian, 2014; Teixeira et al., 2016, Mendy et al., 2017). A growing number of PTI-suppressing effectors have also been characterized (Lozano-Torres et al., 2014; Mantelin et al., 2015). Even so, the role of OGs, PGs, and PGIPs in plant-nematode interactions remained mostly obscured. In this study, we established a molecular framework for regulation and downstream signalling for PGIPs in cyst and in RKN parasitism.

Cyst and root-knot nematodes are both biotrophic pathogens with a sedentary mode of parasitism, however, once inside the root, their style of migration differs. Cyst nematodes migrate destructively and intracellularly through the cortical cells of the roots to reach vascular cylinder, there forming a permanent feeding site called syncytium (Jones and Northcote, 1972; Golinowski et al., 1996; Niblack et al., 2006). In contrast to cyst nematodes, root-knot nematodes migrate intercellularly towards the root tip, then turn to enter the vascular cylinder where they establish a feeding site comprised of 5-7 giant cells. Based on our data, we propose that cyst nematodes invasion and subsequent migration inside the *Arabidopsis* root activates the expression of *PGIP1* and *PGIP2*, which in turn triggers the OG-mediated host basal (PTI) defence responses, thereby limiting further colonization of the roots. This hypothesis is supported by results from infection assays where knocking out *PGIP1* increases *Arabidopsis* susceptibility to cyst nematodes while exogenous OG-treatment leads to a strong decrease in host

susceptibility to the cyst nematode *Heterodera schachtii*. Also there was a strong staining observed in *PGIP1::GUS* and *PGIP2::GUS* lines during the migration (1dpi) of *H. schachtii*, which further confirmed that both these genes are induced as a result of intracellular movement of *H. schachtii*. Unlike cyst nematodes, invasion of roots by root-knot nematode *Meloidogyne incognita* does not induce *PGIPs* expression at 1dpi (migratory stage). Similarly no staining was observed in *PGIP1::GUS* and *PGIP2::GUS* lines upon infection with *M. incognita*. Considering a strong wound-induced induction of *PGIPs* (Ferrari et al., 2003 and our own observations), this difference in *PGIP* expression pattern is likely due to a difference in migration style between cyst nematode and root-knot nematode. The view that RKN do not cause damage during their migration inside the root is supported by a recent study showing that *Arabidopsis* lines altered in DAMP perception do not show any change in susceptibility to RKN (Teixeira et al., 2016). *M. incognita* avoidance to cause damage to the host roots would result in avoiding the activation of damage-related defence responses, which may be one of the reason behind their unparalleled success with a potential host range encompass almost all cultivated crops (Sasser, 1979; Trudgill and Blok, 2001). Unlike *M. incognita, H. schachtii* has a narrow host range and this may well be due to activation of damage-related responses, which it activates. In future, it would be interesting to investigate *Arabidopsis* DAMP-perception mechanisms against *H. schachtii*. This may lead to identification of novel DAMPs receptors that are able to recognize the cyst nematodes during their early migration and parasitism.

Cell wall is the first barrier that is encountered by the invading pathogens to carry out a successful parasitism. For this purpose, pathogens secrete an arsenal of enzymes that are able to degrade the major structural components of polysaccharides that includes cellulose, xylan and pectins. Pectin is the major component of cell wall (Mohnen, 2002) and pathogens secrete certain pectinases enzymes for its degradation. These pectinases include polygalacuronases, pectate lyase, pectin lyase and pectin methyl esterase. polygalacturonase is an important pectinases that is able to degrade the homogalacturanan component of the cell wall providing access to the pathogens (Annis and Goodwin, 1997; Girard and Jouanin, 1999; De Lorenzo and Ferrari, 2002). Polygalacturonases are secreted by pathogens like fungi, bacteria, insects and nematodes and can be classified into an endo-PGs or exo-PGs depending on the mode of action and removal of galacturonic acid residues (Kars and van Kan, 2007; Jayani et al., 2005). Endo-PGs can cleave the alpha-1, 4 glycosidic bonds between the galacturonic acid of pectins in a random manner

leading to the production of small fragments called oligogalacturonides (Nothnagel et al., 1983; Ferrari et al., 2013). On the other hand, exo-PGs can hydrolyse the non-reducing end of galacturonic acid and results in monosaccharide galacturonic acid formation. It has been previously shown that exo-PGs can degrade oligogalacturonides produced as a result of pectin degradation by the endo-PGs and are therefore not inhibited by the plant *PGIPs* (Cervone et al., 1990). Among nematodes, *M. incognita* encodes a PG (*MI-PG-1*), which is secreted into the host tissue to weaken the cell wall during penetration and intercellular migration of the nematodes (Jaubert et al., 2002). In comparison to RKN, existence of a functional PG in cyst nematodes remains questionable. These observations raise the question of whether the secretion of PGs by nematodes (if any) plays a role in activation of *PGIP* expression during nematode infection of the roots. Because we did not see any *PGIP* expression during migratory stage of RKN infection and because CN do not seem to encode a PG, we postulate that PGIP induction during nematode infection (at least during the migration stage) is independent of nematode-derived PGs. This hypothesis is in line with observations that *MI-PG-1* is an exo-PG, which are usually not inhibited by PGIPs (Jaubert et al., 2002; Schacht et al., 2011). This also shows that PG-PGIP interaction is not obligatory for the activation of defence responses by the plants. Our results also showed that overexpression of *PGIP1* leads to a decrease in susceptibility to *H. schachtii* infection indicating a role in plant defence even though no exo-PGs or endo-PGs were observed in this nematode. This agreement is also in line with the observation as no PG was observed in cyst nematode *Heterodera goettingiana*, yet *PGIP* is differentially expressed in susceptible and resistant pea varieties, suggesting a role of this defence protein in plant nematode interaction (Veronico et al., 2011).

The production of a burst of apoplastic reactive oxygen species (ROS) is one of the first defence strategy adopted by plants against biotic and abiotic stresses (Torres, 2010). The primary sources of ROS burst in *Arabidopsis* are NADPH-oxidases, also called respiratory burst oxidase homolog (RBOH). These are plasma membrane localised and generate superoxide (membrane impermeable) or peroxidases in the apoplast, which is dismutated to produce hydrogen peroxide (H_2O_2). H_2O_2 is membrane permeable and can enter the cytosol and different cellular organelles (Lamb and Dixon, 1997; Bolwell, 1999; Nuhse et al., 2007; Suzuki et al., 2011). The production of ROS burst was observed first time in carrot culture cells and since then the mechanism has been well studied in *Arabidopsis* and other crop species (Bach et al., 1993; Bindschedler et al.,

2006; Davies et al., 2006; Martinez et al., 1998; Bolwell et al., 1998; Bolwell, 1999; Choi et al., 2007). Several PAMPs and DAMPs such as flg22, elf18 and oligogalacturonides respectively, are able to elicit apoplastic ROS burst and can induce PTI responses (Nuhse et al., 2007; Mersmann et al., 2010; Galletti et al 2007; Gramagena et al., 2016).

Considering the omnipresent role of ROS in PTI responses, we asked whether the reduced susceptibility of *pgip1* (to nematodes) is due to impaired production of ROS burst in response to PAMPs. Our results concluded that although *pgip1* is hypersusceptible to cyst nematode infection, yet elicitor-induced production of ROS showed no significant difference compared to Col-0. The reason for this may be that *PGIPs* are activated when plants are wounded and subjected to damage, and the absence of *PGIP* has no influence on PAMP-induced ROS production. However, it is also possible that two *PGIP* genes may have a redundant role due to high homology and we do not see difference in ROS production in single mutants (Ferrari et al., 2003).

A recent transcriptomic analysis for a double *rboh* mutant (*rbohD/F*) during migratory stages of cyst nematode infection showed no change in expression of *PGIP2* and *PGIP2* in *Arabidopsis* (Chopra and Hasan et al., unpublished). These data also suggest that expression of PGIPs is independent of ROS production upon damage by the nematodes. This is also in an agreement with the observation that OG-induced resistance does not necessarily involve AtrbohD and that oxidative burst is not involved during early expression of OG-induced marker genes (*PAD3, RetOx, AtPGIP1, WRKY40 and CYP81F2*) (Galletti et al., 2008).

Because plants are sessile in nature, they have evolved several strategies to overcome biotic stresses. Activating large number of defence-related genes is one of the early plant responses during a pathogen attack (Bowles, 1990), which may inhibit the pathogens and protect the plants from successful colonization.Upon coming in contact with a pathogen, the plants can activate two kinds of mechanisms. One is called systemic acquired resistance (SAR), which provides long-lasting protection for the plants through the endogenous signalling molecule, salicylic acid, and results in the induction of a large number of pathogenesis-related (PR) genes (Ward et al., 1991; Cao et al., 1994; Penninckx et al., 1996; Loon and Strien, 1999; Durrant and Dong, 2004). The other mechanism is called induced systemic resistance (ISR), and does not depend upon PR genes and salicylic acid, but utilises pathways regulated by jasmonic acid and ethylene (Yan et

al., 2002; Berrocal et al., 2002; Lorenzo et al., 2004; Shinshi, 2008; Shoresh et al., 2010). In addition to SA, JA, and ethylene production, plants also activate a number of secondary metabolites such as phytoalexins in response to pathogen attack (Paxton, 1981).

Camalexin and indole-glucosinolates (IGs) are the phytoalexins that play the major role in biotic stress (Ausubel et al., 1995; Glawischnig, 2007; Bednarek & Osbourn, 2009; Zhao et al., 2015). There is enough genetic evidence regarding their role in restricting the parasitism of a number of pathogens such as bacteria, fungi and nematodes (Ferrari et al., 2003, 2007; Clay et al., 2009; Thomma et al., 1999; San-chez-Vallet et al., 2010; Ali et al., 2013; Frerigmann et al., 2016; Teixeira et al., 2016). The regulation and biosynthesis of camalexin and IGs is well studied in *Arabidopsis*, which starts from the conversion of tryptophan to indole-3-acetaldoxime (IAOx) by two cytochrome P450 enzymes, *CYP79B2* and *CYP79B3* (Hull et al., 2000). IAOx serves as a precursor for the synthesis of camalexin, IGs and auxin (Mikkelsen et al., 2000; Böttcher et al., 2014). For camalexin biosynthesis, IAOx is dehydrated to indole 3 acetonitrile (IAN) by other cytochrome P450 enzymes, *CYP71A12* and *CYP71A13* (Nafisi et al., 2007; Millet et al., 2010; Müller et al., 2015). This IAN is then conjugated to glutathione by the glutathione-S-transferase GSTF6 to make GSH (IAN) (Su et al., 2011), which then metabolizes to Cys (IAN) in the presence of γ-glutamyl peptidase 1 (GGP1) and GGP3 (Geu-Flores et al., 2011). In the final step, Cys (IAN) is converted to camalexin through the cytochrome P450 enzyme *PAD3/CYP71B15* (Zhou et al., 1999; Schuhegger et al., 2006; Böttcher et al., 2009). For the biosynthesis of IGs, IAOx is converted to 1-aci-nitro-2-indolyl-ethane by yet another cytochrome P450 enzyme *CYP83B1* that, in several more steps, results in the production of IGs (Figure 16; Bak et al., 2001).

In order to suppress such kind of responses, plant parasitic nematodes try to manipulate the cellular biology of the host plant to minimize PTI responses, thus helping the nematode in establishing a feeding site (Wubben et al., 2008; Kammerhofer et al., 2015). As *PGIP* is known to play a role in defence against different pathogens, yet the mechanism regulating downstream signaling of *PGIP* is not fully investigated. We assume that the susceptibility and resistant in the loss of function *pgip1* mutant and overexpression lines respectively against cyst nematode may be due to altered regulation of defence related genes. Recently, a transcriptomic profiling was done during migratory stage (10 hpi) of cyst nematode *Heterodera schachtii* in our lab (Mendy

et al., 2017). The data analysis showed many genes induced upon nematode infection including both *PGIP1* and *PGIP2*. The highly upregulated genes were related to both SA and JA acid signaling. There were also a group of genes involved in camalexin and indole glucosinolates biosynthesis that showed a significant increase in transcript level after nematode infection. In the present study, we investigated the upregulation of these genes in *pgip* mutants upon cyst nematode infection. A previous study showed that OG-mediated resistance to the necrotrophic fungal pathogen *Botrytis cinerea* was independent of SA, JA or ethylene but required *PAD3* (Ferrari et al., 2007). A recent study also revealed that transgenic *Arabidopsis* lines overexpressing cotton *GhPGIP1* showed an upregulation of SA responsive genes upon infection with *V. Dahlia* (Liu et al., 2017). However, regulation of camalexin and glucosinolate pathways by *PGIP* was not shown. We propose that activation of *PGIP* expression to cyst nematode infection promote the formation of elicitors-active OGs, which in turn activate the expression of genes involved in glucosinolate and camalexin biosynthesis. In support of this hypothesis, we could show that upregulation of three key glucosinolate and camalexin biosynthesis genes (*CYP71B15/PAD3*, *CYP71A12*, *CYP81F2*) upon nematode infection was impaired in *pgip* mutants as compared to Col-0, especially in *pgip1*. Furthermore, we also found that prior treatment of plants with OGs leads to a significant decrease in nematode infection. As upon infection, both *pgip* mutants showed impaired induction of camalexin and indole glucosinolates, so we selected a double mutant *cyp79b2/b3*, which lack the accumulation of both the secondary metabolites. As expected, the double mutant showed enhances susceptibility to CN infection. The relevance of camalexin in cyst nematode infection has already been shown in a previous study where loss-of-function *pad3* mutants increases suceptibilty to *H. schachtii* (Ali et al., 2013) Nevertheless, impairment of upregulation of camalexin and glucosinolate genes is only partial in *pgip* mutants, which is likely due to the functional redundancy in this gene family. It is also plausible that these genes are regulated in a *PGIP*-dependent as well as in *PGIP* independent manner during cyst nematode parasitism. Intriguingly, RKN invasion of the *Arabidopsis* root has also been shown to induce the expression of *PAD3* during migratory stages of infection. Mutants that are impaired either in glucosinolate or camalexin biosynthesis are hypersusceptible to RKN (Teixeira et al., 2016). These previous observation together with the fact that we do not see any *PGIP* expression during early stages of infection point to the regulation of camalexin and glucosinolate biosynthesis in a *PGIP*-independent manner during plant-RKN interaction.

Nematode induced syncytia and giant cells are the permanent feeding sites for both cyst and RKN nematodes, respectively. They are highly metabolically active and provide nutrients to the nematode throughout their life cycle (Jones and Northcote, 1972a, b; Golinowski et al., 1996; Hussey and Grundler, 1998; Gheysen and Mitchum, 2011). A consistent expression of *PGIP* genes in syncytia as well as in giant cells during biotrophic stages of parasitism suggests that these genes may play a role in nematode parasitism other than activation of PTI-like defence responses.

Pectin is one of the most abundant macro molecules in the primary cell wall of plants, constituting 30-50% of cell wall matrix (Zablackis et al., 1995). It consists of a linear chain of homogalacturonan (HG), which is made up of 100-200 galacturonic acid residues. The main chain of HG is joined by branches of rhamnogalacturonan I and II (Mohnen, 2008; Ridley et al., 2001). HG is highly methylesterified during its biosynthesis in the endomembrane system and is de-methylesterified after its secretion in the cell wall through the action of pectin methylesterases (PME) (Micheli, 2001). Stretches of de-esterified HG can then form cross-linkages with Ca+ ions, which may lead to a reduced porosity and flexibility of the cell wall. Moreover, pectin-degrading enzymes (pectate lyases and PGs) are unable to hydrolyse HG, which is methylesterified. Therefore, the extent and pattern of methylesterification strongly influences on mechanical properties of the cell wall. Previous studies have shown that HG in the cell walls of younger syncytia (5dpi) is highly de-esterified as compared to older syncytia (15 dpi). In contrast, highly methylesterified HG was abundant in cell wall of younger (7dpi) as well as older (14 dpi) giant cell (Davies et al., 2012; Wieczorek et al., 2014). The differences in methyle sterification of younger feeding sites associated to CN or RKN may be due to their different ontogeny. Syncytium expands through dissolution of cell wall and fusion of root cells. During their expansion, walls have to be locally digested and modified, which leads to their strengthening and thickening. In comparison, giant cells grow with repeated nuclear division without cytokinesis. Their cell walls are modified to become thick and strong without being digested. Therefore, a high de-esterification of cell wall at 5dpi may facilitate their digestion and help in expansion of syncytium. On the other hand, a higher level of methylester in older feeding sites of both CN and RKN may provide a higher strength and flexibility to the cell wall, which may contribute to the capacity of these feeding sites to sustain high turgor pressure during parasitism (Böckenhoff and Grundler, 1994). *PGIPs* have been shown to interact with partially

or completely de-esterified HG, thus shielding it from the hydrolysing activity of plant or pathogen PGs (Spadoni et al., 2006). The level of *PGIP* expression therefore contributes to some of the mechanical properties of cell wall related to growth and development. We propose that the intense expression of *PGIPs* in younger syncytium at 5dpi controls cell wall degradation by not only directly binding to PGs (of plant or nematode origin) but also to HG, thus protecting it from further degradation. This hypothesis is in line with our observations that knocking out or overexpression of *PGIP1* results in a significant increase or decrease in the average size of syncytium. As the syncytium expands and reaches its maximum size, cell wall degradation is slowed down, which is accompanied by a decrease in *PGIP* expression as well. Unlike syncytium, *PGIPs* expression in giant cells is present throughout sedentary stages of nematode development, which may protect the walls of giant cells from being degraded by blocking de-esterified stretches of HG. However, no visible phenotypes for RKN infection were observed in any of the tested lines in this work, which makes it unlikely that *PGIP* plays any role in RKN parasitism.

In conclusion, our results provide a molecular mechanism underlying the PGIP-mediated damage-associated responses during cyst and RKN parasitism of plants. We showed that the differential regulation of *PGIPs* during cyst and RKN invasion of roots reflect the differences in their migration and feeding habits. Moreover, regulation of camalexin and glucosinolate pathways by *PGIP* in an infection-specific manner is demonstrated. This study also shed light on mechanism behind the unparalleled success of *M. incognita* parasitism. Clarifying further details of DAMP-associated pathways in plant-nematode interaction may lead to novel control measures against this important plant parasite.

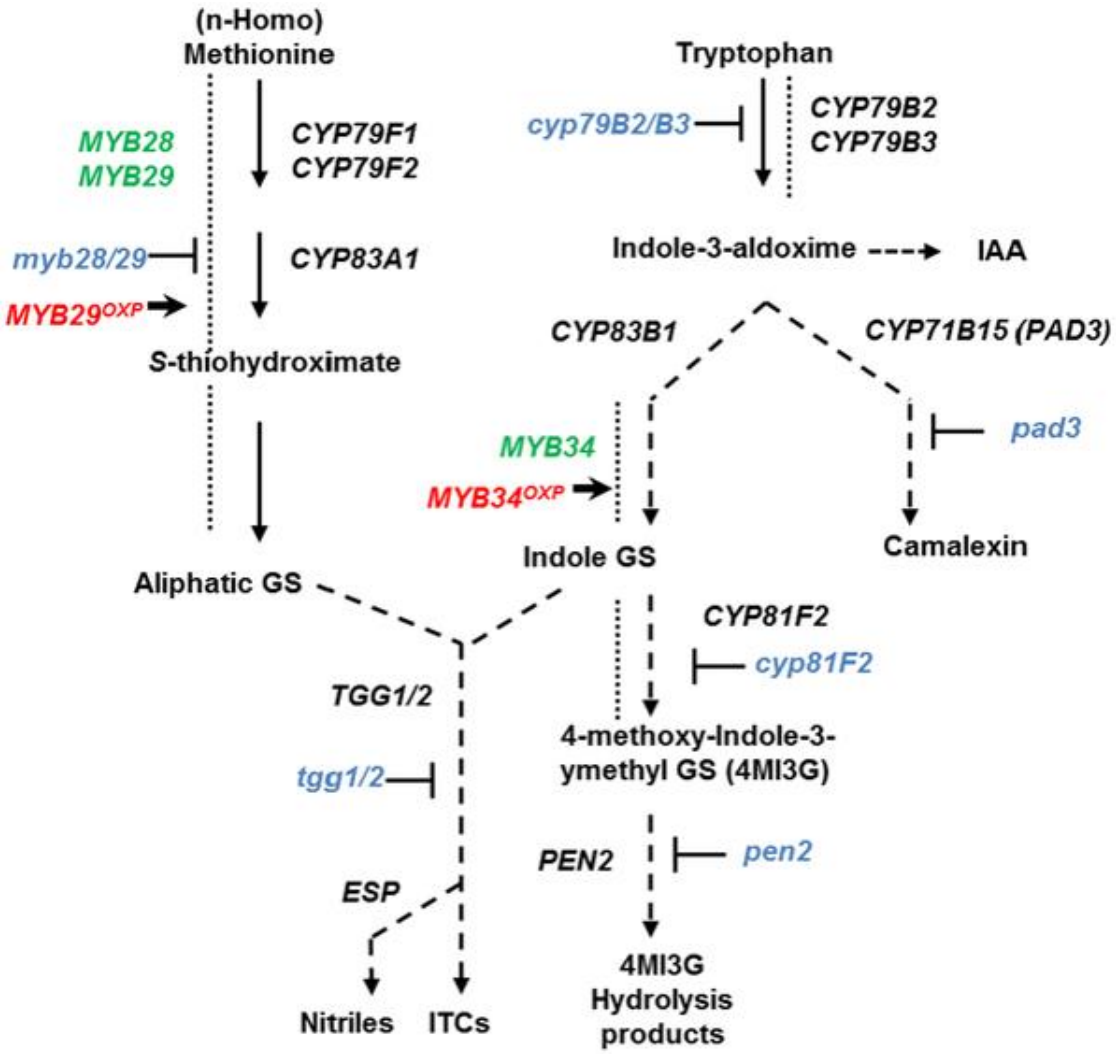


Figure 16: Schematic representation of methionine and tryptophan derived glucosinolates and camalexin in *Arabidopsis*. Black letters indicate genes involved in biosynthesis of camlalexin and glucosinolates, blue letters shows mutations. *MYB28/29* (green letters) are the enzymes involved in the conversion of methionine to aliphatic glucosinolates. *CYP79B2/CYP79B3* are involved in the the conversion of tryptophan to indole-3-acetaldoxime (IAOx) which serves as a precursor for the synthesis of camalexin, Indole glucosinolates and auxin (Buxdorf et al., 2013).

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6. Annex

Supplementary Data

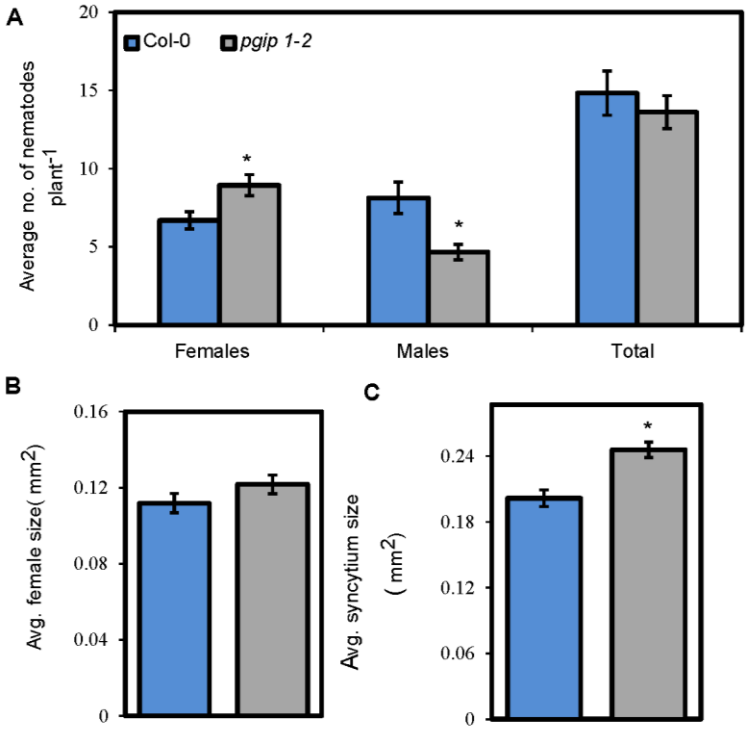
Supplementary Table1: Expression of selected marker genes during migratory stage of cyst nematode infection.

Locus	Gene	Root vs migratory stage (10 hpi) (Mendy et al., 2017)	Biological function
At5g57220	*CYP81F2*	16.66	Involved in indolicglucosinolates synthesis
At3g26830	*CYP71B15*	16.81	Catalyzes the final step in camalexin biosynthesis
AT2G30750	*CYP71A12*	15.71	Involved in camalexin biosynthesis
At1g30135	*JAZ8*	15.83	JA signaling marker gene
At4g26120	*NPR2*	4.06	SA marker gene
At5g64900	*PROPEP1*	3.17	Wound induced marker

Supplementary Table 2: Efficiency check of the primers used through qPCR.

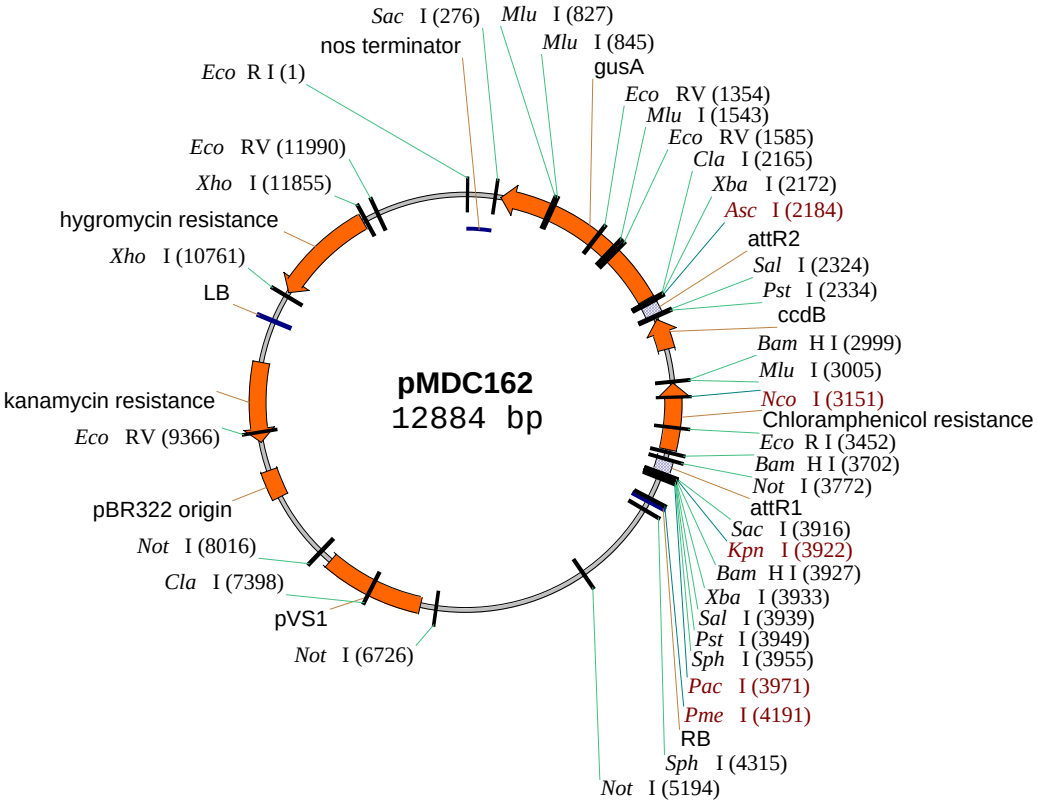
locus	Gene symbol	Primer efficiency
At5g57220	*CYP81F2*	95
At3g26830	*CYP71B15*	103
AT2G30750	*CYP71A12*	98
At1g30135	*JAZ8*	98
At4g26120	*NPR2*	106

At5g64900	*PROPEP1*	102

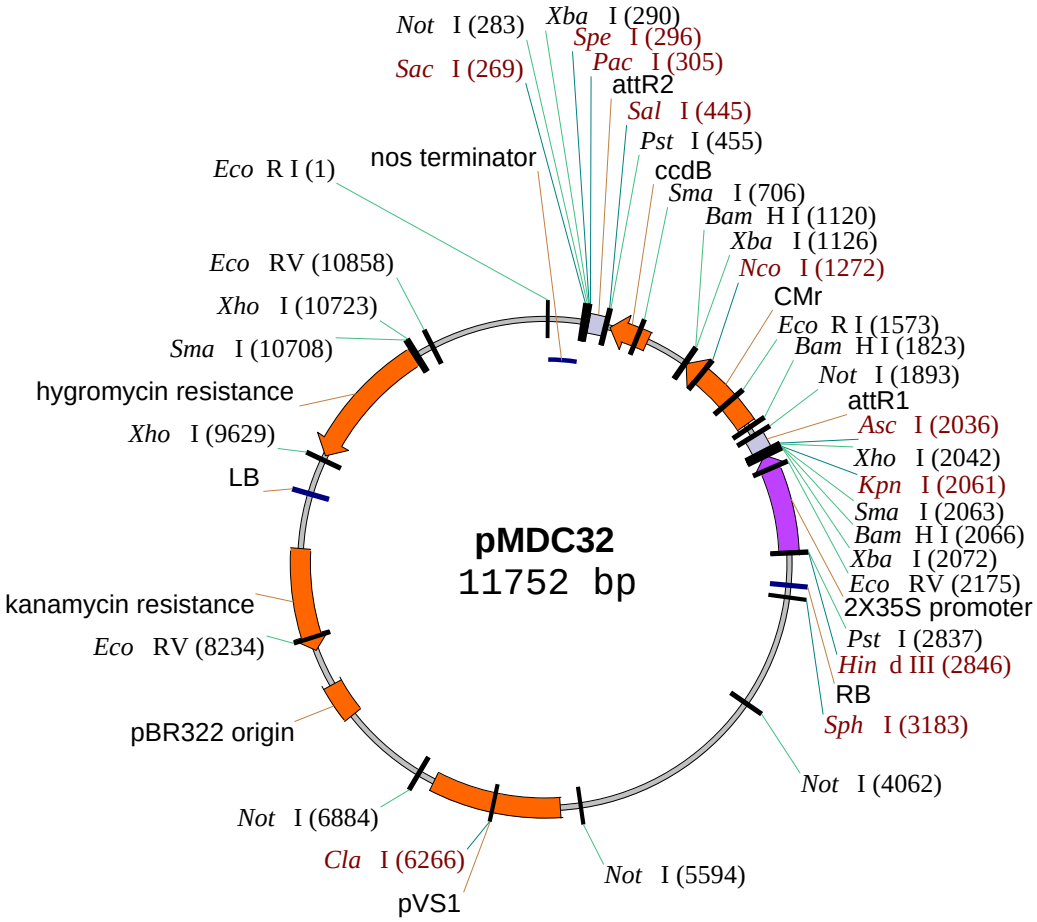


Supplementary Figure 1: Nematode infection assays in *pgip1*1 (*pgip1-2*) mutant lines. (A) Average number of females and males per plant present in Col-0 and *pgip1-2* mutant lines at 14 dai. (B, C) Average females sizes (B) and plant syncytia (C) in Col-0 and *pgip1-2* mutant lines. Bars represent mean ± SE for three independent biological replicates. Data were analysed using student's T-test ($P< 0.05$). Asterisks represent statistically significant difference to corresponding Col-0.

Supplementary Figure 2: Vector map for the gateway destination vector pMDC 162 (www.arabidopsis.org).



Supplementary Figure 3: Vector map for the gateway destination vector pMDC 32 (www.arabidopsis.org).



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8. Curriculum vitae

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2013- Present date	Ph.D from INRES, Molecular Phyomedicine, University of Bonn, Germany.
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Work Experience:

- Six months field research at The University of Agriculture Peshawar as a Research Fellow under the Project title "Modified Double Cross Maize Hybrid Production as a Source of low Cost Seed" from June to December 2008.
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- Syed Jehangir Shah, Muhammad Shahzad Anjam, Muhammad Arsalan Anwar, Shahid Siddiqui and Florian.M.W. Grundler. 2017. Damage-associated responses of the host contributes to defence against cyst but not root-knot nematode infection. **Journal of Experimental Botany (Submitted).**
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