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Studies on nematode factors activating the plant immune system

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“This work is dedicated to my grandfather **Bisenty Nafichar Mendy** (May his soul continues to rest in perfect peace)”

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Abstract

Plant-parasitic nematodes are destructive pests causing crop losses accounting for billions of dollars annually. To defend against invading pathogens plants rely on innate immune system which involves the recognition of microbe/pathogen associated molecular pattern (MAMPs/PAMPs) and endogenous damage associated molecular pattern (DAMPs), by plasma membrane localized pattern recognition receptors (PRRs). Recognition of these molecular signatures activates responses as Pathogen Associated Molecular Pattern-Triggered immunity (PTI). The role of PTI during plant pathogen interaction has been well studied for many microbes of pathological importance, but not well described for plant nematode interactions.

Here we examined the role of PTI at early stages of nematode infection on plants. We describe the activation of PTI responses in *Arabidopsis thaliana* by a nematode aqueous *diffusate* termed NemaWater in a manner depending on a common co-receptor BAK1. Experiments performed after treatment of NemaWater with proteinase K and also with heating, reduce the PTI-like responses observed in untreated NemaWater samples. These results further indicate that the elicitor/s contained in NemaWater is/are of proteinaceous in nature. Considering the role played by BAK1 as co-receptor specifically for those PRRs that recognizes proteinaceous ligands, we identified a leucine-rich repeat receptor-like kinase, termed NILR1 that was specifically regulated upon infection by nematodes. Loss-of-function mutants of NILR1 were hypersusceptible to several nematode species and exhibited impaired PTI responses triggered by NemaWater. We show that NILR1 is essential for PTI responses initiated by nematodes (Chapter 2).

NemaWater protein fraction analysis revealed the presence of nematode proteins components including surface coat associated proteins (Chapter 3). We hypothesize that plants are able to recognize nematode through an unknown conserved protein molecule possibly exposed on the surface of the nematode cuticle.

Apart from recognition of conserved nematode associated molecular patterns by PRRs, plant innate immunity can also be activated as a result of cell damage and subsequent release of endogenous DAMP molecules. Plant invasion by nematodes and migration through cells causes cell-damage and possible release of cell wall fragments either in the form of oligogalacturionides (OGs), ATP, or small peptides that can act as DAMPs and activate host defence responses. These

molecular mechanisms mediating damage responses during plant-nematodes interactions are not well understood. Here we report that polygalacturonase-inhibiting proteins (PGIPs) genes involved in the formation of active OG elicitors in Arabidopsis were strongly induced in response to cyst nematodes. Experiments with loss-of-function mutants and overexpression lines showed an increased and reduced cyst nematode infection, respectively. These finding suggest that cyst nematode during migration within the root cause cell damage which as a result induce camalexin and indole-glucosinolate biosynthesis pathways in a PGIP- dependent manner thereby restricting nematode establishment and development (Chapter 4). The exact ligands that interact directly with PGIPs and how active OGs are formed and act as elicitors of defense during nematode infection of plants are still elusive.

In Chapter 5, we studied the Arabidopsis peptide receptor (AtPEPRs) and their role in defense responses against nematode. Our result showed a high susceptibility of *pepr1/2* double mutant to cyst nematode. In contrast, we did not observe significant differences in root-knot nematode infection of *pepr1/2* mutant compared to control.

This study clearly indicates that plants have a recognition mechanism for nematode elicitors as well as host derived elicitors released as a result of cell damage caused by nematodes. Understanding how both defense regulation pathways function together will provide valuable information for engineering durable crop resistance against plant parasitic nematodes and increase crop yield.

Zusammenfassung

Pflanzenparasitäre Nematoden sind wichtige Schaderreger, die jährlich Ertragsverluste von vielen Milliarden Dollar verursachen. Pflanzen verteidigen sich gegen eindringende Pathogene mit Hilfe ihres Immunsystems, das auf der Erkennung von „Microbe/Pathogen Associated Molecular Patterns“ (MAMPs/PAMPs) und endogenen „Damage Associated Molecular Patterns“ (DAMPs) durch in der Plasmamembran lokalisierte Pattern Recognition Receptors (PRR) beruht. Die Erkennung der molekularen Signaturen führt zur Aktivierung der „Pattern-Triggered Immunity“ (PTI). Die Rolle der PTI ist für eine Reihe von wichtigen Pflanzen-Pathogen-Interaktionen gut untersucht, für die Interaktion zwischen Pflanzen und Nematoden aber kaum bekannt.

Ziel der vorliegenden Arbeit war es, die Rolle von PTI in der frühen Infektionsphase zu untersuchen. Es zeigte sich , dass PTI in *Arabidopsis thaliana* durch eine wässrige Lösung von Nematodenausscheidungen, die als NemaWater bezeichnet wurde, in Abhängigkeit vom Ko-Rezeptor BAK1 ausgelöst wird. Die Behandlung von NemaWater mit Proteinase K und Hitze reduzierten die PTI-artigen Pflanzenreaktionen, die mit unbehandeltem NemaWater ausgelöst werden. Daraus kann geschlossen werden, dass in NematWater enthaltene Elizitoren aus Protein bestehen. Unter Einbeziehung der Rolle, die der BAK1 Korezeptor spezifisch zusammen mit den PRR spielt, die Proteinliganden erkennen, gelang es uns, eine nematoden responsive Leucine-Rich Repeat Receptor-like Kinase zu identifizieren, die wir als NILR1 bezeichneten. Funktionsverlust-Mutanten von NILR1 waren gegenüber mehreren Nematodenarten hypersuszeptibel und zeigten nach NemaWater-Behandlung eine eingeschränkte PTI-Reaktion. Wir konnten zeigen, dass NILR1 eine essentielle Rolle für die Auslösung von PTI durch Nematoden spielt (Kapitel 2).

Analysen der Proteinfraktion von NemaWater ergaben, dass sich darin verschiedene Nematodenproteine einschließlich Proteine der Nematodenoberfläche (Kapitel 3) befanden. Daher stellen wir die Hypothese auf, dass Pflanzen in der Lage sind, Nematoden anhand einer bisher unbekannten konservierten Proteinmoleküls zu erkennen, das möglicherweise auf der Oberfläche der Kutikula präsentiert wird.

Neben der Erkennung von „Nematode Associated Molecular Patterns“ durch PRR kann das pflanzliche Immunsystem auch durch geschädigte Zellen und nachfolgend freigesetzte endogene DAMP Moleküle aktiviert werden.

Wenn Nematoden in Pflanzen eindringen und durch Zellen wandern verursachen sie Zellschäden und damit möglicherweise die Freisetzung von Zellwandbestandteilen entweder in Form von Oligogalacturoniden (OGs), kleinen Peptiden oder ATP-Molekülen, die als DAMPs die Wirtsabwehr aktivieren können. Die molekularen Abläufe während der Interaktion zwischen Pflanzen und Nematoden im Zusammenhang mit der Reaktion auf Zellschäden sind weitgehend unbekannt. Wir konnten zeigen, dass Gene, die für Polygalacturonase-Inhibiting Proteins (PGIPs) kodieren und für die Bildung von OG-Elizitoren in Arabidopsis verantwortlich sind, im Verlauf der Infektion durch Zystennematoden stark aufreguliert sind. Experimente mit Funktionsverlust-Mutanten und Überexpressionslinien zeigten eine gesteigerte bzw. reduzierte Nematodeninfektion. Diese Ergebnisse zeigen, dass Zystennematoden während der Wanderung durch die Wurzel Zellschäden verursachen und abhängig von PIGPs die Biosynthesewege von Camalexin und Indol-Glukosinolaten aktivieren, wodurch sie die Etablierung und die Entwicklung von Nematoden einschränken (Kapitel 4). Allerdings ist noch nicht bekannt, welche Moleküle als Liganden der PGIPs agieren, wie OGs gebildet werden und wie diese als Elizitoren der Abwehrreaktion gegen Nematoden agieren.

In Kapitel 5 wird beschrieben, wie die Rolle von Peptidrezeptoren in Arabidopsis (AtPEPRs) bei der Abwehr gegen Nematoden untersucht wurde. Die Doppelmutante *pepr1/2* war gegenüber Zystennematoden hoch anfällig, zeigte aber in der Reaktion auf den Befall von Wurzelgallennematoden keine signifikanten Unterschiede zur Kontrolle.

Die hier beschriebenen Untersuchungen zeigen, dass Pflanzen einen molekularen Mechanismus besitzen, mit dem sie aus Nematoden stammende Elizitoren und pflanzliche Elizitoren, die durch Nematoden verursachte Zellschäden freigesetzt werden, erkennen können. Erkenntnisse, die dazu führen, zu verstehen, wie beide Abwehrwege zusammenspielen, geben wertvolle Informationen zur Entwicklung von dauerhafter Resistenz gegen pflanzenparasitäre Nematoden.

Acronyms and abbreviations

Ascr#18	Ascarosides #18. Nematodes
Ax21	Activator of XA21
BAK1	BRASSINOSTEROID INSENSITIVE 1-Associated receptor Kinase 1
CERK1	Chitin Elicitor Receptor Kinase 1
CEBiP	Chitin Elicitor Binding Protein
DNA	Deoxyribonucleic Acid
EFR	Elongation Factor -Tu receptor
EF-Tu	Elongation Factor Tu
EGF	Epidermal Growth Factor
EIX1	Ethylene biosynthesis-Inducing Xylanase
Flg22	Bacterial PAMP Flagellin 22 amino acid epitope
FLS2	Flagellin Sensitive 2
GBP	β-Glucan Binding Protein
INF1	*Phytophthora infestans* elicitin 1
J2s	Second stage juveniles
LeEIX1	Ethylene biosynthesis-Inducing Xylanase receptor 1
LeEIX2	Ethylene biosynthesis-Inducing Xylanase receptor 2
LORE	LipoOligosaccharide-specific Reduced Elicitation
LRR	Leucine-Rich Repeat
LRR-RLK	Leucine-Rich Repeat Receptor like Kinase
LRR-RLP	Leucine -Rich Repeat Receptor like Protein
LysM-RLK	Lysin Motif receptor-like kinase
LysM-RLP	Lysin Motif receptor-like Protein
MAMPs	Microbe Associated Molecular Pattern

MBL	Mannose-Binding Lectin
NAMPs	Nematode Associated Molecular Pattern
NbLRK1	*Nicotiana benthamiana* Lectin-like Receptor Kinase 1
NgRLK1	*Nicotiana glutinosa Receptor*-Like Kinase 1
NILR1	Nematode Induce Leucine rich repeat Receptor 1
NTI	NAMP-Triggered immunity
OG	Oligogalacturonides
PAMPs	Pathogen Associated Molecular Patterns
PEPR1	Peptide Receptor 1
PEPR2	Peptide Receptor 2
Pep1	DAMP Peptide 1 in Arabidopsis
Pep2	DAMP Peptide 2 in Arabidopsis
PG	Polygalacturonase
PGIP	Polygalacturonase-Inhibiting Proteins
PGN	Peptidoglycan
PM	Plasma Membrane
PPN	Plant Parasitic Nematodes
PR5	Pathogenesis-Related protein 5
PROPEP	Precursor of Peptide
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
RKNs	Root Knot Nematodes
RLKs	Receptor-Like kinase
RLPKs	Receptor-Like Protein Kinases
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

SC	Surface Coat
SlSERK3A	*Solanum lycopersicum* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3A
SlSERK3B	*Solanum lycopersicum* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3B
SRK	S-locus Receptor Kinase
TNFR	Tumor-Necrosis Factor Receptor
WAK1	Wall-Associated receptor Kinase 1
XA21	*Xanthomonas* resistance 21

Chapter 1 Introduction

1. Nematodes

Nematodes are small round worms belonging to the kingdom Animalia and phylum Nematoda. They are known to be the most abundant animals on the planet (Adams *et al*., 2006). More than 28,000 nematode species have been described (Hugot *et al*., 2001; Wyss, 2002), which is less than 3% of the total nematode species assumed to exist (De Deyn, Raaijmakers *et al*., 2003). Majority of nematode species are free living, but an estimated 15% (more than 4100 nematode species in 197 genera) are described as plant parasitic (Decraemer & Hunt, 2006; Decraemer & Hunt, 2013; Blok *et al*., 2008). Apart from being plants parasitic, some nematodes parasitize animals and are a threat to human lives as diseases causing agents (Hotez *et al.,* 2008; Chan *et al*., 1994). Other types of nematodes are beneficial as decomposers of organic matter and are involved in nitrogen mineralization processes (Ferris *et al*., 1998; Beare, 1997). Nematode species are biologically diverse and versatile, occupying diverse habitats and constitute nearly 90% of all metazoans in number (Hugot *et al*. 2001). One of the best known and well-studied animals is the bacterial feeding nematode *Caenorhabditis elegans*. Being the first animal to have its DNA sequenced completely (Riddle *et al.,* 1997), *C. elegans* helped scientist understand function of individual cells in nematode development. Further, it also provided insights into the nematode biology, neural development and behavior (Riddle *et al.,* 1997; Sulston *et al*., 1988; Kenyon, 1988).

1.1. Nematode cuticle structure and surface coat

The cuticle of nematodes is a very important component having multiple roles, ranging from protection against the harsh environment to acting as an exoskeleton for the attachment of muscles for locomotion and maintenance of post-embryonic body shape (Wright, 1987). In parasitic species, the cuticle represents the site of contact with the host's immune responses (Kennedy & Harnett (Eds.), 2013). Based on microscopic observation of the internal anatomy, the nematode cuticle is divided into three distinct layers and transverse structures as illustrated by Davies & Curtis, 2011 **Fig.1**. These layers are the cortical, medial, and basal layer (Bird & Bird, 1991; Lee, 2002; Baldwin & Perry, 2004).

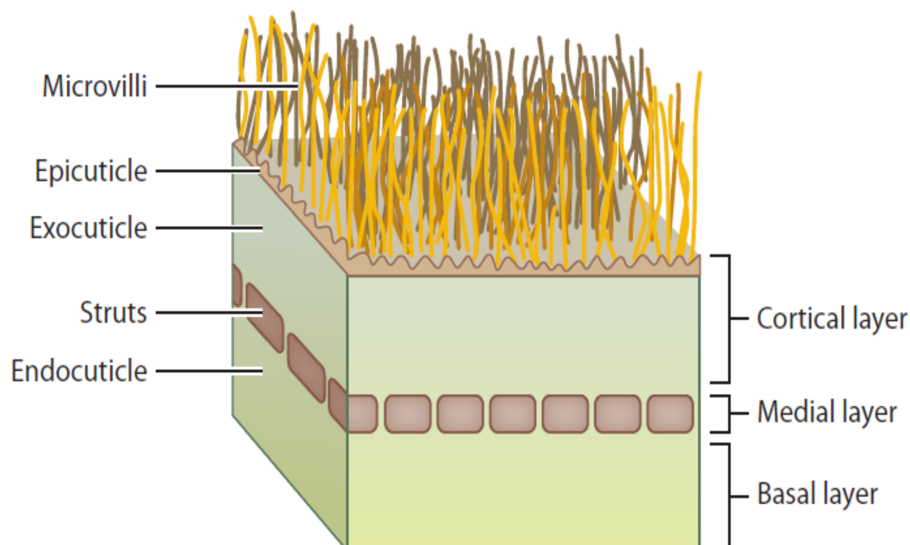

Figure 1: The internal anatomy of the nematode cuticle. The basal layer (endocuticle) is striated and contains collagen. The medial layer also contains collagen but can be variable and often contains struts. The cortical layer(the epicuticle) lies above the medial layer and possesses a number of specialized features such as annulations and alae which are made up of cuticlins, lipids, glycolipids, proteins, and glycoprotein's. Overlying the epicuticle is an amorphous fuzzy structure, the microvilli, that is carbohydrate rich and contains mucins which make up the surface coat or glycocalyx (Davies & Curtis, 2011).

The basal layer is striated and made of collagens. The medial layer possesses struts, which connect the basal and cortical layers together (Edgar *et al*., 1982). Above the median layer lies the cortical layer possessing a number of specialized features such as annulations and prominently made of cuticlins, lipids, protein, and surface associated carbohydrates (Blaxter & Robertson, 1998; Cox *et al*., 1981). The outer layer is the epicuticle overlaid by an amorphous carbohydrate-rich surface coat (SC) or glycocalyx **Fig.1; (**Himmelhoch & Zuckerman, 1978; Zuckerman *et al*., 1979). Hence the cuticle serves as the first point of contact between the nematode and the host organism. The most widely studied cuticle is that of *C. elegans* (Cox *et al*., 1981; Edgar *et al*., 1982). There are conflicting arguments concerning the suitability of *C. elegans* cuticle to be used as a model to study cuticle of plant-parasitic nematodes (PPN). Nevertheless, it is still used to understand aspects of host-pathogen interactions that involve studying cuticle and its surface.

1.2. Plant parasitic nematodes

Different species of Plant parasitic nematodes (PPN) parasitize different plant organs such as seeds, stem and leaves, but majority of them are parasites of roots. PPNs can be grouped in various classes depending on their mode of parasitism (Perry & Moens, 2011). Majority PPN feed outside without entering the root tissues and migrates from one cell after the other causing damage (Bridge & Starr, 2007). This class of PPN is referred to as migratory ectoparasites (e.g. *Tylenchorhynchus spp*. and *Longidorus spp*.). Sedentary ectoparasites contrary to migratory ectoparasites feed on same root tissue for few days before moving to another (e.g. *Criconemilla spp*., and *Paratylenchus spp*.). However, *Hoplolaimus spp*. and *Helicotylenchus spp* are semi-endoparasites feeding both outside of root tissue and partially entering the root cortical or outer stellar cells (Yeates *et al*., 1993). By contrast to ectoparasites, endoparasitic nematodes feed inside the root tissues. They can be either migratory endoparasites, e.g. the root lesion nematode *Pratylenchus spp*, *Hirschmaniella oryzae*, and *Radopholus spp*. or sedentary endoparasites, which is the economically most important and widely studied group of PPN and includes root-knot nematodes (*Meloidogyne spp*.) and cyst nematodes (*Heterodera spp*. and *Globodera spp.)* (Perry & Moens, 2011). Because most plant parasitic nematodes live in the soil, the damage caused by them is difficult to identify, estimate and control (Stirling *et al*., 1998). Farmers, agronomists and pest management consultants have long underestimated the impact caused by plant parasitic nematodes. It has been estimated that about 10 % of world crop production is lost as a result of plant nematode damage **Table 1**(Sasser & Freckman, 1987). Monetary crop losses due to nematode exceeds $100 billion annually (McCarter, 2009; Koenning *et al.,* 1999), making them a major threat to agricultural production and food security worldwide.

Table 1: Worldwide estimated losses due to damage by plant parasitic nematodes (Sasser & Freckman, 1987)

Life sustaining crops	Annual Loss (%)	Economically important crops	Annual Loss (%)
Banana	19.7	Cacao	10.5
Barley	6.3	Citrus	14.2
Cassava	8.4	Coffee	15
Chickpea	13.7	Cotton	10.7
Coconut	17.1	Cowpea	15.1
Corn	10.2	Eggplant	16.9
Field bean	10.9	Forages	8.2
Millet	11.8	Grapes	12.5
Oat	4.2	Guava	10.8
Peanut	12	Melons	13.8
Pigeon pea	13.2	Misc. Other	17.3
Potato	12.2	Okra	20.4
Rice	10	Ornamentals	11.1
Rye	3.3	Papaya	15.1
Sorghum	6.9	Pepper	12.2
Soybean	10.6	Pineapple	14.9
Sugar beet	10.9	Tea	8.2
Sugar cane	15.3	Tobacco	14.7
Sweet potato	10.2	Tomato	20.6
Wheat	7	Yam	17.6
Average	**10.70%**	**Average**	**14.00%**
	Overall average 12.3%		

1.3. Cyst Nematodes

The cyst nematodes are important parasites with a highly specialized interaction with plants. They induce the formation of a syncytial structure within the roots of their host, which serves as nutrient source for the developing nematode (Moen *et al*., 2018; Wieczorek & Grundler, 2006; Hofmann & Grundler, 2007). Cyst nematode are grouped into eight genera within the subfamily Heteroderinae e.g. *Heterodera, Globodera, Paradolichodera, Dolichodera, Cactodera, Betulodera, Punctodera* and *Vittatidera*, (Subbotin *et al*., 2010[a, b]). The most economically important species belong to *Globodera* and *Heterodera* genera (Moens *et al*., 2018). The cyst nematodes have a unique and common feature in that, the adult female turn her cuticle to a

strong and durable protective cover for the eggs. This cover helps the eggs and juveniles to persist in the soil for a long period of time till the environment is deemed favorable to hatch.

1.4. Sugar Beet cyst nematode – *Heterodera schachtii*

Beet cyst nematode (*Heterodera schachtii* Schmidt) was first observed in 1859 by Hermann Schacht near Halle, Germany and later described by Schmidt in 1871. *H. schachtii* is a major pest in sugar beet (*Beta vulgaris*) production with a wide host range within 95 genera from 23 different plant families including *Chenopodiaceae* and *Cruciferae* (Steele, 1965; Grundler *et al*., 1997). The impact includes yield losses and decrease in sugar content of sugar beet. Due to its low mobility, *H. schachtii* depends on soil moisture to spread (Wallace, 1958). Symptoms occur in patches in the field. Infected plant shows stunted growth with leaves of severely affected plant turning yellow due to decreased chlorophyll contents (Hillnhünter *et al*., 2012). Distribution of cyst nematodes within and among fields can be by irrigation water, vegetative plant parts, and soil infested with eggs or larvae, which adhere to farm implements, animals, or humans (Riggs, 1977; Gray *et al*., 1992). Annual yield losses as a result of *H. schachtii* are estimated at $90 million annually (Müller, 1999).

1.4.1. Life cycle

Eggs of *H. schachtii* are embedded in the dead body of the female known as the cyst and might stay in the soil for several years until the environmental conditions become favorable for the second stage juvenile (J2) to hatch. Several factors can contribute in triggering the J2 to hatch including contact with roots of the host plant (Harveson & Jackson, 2008), adequate soil moisture and temperature above 50ºC. The root exudates also stimulate the J2 to hatch (Harveson & Jackson, 2008). Plant volatiles, temperature, pH, CO_2 concentrations guide the J2s to move towards the root of host plant (Perry, 1997; Fenoll *et al.*, 1997).

The infective J2s penetrate the root in the elongation zone above the root tip (Wyss & Grundler, 1992; Lilley *et al.,* 2005*)* and migrate towards the vascular cylinder where they selects a single cell to induce an initial syncytial cell without destroying the plasma lemma **(Fig. 2).** Afterwards, this single cell develops into a multinucleate syncytium through local dissolution of cell wall, and the formation of a large multinucleate feeding structure (Gheysen & Jones, 2006). The mechanism of selecting an initial syncytia cell is not known but the secretions from the nematode glands trigger the host cell to undergo structural modification, which involves a massive

reprogramming of root cell development, and expand through the incorporation of neighboring cells. The protoplast becomes fused thus forming a syncytium. During the following weeks, juveniles undergo three moults (J3, J4, and adult) (Wyss & Grundler, 1992) and sex can be determined before the J2 moult to J3 stage (Grundler, 1988; Wyss, 1992). After the fourth moult, females become lemon-shaped, which are visible as white dots attached to the roots (**Fig. 2**). The swollen bodies in the adult stage become filled with eggs. Due to extensive growth during maturation, the swollen females rupture the root and only their anterior part remains embedded in the root tissue (Perry, 1989; Sijmons *et al*., 1991). Cyst nematodes are sexually dimorphic with the swollen lemon shaped bodies of mature female filled with about 200-250 eggs, becoming sedentary for the rest of their life.

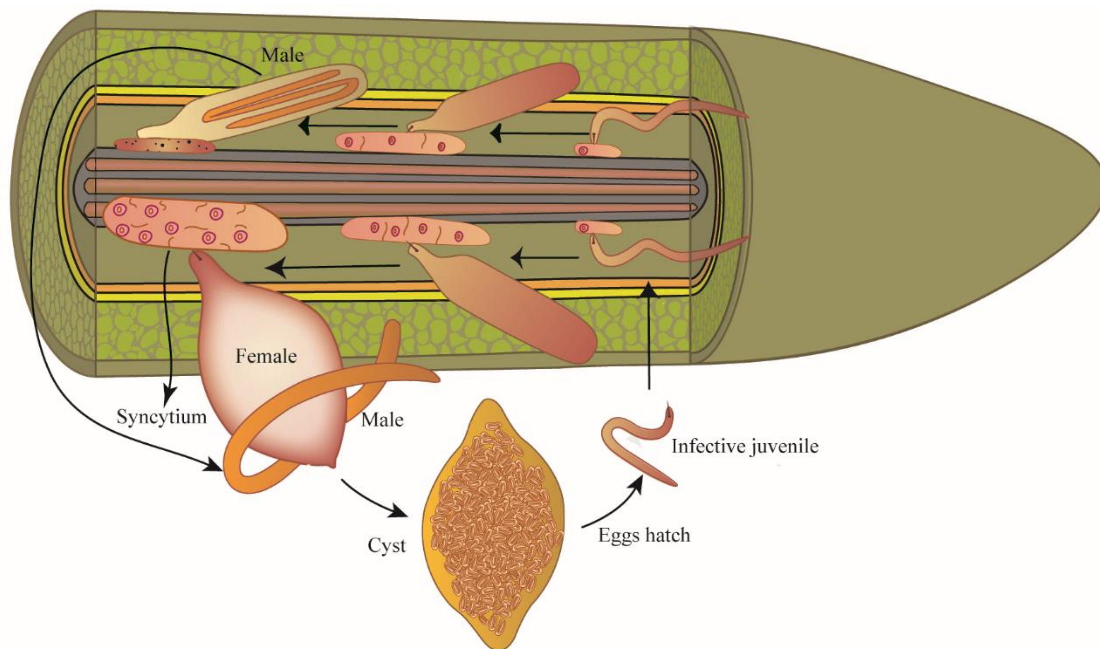

Figure 2: Life cycle of a cyst nematode (*H. schachtii*). It highlights the initiation of feeding site by second stage juvenile (J2s) and subsequent development into mature syncytia while the nematode becomes either male or female (Siddique & Grundler, 2018).The feeding structure of female nematodes is bigger in comparison to male which correlates to the size and amount of nutrient the female needs.

Males however, do not swell to the same extent and after molting to J4, they regain the vermiform body shape inside the J3 cuticle (**Fig.2**). The adult male emerges from the cuticle and migrates to a female for mating, attracted by sexual pheromones. After fertilization, the female

dies and becomes a robust brown cyst protecting up to several hundred eggs. In the absence of a host, juveniles within the eggs can persist in the cyst for several years (Sijmons *et al*., 1991). The mechanism of sex determination by *H. schachtii* is not well understood. However under favorable conditions, more J2 develop into mature female than male. In the event that host plants are less-susceptible, more males are developed as compared to female (Trugdill, 1967). High nutrient availability can promote female nematode development of *H. schachtii* while scarcities increase male development (Betka *et al.,* 1991). Mueller *et al*., 1981, reported that male J2 consume 29 times less food than female. Nevertheless, the host plants can influence the nematode size, penetration and establishment rate, number of eggs and syncytium size (Siddique *et al*., 2009). Defense gene activation and hypersensitive response of host cell could alter the development of nematode (Holbein *et al*., 2016).

1.5. Root-knot nematodes

Root-knot nematodes (RKNs) cause most significant economic damage in food crop production. The nematode was first described in 1887 by Göldi (*Meloidogyne exigua*) as the cause of root galling on coffee plants in Brazil (Chitwood, 1949). Göldi, 1892 described them as member of the genus *Meloidogyne* which is of Greek origin meaning, ‘apple- shaped female’. Based on temperature requirements, RKN are placed into two groups of species known as thermophils and cryophils (Moens *et al*., 2009). Thermophils species of *Meloidogyne* cannot survive soil temperature below 10 °C (e.g. *M. incognita, M. arenaria, M. javanica, M. exigua*), they are mostly predominant in the tropical and subtropical climatic conditions (Moens *et al*., 2009), whereas cryophils (e.g. *M. hapla, M. chitwoodi*), are able to survive below 10 °C soil temperatures (Moens *et al*., 2009). The asexually reproducing nematode (*Meloidogyne spp)* is the most widespread and probably the most economically important plant parasitic nematode pest of tropical and subtropical regions throughout the world. It occurs as a pest on a very wide range of crops (e.g. tomato, carrot, groundnut, etc.). Global annual crop losses of up to 5% are incurred due to RKN (Sasser & Carter, 1985).

1.5.1. *Meloidogyne incognita*

The southern root-knot nematode *Meloidogyne incognita*, is the most widespread and probably the most devastating plant parasitic nematode pest throughout the world (Sasser, 1979), especially in the tropical and subtropical regions. It belongs to the thermophils group of species of *Meloidogyne* which strive well in soil temperature above 10 ºC (Moens *et al*., 2009). The species is highly polyphagous with a broad host range. It's able to parasitize vegetable crops, fruit trees and ornamental plants. Global crop losses due to *M. incognita* is estimated at $78 billion (Chen *et al*., 2004), especially on cucurbitaceous and solanaceous plants. The assembled genome sequence of *M. incognita* is up to 86 Mb mostly existing in pairs of homologous and diverse segments (Abad *et al.,* 2008). The RKN, *M. incognita* is becoming a key model system for understanding plant metazoan interaction; not only because the genome is sequenced but also its ability to effectively infect *Arabidopsis thaliana*.

1.5.2. Life cycle

Mature females of RKN deposit eggs (up to 1000 or more) in a gelatinous matrix (egg sac or egg mass), which can be observed attached to the protruding posterior end of the females on the root surface (**Fig. 3a**). This sac protects the eggs from dehydration (Mitkowski & Abawi, 2003). The first stage juvenile develops in the egg, and the first moult usually occurs within the eggshell, giving rise to the second-stage juvenile, which emerges free into the soil or plant tissue (Bird, 2004). The infective second stage juveniles' hatch from the eggs and move through the soil in search of roots of suitable host plants (Bird, 2004). The J2 usually penetrate host roots just behind the root tip region (**Fig.4),** and establish their special permanent feeding sites (giant cells) in the vascular tissues of the root (**Fig. 3b;** Cabello *et al.*, 2014). The formation of giant cell during compatible interactions involved the reprogramming of selected cells and promoting mitosis and cell expansion with the aid of effector molecules secreted from the esophageal glands of the nematode (Mitchum *et al*., 2013; Marella *et al*., 2013; Cabello *et al*., 2014). Once the nematode begins feeding on tissue of a favorable host, the second, third and fourth moults occurs giving rise to the third, fourth and adult stages respectively (Mitkowski & Abawi, 2003). Between moults, there is further growth and development of the nematode, with concurrent development of the reproductive systems in the two sexes. Upon maturity, the female deposits eggs and the life cycle is repeated. The giant cells provide nutrients for the nourishment of sedentary nematodes throughout its lifecycle (Mitkowski & Abawi, 2003). Root cells around the

feeding sites are induced to enlarge and form galls (knots) and often involves an extensive secondary root formation and branching of the main root (**Fig. 3c)**.

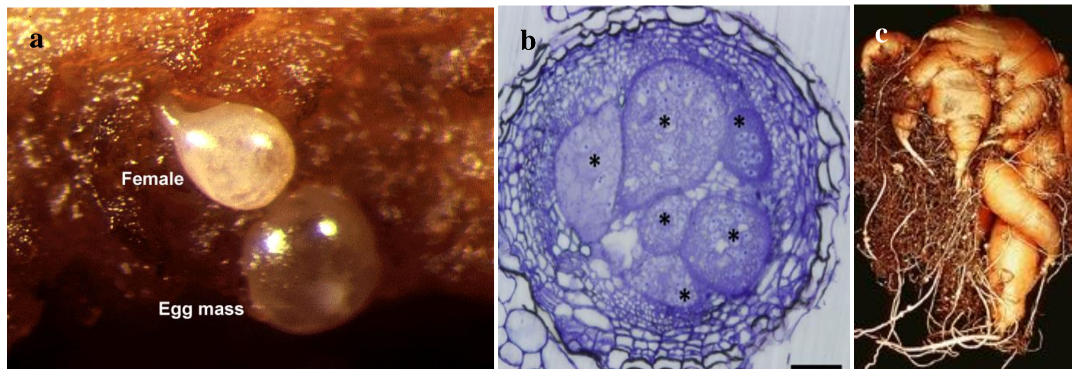

Figure 3: **Physical symptom of RKN crop infection a)** Root-knot nematode female and egg mass (Sardanelli, 2010), **b)** RKN feeding sites with group of cells known as the giant-cell (Escobar *et al.,* 2015), **c)** Carrot typically undergoes severe folding with galling predominantly found on lateral roots (Mitkowski & Abawi, 2003).

Depending upon the host and soil temperature, the entire life cycle of RKN may be completed within 17 to 57 days (Mitkowski & Abawi, 2003). The degree of root galling generally depends on three factors: nematode population density, *Meloidogyne* species and 'race', and host plant species or cultivar (Jones *et al*., 1967; Barker & Olthof, 1976). As the density of nematodes increases in a particular field, the number of galls per plant also increases. Large numbers of nematodes penetrating roots in close proximity also will result in larger galls. Finally, each crop responds differently to root-knot nematodes infection.

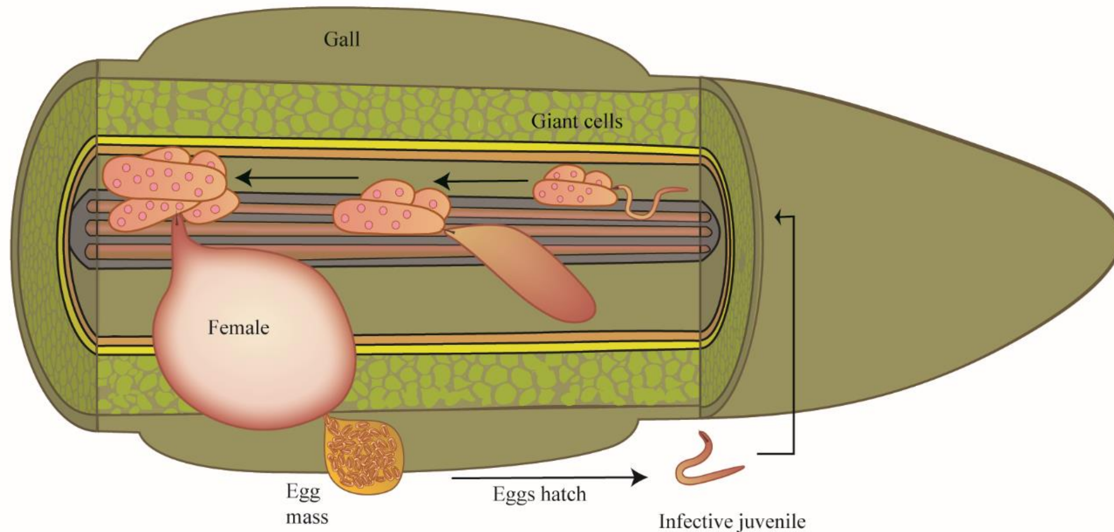

Figure 4: **Life cycle of root-knot nematodes (*M. incognita*)**. Pre-parasitic second stage juvenile hatches, guided by many factors find root and enter usually near the root tip region. After entry the nematode initiate the formation of permanent feeding structure (Giant cells), become sedentary and starts feeding. Manipulation of the cell cycle by the larva result in formation of galls and the nematode develop into adult female. Mature female produce egg mass which then develop into next generation (Siddique & Grundler, 2018)

1.6. *Arabidopsis thaliana* - a model plant

The small crucifer *A. thaliana* is a common model plant in molecular sciences because of the small genome size, short life cycle, and ease of propagation with a well-developed classical genetics of the species (Sijmons *et al*., 1991). It belongs to the *Brassicaceae* family with approximately 6 weeks of life cycle. The genome of *A. thaliana* composes of 125Mb with 115.4Mb sequenced regions. The genome consists of 25,498 genes encoding proteins from 11,000 different protein families (Arabidopsis Genome Initiative, 2000). *A. thaliana* was long thought to have the smallest genome among higher plant with only 5 chromosomes (2n=10), but the smallest known flowering plants' genomes recently is said to belong to plants in the genus *Genlisea*, order Lamiales, species includes *Genlisea margaretae*, a carnivorous plant, with a genome size of 63.4 Mbp (Greilhber *et al*., 2006), and *G. tuberose* has 61 Mbp (Fleischmann *et al*., 2014). *A. thaliana* genome was the first fully sequenced genome among plant species (Arabidopsis Genome Initiative, 2000). Colombia-0 ecotype is the most commonly used in

molecular research and mutants can be ordered from Stock centers such as Arabidopsis Biological Resource Center (ABRC) and Nottingham Arabidopsis Stock Centre (NASC). Advantage of *A. thaliana* as a model are numerous and include ease of transforming plants with *Agrobacterium tumefaciens* (Somerville & Koornneef, 2002), as well as by chemical or radiation mutagenesis to generate mutants. *Arabidopsis* is susceptible to many pathogen including bacteria and fungi (Dangl, 1993). Furthermore, *A. thaliana* is suitable host for a few root-parasitic nematodes (Sijmons *et al*., 1991; Wyss & Grundler, 1992; Grundler *et al* 1998; Wang *et al.,* 2016). In combination with *H. schachtii*, and *M. incognita,* this plant therefore served as model system during this study.

1.7. Plant defense strategies against pathogens

Plants are sessile and cannot evade pathogenic microbe or pest attack and therefore rely exclusively on the innate immune system for protection (Ausubel, 2005; Nürnberger *et al*., 2004; Zipfel & Felix, 2005; Jones and Dangl, 2006). Plants lack the adaptive immune system (Nürnberger *et al.,* 2009), including specialized immune cells, as present in higher animals (Medzhitov, 2007). Innate Immunity is a basal defense system against pathogen, which exists in both plant and animal kingdom, with conserved signaling components (Nürnberger *et al*., 2004; Zipfel *et al*., 2004; Nürnberger & Brunner, 2002). Since plant's entire immune response is not based on an adaptive/acquired system as seen in mammals (Nürnberger *et al.,* 2009; (Medzhitov, 2007), it would appear to be an evolutionary ancient defense mechanism able to genetically distinguish 'self' from 'non-self' (Medzhitov, 2001), and result in downstream cascades to counter or eliminate pathogen attack.

Pathogen associated molecular patterns (PAMPs) are recognized as conserved molecular signatures (non-self) by membrane localized pattern-recognition receptors (PRRs) of host cell serving as a surveillance system against invasion of pathogens (Akira & Hemmi, 2003; Boller & Felix, 2009). Recognition of these PAMPs initiates pattern-triggered immunity (PTI), the first line of defense in host plants as illustrated in the Zig-Zag model by Jones and Dangl, 2006 and in many other reviews (Zvereva *et al*., 2012). PAMPs are very unique to microbes and therefore are not present in the host organism (Medzhitov & Janeway, 1997).

Several microbes conserved molecular signature recognized by plant PRRs have been described (**Table 2.**), and includes bacterial flagellin (Gómez-Gómez *et al*., 1999; Felix *et al*., 1999;

Gómez-Gómez & Boller, 2002), lipopolysaccride (Aderem & Ulevitch, 2000; Medzhitov & Janeway, 2002; Newman *et al*., 2007; Livaja *et al*., 2008; Loucks *et al*., 2013), peptidoglycan PGN (Girardin *et al*., 2003; Gust *et al*., 2007), elongation factor Tu (Kunze *et al*., 2004; Zipfel *et al*., 2006), fungal chitin (Felix *et al*., 1993; Boller, 1995; Nürnberger & Brunner, 2002), and nematode pheromones Ascarosides (Manosalva *et al.*, 2015). Pathogens possess a wide array of PAMPs of diverse chemical structures and a single class of pathogen can have or secrete multiple PAMPs (Aderem & Ulevitch, 2000). The number of PAMPs presence in the plant-pathogen interaction sites may determine the intensity of induced gene expression. Different PAMPs may induce the same signaling system but the intensity of the defense signaling gene expression may differ (Zipfel *et al*., 2004). Also a single PAMP may not be able to activate all the defense signaling related genes and multiple of these conserved molecules maybe required to activate a complex signaling system.

1.8. Receptor-like kinases in PAMPs perception and defense activation

Receptor-like kinases (RLKs) are one of the largest gene families in plant (Melissa *et al*., 2012). John Walker and Ren Zhang, 1990 were the first to report and clone the protein kinases resembling animal receptor 'tyrosine' kinase from maize. As for them the clone contains a putative extracellular domain characterized by a signal sequence and a hydrophobic transmembrane region manifesting the phenotypic structure of RLKs (Walker & Zhang, 1990). Following that, a series of genetic research and phenotype studies by many scientists revealed the diverse roles of RLKs ranging from control of organisms growth and development (Shpak *et al*., 2005; Postel *et al*., 2010; Van Norman *et al*., 2011; Dao *et al*., 2018), to stress responses including both biotic and abiotic related stress (Walker, 1994; Krishna *et al*., 2003; Morillo & Tax, 2006; Osakabe *et al*., 2013). RLKs in developmental functions includes epidermal differentiation by CRINKLY-4 gene reported in maize (Bencraft *et al*., 1996), morphogenesis and determining organ shape by ERECTA gene in Arabidopsis (Torii *et al*., 1996), and maintenance of stem cells in the shoot apical meristems mediated by CLAVATA-1 gene in Arabidopsis (Tax & Kemmerling, 2012).

Table 2: Structure of plant pattern recognition receptors and their corresponding ligand

PAMP/Endogenous elicitor	Origin	PRR	Structure	Reference
Flg22	Gram-negative bacteria	FLS2	LRR-RLK	Felix *et al*., 1999; Gómez-Gómez*et al.*, 2001
EFTu	Gram-negative bacteria	EFR	LRR-RLK	kunze *et al*., 2004
Ax21	*Xanthomonas oryzae*	XA21	LRR-RLK	Song *et al*., 1995; Lee *et al*., 2009
Pep1	Plant DAMP	PEPR1	LRR-RLK	Huffaker *et al.,* 2006; Yamaguchi *et al*., 2006
Pep2	Plant DAMP	PEPR2	LRR-RLK	Yamaguchi *et al*., 2010
Xylanase (EIX1)	Fungi	LeEIX1, LeEIX2	LRR-RLP	Bailey *et al*., 1990; Ron & Avni, 2004
Chitin	All fungi	CERK1 (Arabidopsis)	LysM-RLK	Felix *et al*., 1993; Kaku *et al*., 2006; Miya *et al*., 2007
Chitin	All fungi	CEBiP (rice)	LysM-RLP	Shimizu *et al*., 2010
ß-glucans	fungi (*Pyricularia oryzae*), Oomycetes (*Phytophthora spp*)	GBP	Glycoside hydrolases	Umemoto *et al*., 1997
Mannose	Gram-negative bacteria	MBL	Lectin receptor kinase	Eddie *et al*., 2009
Oligogalacturonids	Plant DAMPs	WAK1	Wall-associated RLK	Brutus *et al*., 2010
INF1 elicitin	Phytophthora infestans	NbLRK1	Lectin-like receptor kinase	Kanzaki *et al*., 2008
Capsicein elicitin	Phytophthora capsici	NgRLK1	PR5 protein kinase	Kim *et al*., 2010
Lipopolysaccharides	Gram-negative bacteria(*Xanthomonas, Pseudomonas*)	LORE	Lectin S-domain RLK	Newman *et al*., 1995; Ranf *et al*., 2015
Peptidoglycan	Gram-positive and Gram-negative bacteria	Lym1 & Lym3	LysM-receptor kinase	Gust *et al*., 2007; Willmann *et al*., 2011
Ascarosides (Ascr#18)	Nematodes	Unknown	Unknown	Manosalva *et al*., 2015

More than 600 RLKs protein have been reported in *A. thaliana* (Shiu & Bleecker, 2001; Torii. 2004), and rice have more than 1131 RLK protein (Shiu *et al*., 2004). Belvin and Anderson, (1996), reported that the plant RLKs are closely related to RLK/*pelle* family of *Drosophila melanogaster* fruit flies. Cao *et al*., 1996, also reported their close relation to mammalian Interleukin Receptor-Associated Kinases (IRAK), which has a small family of cytoplasmic kinases without extracellular domain (ECD) or transmembrane region.

Receptor-like cytoplasmic kinases (RLCKs) belong to the RLK family lacking extracellular domain and transmembrane regions (Shin & Blecker, 2001). Both RLKs in plant and mammalian IRAKs are likely to be orthologs inherited from an ancestral kinase that is present in the common ancestor of plants and animals. The related kinases make them to be classified together as members of the RLK/*Pelle* family (Shin & Blecker, 2001).

RLKs are well characterized PRRs common features including the presence of an N-terminal signal sequence at the extracellular domain that varies in structure, a single membrane-spanning region linking the ligand binding region to the downstream kinase domain, and a cytoplasmic protein kinase catalytic domain with almost similar structures (Fig. 5.). Receptor-like protein (RLP) unlike RLK lack intracellular kinase domain and therefore need an adapter protein for signal transduction (Zipfel, 2009; Zipfel, 2014).

The plant receptor-like protein kinases (RLPKs) are structurally related to the polypeptide growth factor receptors of animals (Carpenter *et al*., 1990; Normanno *et al*., 2006), which consist of a large extracellular domain, a single membrane spanning segment and a cytoplasmic domain of the protein kinase gene family (Walker, 1994; Lease *et al*., 1998). This reveals the close similarities in the structural organization between plant and animal RLK family.

Receptor protein kinases (RPK) are classified into three major subclasses (tyrosine, Serine/threonine and histidine kinases) based on the kinase domain substrates specificity (Becraft, 2002; Afzal *et al*., 2008). Receptor tyrosine kinases (RTK) are involved in the phosphorylation of tyrosine residues and included most of the animal RLK (Becraft, 2002). Serene/threonine kinases (STK) phosphorylates serine and threonine residues (Becraft, 2002), which includes most of the plant RLKs (Hardie, 1999). Histidine kinases receptors are phosphorylated at histidine residue site involved in signaling for growth and development. Examples of histidine kinases includes ethylene receptor ETR1 (Bleecker & Kende, 2000) and cytokinin receptor CRE1 (Inoue *et al*., 2001) that facilitate response to ethylene and cytokinin hormones respectively (Bleecker & Kende, 2000).

Serene/threonine RPKs on the basis of their extracellular ligand-binding domain can also be classified into six groups e.g. S-domain RLK, LRR-RLK class, TNFR (tumor-necrosis factor receptor) class, EGF (epidermal growth factor) class, PR class, and Lectin class (Kohorn *et al*.,

1992, Nasrallah & Nasrallah, 1993, reviewed in Walker, 1994, Becraft *et al*., 1996, Herve *et al*., 1996, Wang *et al*., 1996, Li *et al*., 2002). Among these, the S-locus receptor kinase (SRK) group was the first to be identified with a self-incompatibility nature in *Brassica* reproduction (Katchroo *et al*., 2002, Takayama & Isogai, 2003).

RLKs with leucine-rich repeat (LRR)-containing extracellular domains comprise the largest subfamily of transmembrane RLKs in plants with over 200 members in *A. thaliana* out of the more than 600 RLKs protein (Shiu & Bleecker, 2001; Torii, 2004). An example of these receptor with LRR-domain includes flagellin receptor FLS2 of *A. thaliana* (Table 2.), which perceives a highly conserved domain of bacteria flagellin (Gómez-Gómez & Boller, 2000; Asai *et al*., 2002). FLS2 was first identified as a receptor for flg22 from bacteria in an experiment using the model plant *A. thaliana* (Gómez-Gómez & Boller, 2000). Arabidopsis FLS2 recognize bacteria flagellin by direct binding of an immunogenic protein defined by a conserved length of 22 amino acids hence the name flg22 (**Fig. 5),** which is located close to the N-terminus of flagellin in bacteria (Felix *et al*.,1999; Chinchilla *et al*., 2006; Sun *et al*., 2013). Experiments with mutants from *A. thaliana* lacking the FLS2 receptor were shown to be more susceptible to diseases caused by *Pseudomonas syringae* DC3000 compared to Col-0 (Zipfel *et al*., 2004). Apart from *A. thaliana* FLS2 perception, flg22 is recognized by most higher plant and the functional orthologs of FLS2 have been identified in a wild relative of tobacco *Nicotiana benthamiana* (Hann & Rathjen, 2007), rice *Oryza sativa* (Takai *et al.,* 2008), tomato *Solanum lycopersicum* (Robatzek *et al*., 2007), and grapevine *Vitis vinifera* (Trda *et al*., 2014). This shows that receptors are highly conserved in different genera of plants as it evolves to recognize and defend itself against intruders.

Many proteins that function in plant innate immunity responses reside on, or are, associated with the plasma membrane (PM). The plasma membrane is the cellular interface that regulates the exchange of molecules and information between cells and their environment. The PM is engaged in a range of plant physiologic processes including growth and development, ion and transport of metabolite, with consciousness of environmental changes, and disease resistance (Marmagne *et al*., 2004; Mongrand *et al*., 2010). Several studies have analyzed PM dynamics during pathogen perception and immune signaling including the amplification signaling pathways described by Yadeta *et al*., 2013**.** The kinase domain as can be described is like the central processing unit of a

computer, receiving and accepting relevant inputs from outside extracellular domain and transforming them to generate outputs. Protein phosphorylation at the C-terminal has an extensive role in immune signaling and quantitative proteomics of phosphopeptides enriched from PM fractions isolated from tissue treated with PAMPs has uncovered novel modes of protein regulation during immunity (Benschop *et al*., 2007; Nuhse *et al*., 2007).

Other sub-families of the LRR can function together as co-receptors to signal for a derived peptide of avirulent protein factor. Leucine rich receptor Serine/threonine protein kinase BAK1 as detailed in many studies revealed their function as a co-receptor to perceive flg22 and is required for the full activation of FLS2 and other extracellular containing LRR RLKs (**Table 2**), and thereby triggering immune signaling (Chinchilla *et al*., 2007; Sun, *et al*., 2013; Heese *et al*., 2007; Roux *et al*., 2011). The rice PRR gene, *Xanthomonas resistance 21* (XA21), functions to recognize a conserved sulfated peptide called $AxY^{s}22$ (**Table 2**), derived from the rice *Xanthomonas oryzae* protein Ax21 which catalysis XA21-mediated protection (Lee *et al*., 2009).

The recognition of conserved PAMPs by PRRs RLKs triggers mitogen-associated protein kinases (MAPKs) activation, production of reactive oxygen species (ROS), Ca^{2+} burst, transcriptional reprogramming, hormones biosynthesis, and deposition of callose to strengthen the cell wall (Nürnberger *et al*., 2004; Ronald & Beutler, 2010; Segonzac & Zipfel, 2011).

A lot of researches during last decades have decisively established that plants have a perception system on the cell surface as the first line of defense against unwanted intruders (Boller & Felix, 2009; Zipfel & Robatzek, 2010).

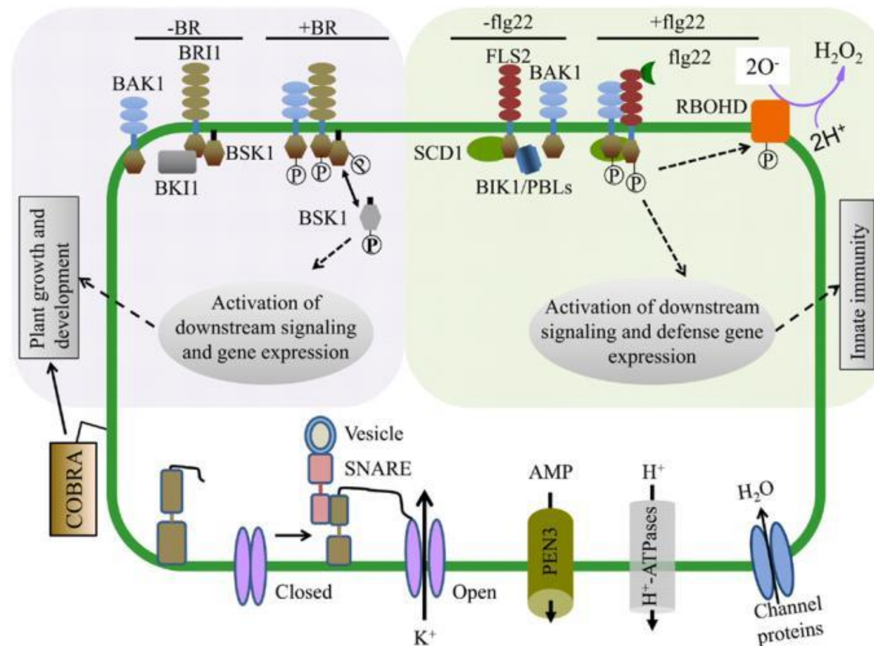

Figure 5: **A model for the amplification of signaling pathways for PAMPs in Arabidopsis**. The FLS2 innate immune receptor recognizes a 22 amino acid epitope of the bacterial PAMP flagellin (flg22). In the presence of flg22, FLS2 form a heterodimer with its co-receptor BAK1 and multiple trans-phosphorylation events occur between the kinase domains of FLS2, BAK1, and BIK1/PBLs, leading to the activation of plant innate immunity and disease resistance. Within minutes of flg22 perception, the NADPH oxidase RBOHD pathway is activated, potassium and calcium ion fluxes occur, and the apoplast is alkalinized. SNARE [soluble NSF (N-ethylmaleimide-sensitive factor) accessory protein receptor] protein complex drives the process of vesicle fusion with the target organelle in membrane trafficking. GPI-anchored protein COBRA controls orientational cell expansion, a potential regulator of cellulose biogenesis. The ABC transporters PEN3 (transports AMP- antimicrobial peptides) and water transporters (Yadeta *et al*., 2013)

Some receptors however, signal in responses to DAMPs (damage associated molecular pattern), peptides release as a result of cell damage or wounding by insects (Boller & Felix, 2009). One good example of DAMP is Arabidopsis cell damage signal peptides and corresponding receptors (PEPR1/2) reported by Krol *et al.,* 2010.

Although PAMPs are described for many pathogens, NAMPs (Nematode-associated molecular patterns) that trigger PTI during nematode-plant interaction have not been well characterized (Holbein *et al*., 2016), and are the focus of several active research programs including this thesis. The only known PAMPs from nematodes to be reported so far is a small signaling molecule known as ascarosides (Manosalva *et al*., 2015), which are widely conserved among nematodes (Choe *et al*., 2012). Ascaroside (Ascr#18), the most abundant in plant parasitic nematodes, is

perceived in nanomolar and picomolar concentration (Manosalva *et al*., 2015). However, the receptor that perceives Ascr#18 as a NAMP is still elusive. Other works also suggest the activation of PTI during nematode plant interaction as evidence by silencing of BAK1 orthologues SISERK3A and or SISERK3B in tomato (*Solanum lycopersicum*), result in susceptibility of plant to root-knot nematode (Peng *et al*., 2014). Teixeira *et al*., 2016, also reported that nematode infection in Arabidopsis triggers PTI responses in a BAK-dependent and also independent manner as they observed *bak1-5* mutant being more susceptible to root-knot in comparison to control.

As nematodes continue to threaten world food security, and most of the chemical control under scrutiny due to environmental concerns (Tytgat *et al*., 2000), effect on human health and nations economic benefit, it will be therefore of utmost important to put focus on engineering resistance cultivars. This is possible via identification of resistance genes or RLKs potentially involved in signaling against nematode and thereby engineering crop to confer resistance in the long run. Sedentary biotrophs nematodes apart from host environmental factors depends on the feeding site formation for their survival, if disrupted by the resistance of a cultivar will help in reducing the nematode population and impact they will cause to crops.

1.9 . Aims and objectives

Previous studies have showed that plant parasitic nematodes during initial infection and migratory stage secrete effectors that contribute in suppressing plant basal immune responses in order to facilitate parasitism. However, the role of plant basal defense during early stages of infection is not well understood. In this thesis we aimed at deciphering plant early defense system against nematodes. The objective was to study the mechanisms of nematodes-induced PTI responses in plants and to screen for potential nematode PAMPs that elicits plant innate immunity. Also we studied the importance of DAMPs responses during plant-nematode interaction.

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Chapter 2

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Arabidopsis leucine-rich repeat receptor-like kinase NILR1 is required for induction of innate immunity to parasitic nematodes

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RESEARCH ARTICLE

Arabidopsis leucine-rich repeat receptor–like kinase NILR1 is required for induction of innate immunity to parasitic nematodes

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Abstract

Plant-parasitic nematodes are destructive pests causing losses of billions of dollars annually. An effective plant defence against pathogens relies on the recognition of pathogen-associated molecular patterns (PAMPs) by surface-localised receptors leading to the activation of PAMP-triggered immunity (PTI). Extensive studies have been conducted to characterise the role of PTI in various models of plant-pathogen interactions. However, far less is known about the role of PTI in roots in general and in plant-nematode interactions in particular. Here we show that nematode-derived proteinaceous elicitor/s is/are capable of inducing PTI in Arabidopsis in a manner dependent on the common immune co-receptor BAK1. Consistent with the role played by BAK1, we identified a leucine-rich repeat receptor-like kinase, termed NILR1 that is specifically regulated upon infection by nematodes. We show that NILR1 is essential for PTI responses initiated by nematodes and *nilr1* loss-of-function mutants are hypersusceptible to a broad category of nematodes. To our knowledge, NILR1 is the first example of an immune receptor that is involved in induction of basal immunity (PTI) in plants or in animals in response to nematodes. Manipulation of NILR1 will provide new options for nematode control in crop plants in future.

Author summary

Host perception of pathogens via receptors leads to the activation of antimicrobial defence responses in all multicellular organisms, including plants. Plant-parasitic nematodes cause significant yield losses in agriculture; therefore resistance is an important trait in crop breeding. However, not much is known about the perception of nematodes in plants. Here we identified an Arabidopsis leucine-rich repeat receptor-like kinase, NILR1 that is specifically activated upon nematode infection. We show that NILR1 is required for the induction of immune responses initiated by nematodes and *nilr1* loss-of-function mutants

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Introduction

Plant-parasitic nematodes attack the majority of economically significant crops, as shown by international surveys indicating an overall yield loss of 12%. In some crops, such as banana, a loss of up to 30% has been reported. Losses amount to $100 billion annually worldwide [1]. The economically most important nematodes belong to the group of sedentary endoparasitic nematodes that includes root-knot nematodes (*Meloidogyne spp*.) and cyst nematodes (*Globodera spp*. and *Heterodera spp*.). Most chemical pesticides used for control of plant-parasitic nematodes are environmentally unfriendly, expensive and ineffective in the long term. Therefore, an increased demand for novel crop cultivars with durable nematode resistance is inevitable [2, 3]. In this context, it is important to identify and characterize the different natural means by which plants defend themselves against nematodes.

The infection cycle for root-knot and cyst nematodes begins when second-stage juveniles (J2) hatch from eggs. J2, the only infective stage, search for roots guided by root exudates. They invade the roots by piercing the epidermal root cells using a hollow spear-like stylet. After entering the roots, they migrate through different cell layers until they reach the vascular cylinder. There, root-knot nematodes induce the formation of several coenocytic giant cells, whereas cyst nematodes induce the formation of a syncytium. Because established juveniles become immobile, the hypermetabolic and hypertrophic feeding sites serve as their sole source of nutrients for the rest of their lives. In a compatible plant-nematode interaction, plant defence responses are either down-regulated or overcome by the nematodes [4–6]. A cocktail of secreted molecules including effectors that are synthesized in the oesophageal glands of the nematodes is purportedly responsible for modulating the plant defences as well as the induction and development of the syncytium [7–10]. Whereas most root-knot nematodes reproduce parthenogenically, cyst nematodes reproduce sexually. Although the mechanism of sex determination in cyst nematodes is not clear, studies have shown that the majority of juveniles develop into females under favourable nutritional conditions. When juveniles are exposed to adverse growth conditions, as it is the case with resistant plants, the number of male nematodes increases considerably [11].

Numerous studies have shown that plants sense microbes through the perception of pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) via surface-localised pattern recognition receptors (PRRs), leading to the activation of PAMP-triggered immunity (PTI). The activation of PTI is accompanied by the induction of an array of downstream immune responses including bursts of calcium and reactive oxygen species (ROS), cell-wall reinforcement, activation of mitogen-associated and calcium-dependent protein kinases (MAPKs and CDPKs), and massive reprogramming of the host transcriptome [12–15]. Together, these downstream responses can fend off the pathogen's infection. PAMPs are typically evolutionary conserved across a class of pathogens and perform an important function in the pathogen life cycle [16].

Plant PRRs are either plasma membrane-localised receptor-like kinases (RLKs) or receptor-like proteins (RLPs) [14]. Both RLKs and RLPs consist of an extracellular receptor domain (ECD) for ligand perception, a single membrane-spanning domain, but only RLKS have a cytoplasmic kinase domain. The major classes of RLKs are leucine-rich repeat (LRR)-RLKs, lysine-motif (LysM)-RLKs, crinkly4 (CR4)-RLKs, wall-associated kinases (WAKs),

pathogenesis-related protein 5 (PR5)-RLKs and lectin-RLKs (LeCRKs). Nevertheless, it is becoming increasingly clear that PRRs do not act alone but are part of multiprotein complexes at the plasma membrane [13]. For example, the LRR-RLK BRASSINOSTEROID INSENSITIVE-1 (BRI1)-ASSOCIATED KINASE 1 (BAK 1) forms receptor complexes with various LRR-containing PRRs to positively regulate PTI [14–15, 17]. In addition to PAMPs, plant PRRs can also perceive endogenous molecules, so-called damage-associated molecular patterns (DAMPs) that are released upon cell damage or pathogenic attack [16].

Although extensive studies have been conducted to characterise the role of PTI response in various models of plant-pathogen interactions, relatively less information is available pertaining to nematode-induced PTI responses in plants. To date, no PRR that recognises a nematode-associated molecular pattern (NAMP) has been identified [18]. However, some recent work suggests that nematode infection triggers PTI responses in host through surface-localised receptors. For example, silencing of the orthologues of BAK1 in tomato (*Solanum lycopersicum*, *Sl*) (*SlSERK3A* or *SlSERK3B)* has been shown to increase the susceptibility of these plants to nematodes due to defects in activation of basal defence [19]. In a more recent publication, it was shown that nematode infection triggers PTI responses in Arabidopsis in a BAK1-dependent and BAK1-independent manners. These authors showed that several PTI-compromised mutants including *bak1-5* were significantly more susceptible to root-knot nematodes as compared to control [20]. However, the identity of ligands and/or receptors involved in BAK1-mediated response remains unknown. As far as NAMP identification is concerned, ascarosides, which are conserved nematode-secreted molecules, have been shown to elicit plant defence responses that lead to reduced susceptibility against various pathogens [21].

In comparison to PTI, Effector-triggered immunity (ETI) during plant-nematode interaction is relatively well studied. A number of host resistance genes (*R-genes*) against nematodes have been described and their mode of action is relatively well investigated [22]. Notably, a host cell-surface immune receptor Cf-2 has been shown to provide dual resistance against a parasitic nematode *Globodera rostochiensis* and a fungus *Cladosporium fulvum* through sensing perturbations of the host-derived protease RCR3 by the venom allergen-like protein of *Globodera rostochiensis* [23]. In the present study, we provide evidence that nematodes induce PTI-like responses in Arabidopsis that rely on the perception of elicitors by membrane-localised LRR-RLKs.

Results

Nematode infection triggers PTI responses in host plants

To reveal changes in gene expression in response to nematodes at and around the infected area, GeneChip analysis was performed. Small root segments (approx. 0.5 cm) containing nematodes that were still in their migratory stage (defined as continuous stylet movement), were cut and compared with corresponding root segments from plants that were not infected. Total RNA was extracted, labelled, and amplified to hybridize with the GeneChip Arabidopsis ATH1 Genome (Affymetrix UK Ltd). The ATH1 Genome Array contains more than 22,500 probe sets representing approximately 24,000 genes. Subsequent analysis of the data showed that approximately 2,110 genes were differentially expressed ($FDR < 0.05$; Fold change > 1.5). Among them, 1,139 were upregulated, whereas 971 were downregulated (S1 Data). To explore regulation of the biological processes, molecular functions, and their distribution across different cellular components, a gene ontology enrichment analysis was performed on significantly upregulated genes. Those categories which were particularly over-represented in the differentially upregulated genes included the immune system response, response to stimulus, death, and the regulation of the biological processes (Fig A in S1 Text). We have previously published

a subset of 62 genes representing selected jasmonic acid (JA), ethylene (ET) and salicylic acid marker (SA), signalling and biosynthesis genes from this GeneChip data, which were also validated by qRT-PCR [24]. In general, transcript levels of genes involved in JA/ET signalling and biosynthesis were increased. However, in comparison to JA/ET, changes in SA-related genes were relatively less pronounced. Nevertheless, a slight increase in a SA biosynthesis (PAL1) and few SA signalling genes (NPR1, NPR3) was also observed (S2 Data). A detailed look at the transcriptomic data indicate that nematode infection triggered the induction of genes previously shown to be induced during PTI (Fig 1A) [25–27].

NemaWater elicits PTI responses in host plants

Our transcriptome data showed the induction of PTI-like responses upon nematode infection, however, it was unclear whether this induction was due to the recognition of nematodes by plant receptors or whether it was the result of wounding due to continuous nematode movement. To clarify this, we established a PTI screening assay involving the measurement of ROS burst, one of the hallmark responses of PTI. For this purpose, we incubated the pre-infective J2 of *H. schachtii* in H_2O for 24 hours at RT. The water obtained after removing the nematodes was termed as NemaWater (*Heterodera schachtii* NemaWater, *Hs*NemaWater; *Meloidogyne incognita* NemaWater, *Mi*NemaWater) and was used to treat Arabidopsis roots (see Methods for details). After treatment, ROS burst was measured using a root-based procedure adapted from a previous work [27]. Flg22 and H_2O treatments were used as positive and negative controls, respectively. Treatment with flg22 as well as with *Hs*NemaWater induced a strong and consistent ROS burst in roots (Fig 1B). The ROS burst with *Hs*NemaWater was, however, slightly delayed as compared to flg22; the ROS burst to flg22 occurs within 10 to 40 min, while that to *Hs*NemaWater occurred after 20 to 120 min. Although *Hs*NemaWater induced a consistent ROS burst in Arabidopsis roots, it was not clear whether this is due to the presence of a NAMP in *Hs*NemaWater or whether it is due to the production of an eliciting-molecule by plants (upon NemaWater treatment), which in turn induced production of ROS burst in roots. Such an eliciting-molecule could be called as DAMP or a NIMP (nematode-induced molecular pattern). One way to address the question of NAMP, or DAMP/NIMP was to dilute the *Hs*NemaWater with H_2O and analysed the production of ROS burst in roots. We hypothesised that if ROS burst is due to production of a DAMP or NIMP, diluting the NemaWater would not only reduce the magnitude of the ROS burst but may also slow its kinetics. However, our data showed that although magnitude of ROS burst was reduced strongly upon dilution, there was no delay in production of ROS between different dilutions (Fig 1C). Next, we incubated the *Hs*NemaWater with Arabidopsis roots for 60 min and then used this *Hs*NemaWater for production of ROS burst on fresh roots. The data showed that prior incubation of *Hs*NemaWater with roots did not cause any significant change in magnitude as well as kinetics of ROS Burst (Fig 1D). Regardless of the nature or origin of elicitor, activation of ROS burst upon *Hs*NemaWater treatment confirmed our observations from transcriptomic studies indicating that PTI-like responses are induced upon nematode detection.

To confirm whether NemaWater from different species of nematodes elicit a similar response, we produced NemaWater from the root-knot nematode species, *Meloidogyne incognita* (*Mi*NemaWater) and performed ROS burst assays. We observed a strong and consistent ROS burst (Fig 1E) similar to that of *H. schachtii* (Fig 1B). A prolonged treatment of young Arabidopsis seedlings with flg22 activated defense responses and leads to growth inhibition [28]. Although the mechanism underlying this growth inhibition is unclear, it is commonly accepted that activation of defense responses may take the resources away from growth. Importantly, this assay has frequently been used to analyse the eliciting capacity of PTI

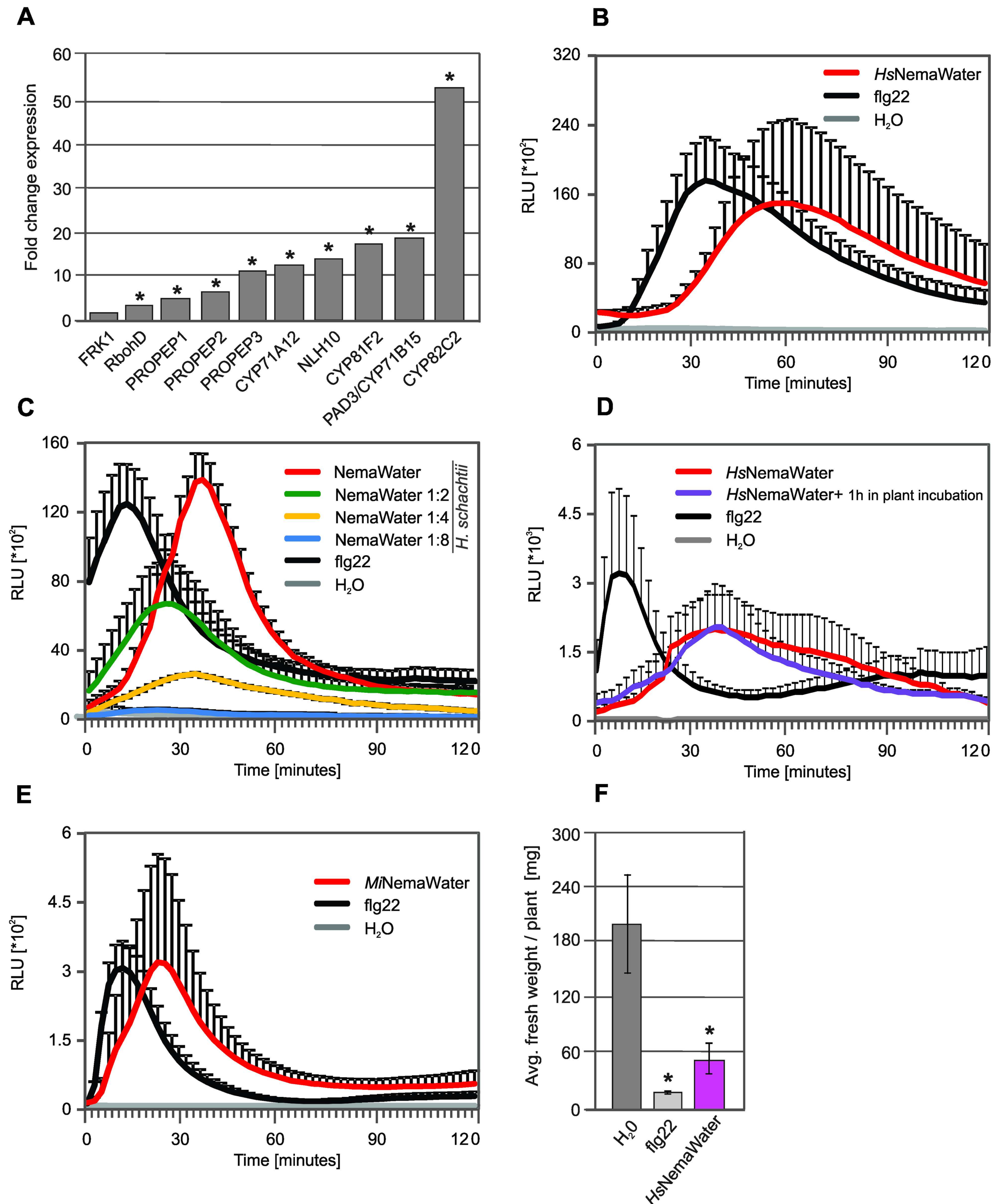

Fig 1. Nematode infection induced defense responses in plants that are characteristics of PTI. (A) Expression of PTI marker genes in microarray analysis upon nematode infection in migratory stage. Root segments from uninfected roots were used as control. Values indicate fold change compared with control. Asterisk indicates significant difference to control (*FDR* <0.05; Fold change >1.5). (**B**) Root segments from Col-0 plants were treated with water, *Hs*NemaWater or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. (**C**) Root segments from Col-0 plants were treated with water, different dilutions of *Hs*NemaWater or flg22 and ROS burst

was measured using L-012 based assay from 0 to 120 min. (**D**) Root segments from Col-0 plants were incubated with *Hs*NemaWater for 1 hour and then this *Hs*NemaWater was used for production of ROS burst on fresh root segments. Water, fresh *Hs*NemaWater or flg22, were used as controls. (**E**) Root segments from Col-0 plants were treated with water, *Mi*NemaWater, or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. (**B-E**) Bars represent mean ± SE for three technical replicates. Experiment was repeated three times with same results. RLU, relative light units. (**F**) 5-day-old Col-0 seedlings were incubated in water, *Hs*NemaWater or flg22 for seven days. Fresh weight was measured at 12 days after germination. Data were analysed using *t-test*. Asterisk represent significant difference to water-treated control root segments (P<0.05). Hs, *Heterodera schachtii*. Mi, *Meloidogyne incognita*.

components [28, 29]. We tested whether NemaWater also caused seedling growth inhibition, and found that both flg22 and *Hs*NemaWater inhibited seedling growth and reduced the root weight to a similar extent (Fig 1F, Fig B in S1 Text). Our results suggest that NemaWater contains potential elicitor/s that is/are recognized by an immune receptor in plants leading to the activation of PTI-like responses. To test this hypothesis, we incubated 12-day-old Arabidopsis seedlings in *Hs*NemaWater for one hour: ddH_2O alone was used as a control. RNA was extracted from the roots of both the non-treated control and NemaWater-treated seedlings. They were subsequently labelled, amplified, and hybridized with a GeneChip, as described above. The data analysis showed that 2,520 genes were differentially expressed, of which, 1,422 were upregulated and 1,098 were downregulated ($FDR < 0.05$; Fold change > 1.5; S3 Data). A gene ontology enrichment analysis for differentially upregulated genes showed the over-representation of categories such as immune system response, response to stimulus, death, signaling and the regulation of the biological processes (Fig C in S1 Text). A look at the expression of hormonal response gene upon *Hs*NemaWater treatment showed the same tendency for upregulation of JA/ET-related genes as observed upon nematode infection as described above (S2 Data). Moreover, a significant increase in the expression of genes characteristics for PTI was detected (Fig 2A). This upregulation in expression of PTI marker genes was very similar to that observed upon infection with nematodes (Fig 2B). Interestingly, expression of camalexin biosynthesis genes (*PAD3/CYP71B15*, *CYP71A12*) was upregulated only in nematode-infected plants but was not regulated upon *Hs*NemaWater treatment (Fig 2B). This was further confirmed by analyzing a reporter line (*pCYP71A12*:GUS) [30] on treatment either with nematodes or with *Hs*NemaWater. We found a strong GUS expression upon nematode infection, whereas such an expression was absent in seedlings treated with *Hs*NemaWater (Fig 2C–2E). We validated the microarray data by measuring the expression of 13 genes via qRT-PCR upon treatment with *Hs*NemaWater. Our analysis showed a similar trend for expression of selected genes as shown by microarray data (Table 1). Together, these results suggest that both nematode infection and NemaWater treatment induce PTI responses including a significant activation of JA pathways. The data analysis also showed that the changes in gene expression triggered upon treatment of seedlings with *Hs*NemaWater were to an extent similar to those that were observed upon nematode infection (Fig 2F and S4 Data). Even so, both treatments induced expression of a distinct set of genes, which may reflect differences in both treatments such as number and concentration of elicitors, duration of treatments, physical damage, etc.

On the basis of our finding that NemaWater triggers PTI responses, we asked whether pre-treatment with NemaWater effects plant responses to nematodes and other pathogens. To test this, plants were pre-treated with *Hs*NemaWater 24 hours prior to inoculation and were then infected with juveniles of *H*. *schachtii or M*. *incognita* or the virulent bacterial pathogen *Pseudomonas syringae* pv. tomato (see Methods for details). We found a strong decrease in number of nematodes in *Hs*NemaWater-treated plants compared with Col-0 (Fig 3A and 3B, Fig D in S1 Text). Similarly, the growth of virulent *P*. *syringae* was also reduced strongly upon *Hs*NemaWater treatment (Fig 3C and 3D).

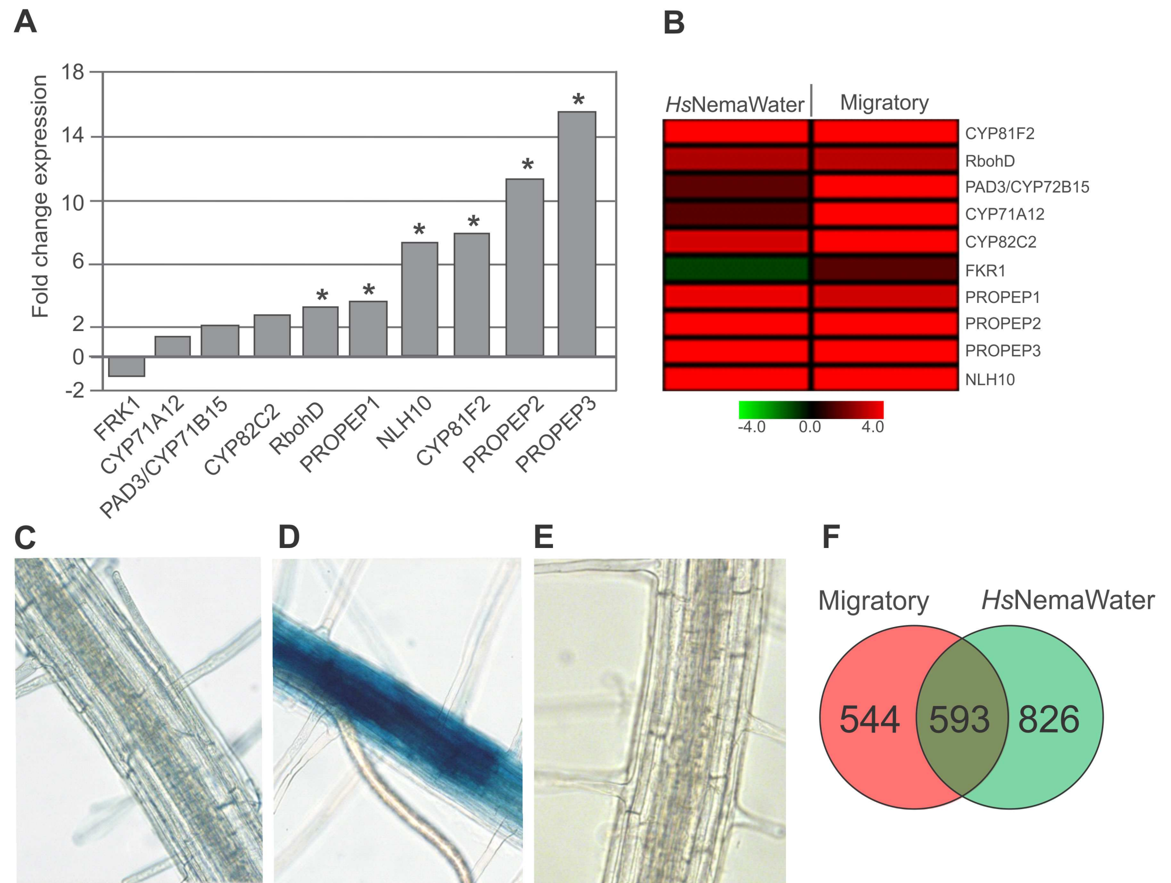

Fig 2. NemaWater treatment induced defense responses in plants that are characteristics of PTI. (A) Expression of PTI marker genes in microarray analysis upon *Hs*NemaWater treatment. Root segments from uninfected roots were used as control. Asterisk indicates significant difference to control *(FDR* <0.05; Fold change >1.5). (**B**) A heatmap showing expression of PTI marker genes upon nematode infection or upon *Hs*NemaWater treatment. (**A-B**) Values represent fold change compared with control. (**C-E**) Expression of glucuronidase (GUS) driven by *pCYP71A12* in control (C), *H. schachtii* infection at migratory stage (D) and *Hs*NemaWater treated plants (E) (**F**) A Venn diagram showing distribution of upregulated genes in Arabidopsis upon nematode infection or upon *Hs*NemaWater treatment.

NemaWater-induced PTI responses are mediated by BAK1

Induction of PTI by NemaWater indicated the presence of putative elicitor(s) in NemaWater. To test whether these elicitors is/are of proteinaceous nature, we added Proteinase K to *Hs*NemaWater and performed a ROS production assay. Duration and intensity of NemaWater-induced ROS burst varied in different experimental batches, which may be due to differences in the concentration of elicitors in different preparations of NemaWater and the possibility that NemaWater may contain more than one elicitor. Therefore, we used total photon count as a more reliable parameter for quantification of ROS burst activation in this study. We observed that the treatment of *Hs*NemaWater with Proteinase K or heat strongly reduced the induction of ROS burst (Fig 4A). These results were further confirmed by seedling growth inhibition assays (Fig 4B). BAK1 has been shown to act as a co-receptor for LRR-RLKs and LRR-RLPs, which typically detect proteinaceous ligands [14, 15]. Considering the data from Proteinase K treatment (Fig 4A and 4B) and recently published data on root-knot nematodes [20], we hypothesized that *bak1* mutants would be more susceptible to cyst nematodes. A nematode infection assay was performed on *bak1-5* and the double mutant *bak1-5 bkk1-1* (BKK1

Table 1. Validation of changes in gene expression upon *Hs*NemaWater treatment via qRT-PCR. The values represent relative fold change in response to NemaWater treatment as compared with control roots. 18S was used as housekeeping gene to normalize the data. All values are means of three biological replicates +/- SD.

Locus	GeneChip	qRT-PCR	Function
	Fold Change Control vs *Hs*NemaWater treated roots		
At3g55950	2.2	3.6 +/- 1.6	Crinkly4 Related 3
At4g21390	8.3	6.9 +/- 2.51	B120: serine/threonine kinase
At1g66880	4.3	5.3 +/- 1.1	Protein kinase superfamily protein
At1g69930	38.4	38.1 +/- 6.2	Glutathione-s-transferase 11
At3g46230	36.4	34.2 +/- 18.7	Heat shock protein 17.4
At2g38470	12.6	10.0 +/- 7.7	WRKY33
At5g25930	6.0	5.22 +/- 0.3	LRR-RLK, Protein phosphorylation
At4g23190	5.2	5.38 +/- 1.1	Cysteine-rich-RLK
At1g74360	4.1	3.28 +/- 2.2	Nematode-Induced-LRR-RLK 1
At5g48540	3.7	3.03 +/- 1.3	RLK-family protein
At1g11050	3.6	2.52 +/- 0.9	ATP-binding protein kinase
At1g61590	-2.4	-1.56 +/- 0.28	Defense response protein kinase
At4g26790	-2.5	-9.3 +/- 6.6	GDSL-motif esterase/lipase

being the closest homolog of BAK1) [31]. Both mutants were significantly more susceptible to nematodes compared with Col-0, as they allowed more females to develop (Fig 4C). We also investigated whether BAK1 is required for PTI-responses upon *Hs*NemaWater treatment and found that the nematode-derived ROS burst was strongly reduced in *bak1-5* mutants (Fig 4D). Similar results were obtained in seedling growth inhibition assays (Fig 4E and Fig E in S1 Text).

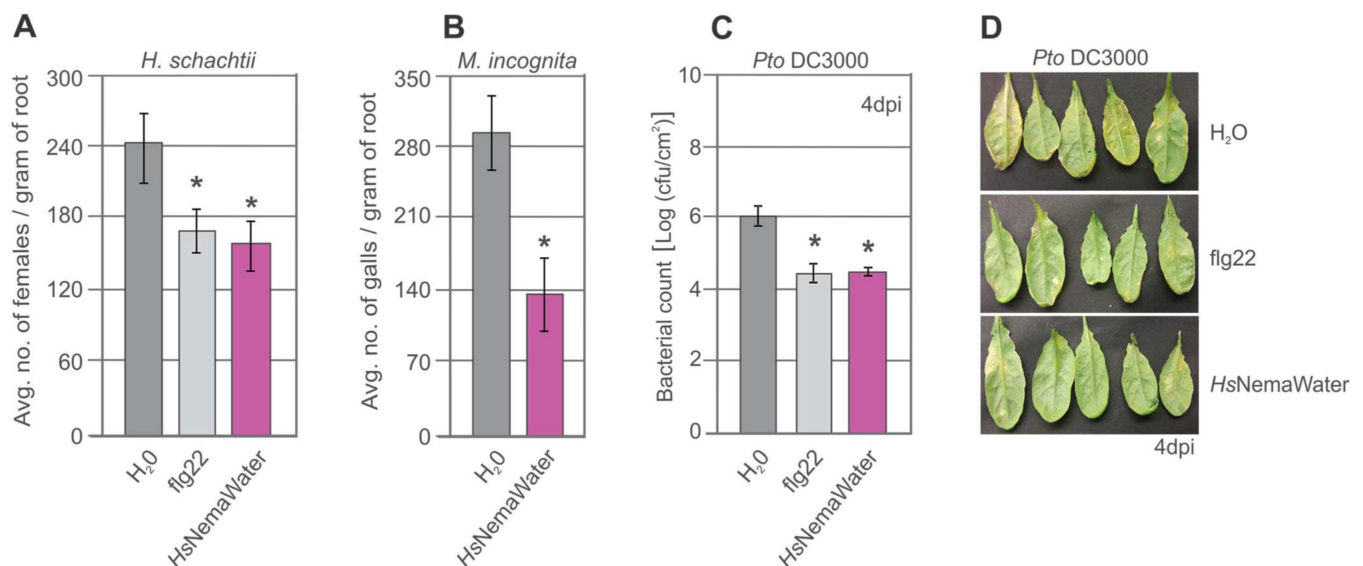

Fig 3. Pre-treatment with NemaWater induces resistance to pathogens. (A-B) Roots of Col-0 plants were treated with water or *Hs*NemaWater prior to infection and number of females were counted at 14 dai for cyst nematodes and number of galls were counted at 19 dai for root-knot nematodes. Bars represent mean ± SE for three independent biological replicates. (C-D) Plants were sprayed with flg22 or *Hs*NemaWater prior to inoculation and C. F.U/cm^2 was counted at 4 dai. Bars represent mean ± SE. Experiments were repeated three times with similar results. Asterisks represent significant difference to water-treated control root segments (P<0.05).

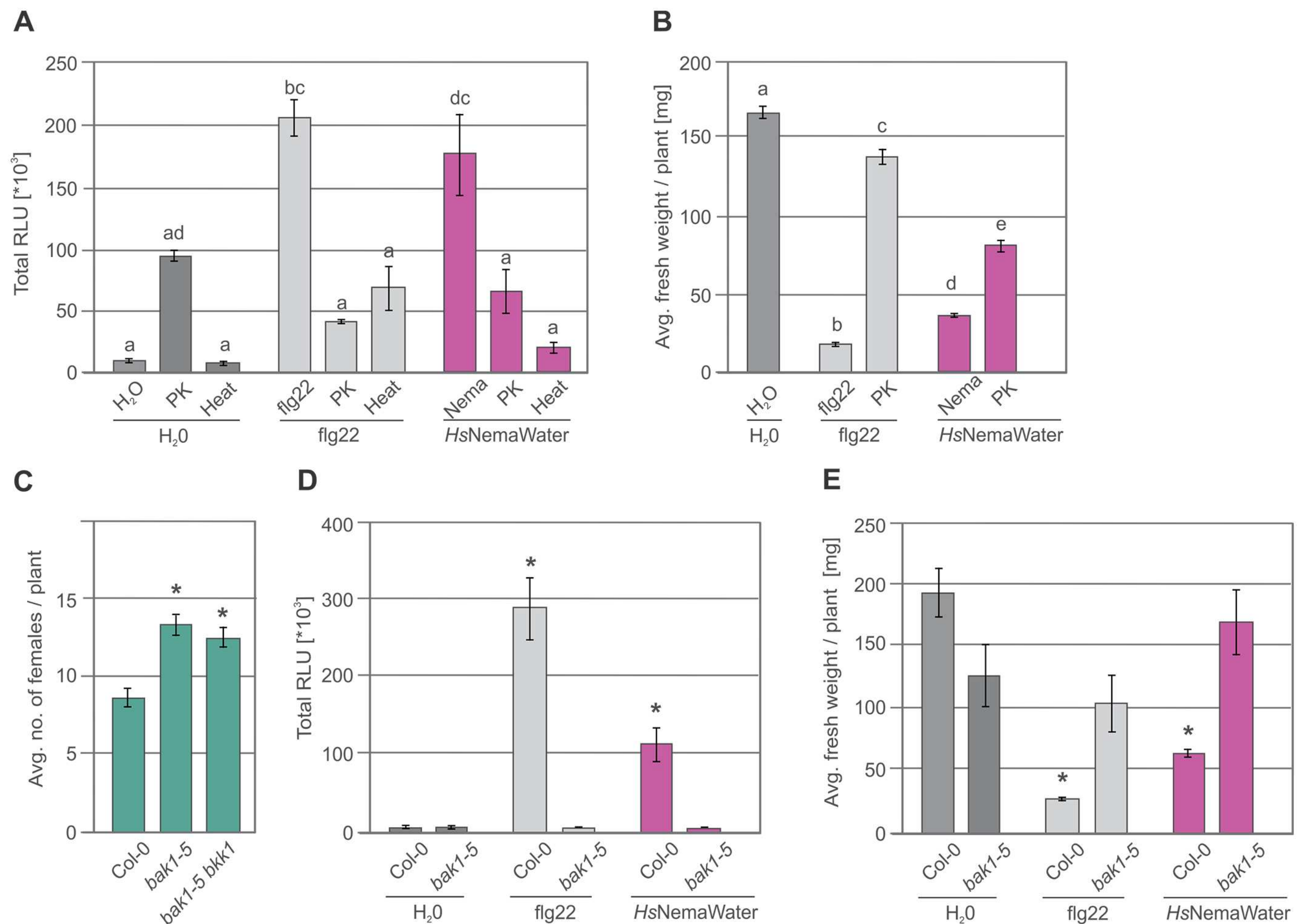

Fig 4. NemaWater treatment induced PTI responses were reduced strongly upon proteinase K, heat treatment, and in *bak1-5* plants. (**A**) Effect of Proteinase K and heat on production of ROS burst in root segments from Col-0 plants treated with water, *Hs*NemaWater or flg22. ROS burst was measured by using L-012 based assay from 0 to 120 min. PK, Proteinase K. Bars represent mean ± SE for two independent biological replicates. Data were analysed using single-factor ANOVA and Tukey's post hoc test (P<0.05). Columns sharing same letter are not statistically different. (**B**) 5-day-old Col-0 seedlings were incubated in water, *Hs*NemaWater, or flg22 with or without Proteinase K for seven days. Fresh weight was measured at 12 days after germination. Bars represent mean ± SE for two independent biological replicates. Data were analysed using single-factor ANOVA and Tukey's post hoc test (P<0.05). Columns sharing same letter are not statistically different. (**C**) Average number of female nematodes per plant in Col-0, *bak1-5* and *bak1-5 bkk1*. (**D**) Root segments from Col-0 and *bak1-5* plants were treated with water, *Hs*NemaWater or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. (**E**) 5-days-old Col-0 and *bak1-5* seedlings were incubated in water, *Hs*NemaWater or flg22 for seven days. Fresh weight was measured at 12 days after germination. (**C-E**) Bars represent mean ± SE for three independent biological replicates. Data were analyzed using single-factor ANOVA and Dunnet post hoc test. Asterisks represent significant difference to control (P<0.05).

Nematode-triggered PTI is mediated by LRR-RLK NILR1

Within the group of 593 commonly upregulated genes between two microarray experiments, 52 genes encoded RLKs (including 11 LRR-RLKs, 7 LeCRKs and 1 LysM-RK) and 2 encoded RLPs (S4 and S5 Data). Out of 52 candidate RLKs, we selected homozygous loss-of-function T-DNA mutants for ten genes (from five different RLK families), including those coding for three LRR-RLKs and one LeCRK. Confirmed loss-of-function mutants were then screened for infection against *H. schachtii*. Of particular interest, we found one LRR-RLK mutant, termed NILR1 (NEMATODE-INDUCED LRR-RLK 1; NILR1, At1g74360), which showed a consistent increase in the number of female nematodes as compared with Col-0 (Fig 5A and Fig F

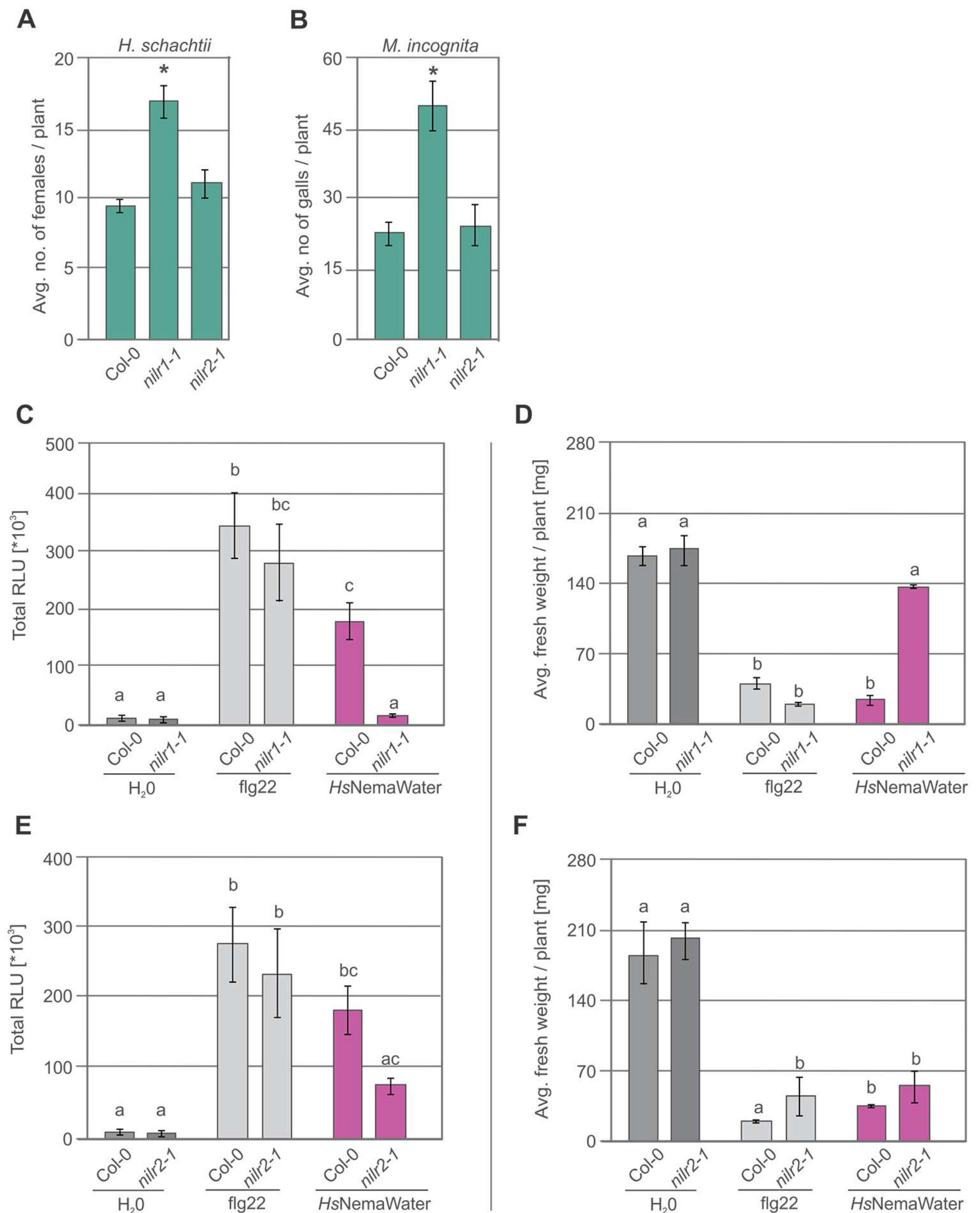

Fig 5. Knock-out *nilr1-1* enhances susceptibility to nematodes. (**A**) Average number of female nematodes induced by *H. schachtii* per plant in Col-0, *nilr1-1* and *nilr2-1*. Bars represent mean ± SE for three biological replicates. (**B**) Average number of galls induced *by M. incognita* per plants in Col-0, *nilr1-1* and *nilr2-1*. Bars represent mean ± SE for three biological replicates. (**C**) Root segments from Col-0, and *nilr1-1* plants were treated with water, *Hs*NemaWater or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for sixteen biological replicates. (**D**) 5-day-old Col-0 and *nilr1-1* seedlings were incubated in water, *Hs*NemaWater, or flg22 for seven days. Fresh weight was measured at 12 days after germination. Bars represent mean ± SE for three independent biological replicates. (**E**) 5-day-old Col-0 and *nilr2-1* seedlings were incubated in water, *Hs*NemaWater, or flg22 for seven days. Fresh weight was measured at 12 days after germination. Bars represent mean ± SE for three independent biological replicates. (**F**) Root segments from Col-0 and *nilr2-1* plants were treated with water, *Hs*NemaWater or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for sixteen biological replicates (**A-E**) Data were analysed using single-factor ANOVA and Tukey's post hoc test ($P<0.05$). Columns sharing same letter are not statistically different.

and G in S1 Text). In comparison to *nilr1-1*, the loss-of-function mutant for *NILR2* (AT1G53430) did not show any change in susceptibility to nematodes (Fig 5A). Based on our data with Proteinase K and BAK1, we hypothesized that NILR1 may be a PRR involved in the perception of nematodes. Therefore, this study focused on the characterization of *NILR1 and NILR2*, while other candidate genes will be described elsewhere.

To test NILR1's involvement in nematode perception other than *H. schachtii*, we analysed *nilr1-1* mutants for infection with root-knot nematode *M. incognita*. Our data showed that *nilr1-1* was significantly more susceptible to *M. incognita* than Col-0. In comparison, there was no change in susceptibility of *nilr2-1* to *M. incognita* (Fig 5B). To investigate whether enhanced susceptibility of *nilr1-1* to nematodes is due to impairment in PTI responses, we performed ROS burst assays on root segments from Col-0 and *nilr1-1* upon treatment with NemaWater from two different nematode species (*H. schachtii* and *M. incognita*). Notably, the Nema-Water-induced ROS burst was strongly reduced in *nilr1-1* (Fig 5C and Fig H in S1 Text). Similar results were obtained in seedling growth inhibition assays (Fig 5D and Fig I in S1 Text). We also tested *nilr2-1* for seedling growth inhibition and ROS burst induction upon treatment with NemaWater. We found that even though ROS production was reduced in *nilr2-1* upon *Hs*NemaWater treatment, the growth of these plants was inhibited to the same extent as Col-0 (Fig 5E and 5F and Fig I in S1 Text). Next, we isolated an additional homozygous knock-out T-DNA line for NILR1 (*nilr1-2*) and analysed it for infection by *H. schachtii* and production of ROS burst upon *Hs*NemaWater treatment (Fig J-L in S1 Text). We observed that *nilr1-2* plants were impaired in ROS production and were also significantly more susceptible to *H. schachtii* as compared to Col-0 (Fig K-L in S1 Text). Together our results show that NILR1 is an important component of host immune responses that are activated upon nematode infection.

NILR1 is widely conserved in dicotyledonous plants

NILR1 is closely related to LRR-RLK BRI1, belonging to the subfamily X of LRR-RLKs [32]. NILR1 encodes a serine/threonine kinase with 1,106 amino acid residues (predicted molecular weight 121.8 kDa) and shows all of the characteristics of an LRR-RLK. NILR1 has been suggested to have an extracellular domain with 22 tandem copies of LRRs, which are interrupted by a 76-amino acid island located between LRR17 and LRR18. The island domain of NILR1 is longer than those of BRI1 and contains a cysteine cluster with the pattern of $Cx_{25}Cx_{16}C$, which is followed by a transmembrane domain and a cytoplasmic kinase domain (Fig M-N in S1 Text) [31]. Moreover, a pair of cysteines at the amino terminal flanks NILR1's LRR domain with the characteristic spacing formerly observed in several plant LRR-RLKs [33]. Previous analysis has shown that NILR1 is presumably localised to the cell membrane, and that homologs are conserved among ten different species of flowering plants [32]. To gain further insights into molecular functions of NILR1, we determined its subcellular localization by confocal microscopy transiently expressing 35S::*NILR1-GFP* in the epidermis of *Nicotianna benthamiana*. We detected a strong GFP signal at the plasma membrane (PM) (Fig 6A). The PM localization of NILR1 was confirmed by co-localization with PM marker (see Methods for details). To investigate the conservation of NILR1, we conducted a BLAST search using ECD's amino acid sequence of NILR1 against non-redundant protein sequences of all land plants. We detected homologues of NILR1 among different species of the *Brassicaceae* family. Additionally, orthologues of NILR1 were found to be widely conserved in the genome of various dicotyledonous as well as monocotyledonous plant species. (Fig O in S1 Text). To further determine whether NILR1 is conserved across the plant kingdom and to test for effects of NemaWater, we measured the ROS burst upon *Hs*NemaWater treatment in the dicotyledonous tomato, sugar beet (*Beta vulgaris*) and tobacco (*Nicotianna benthamiana*), as well as in

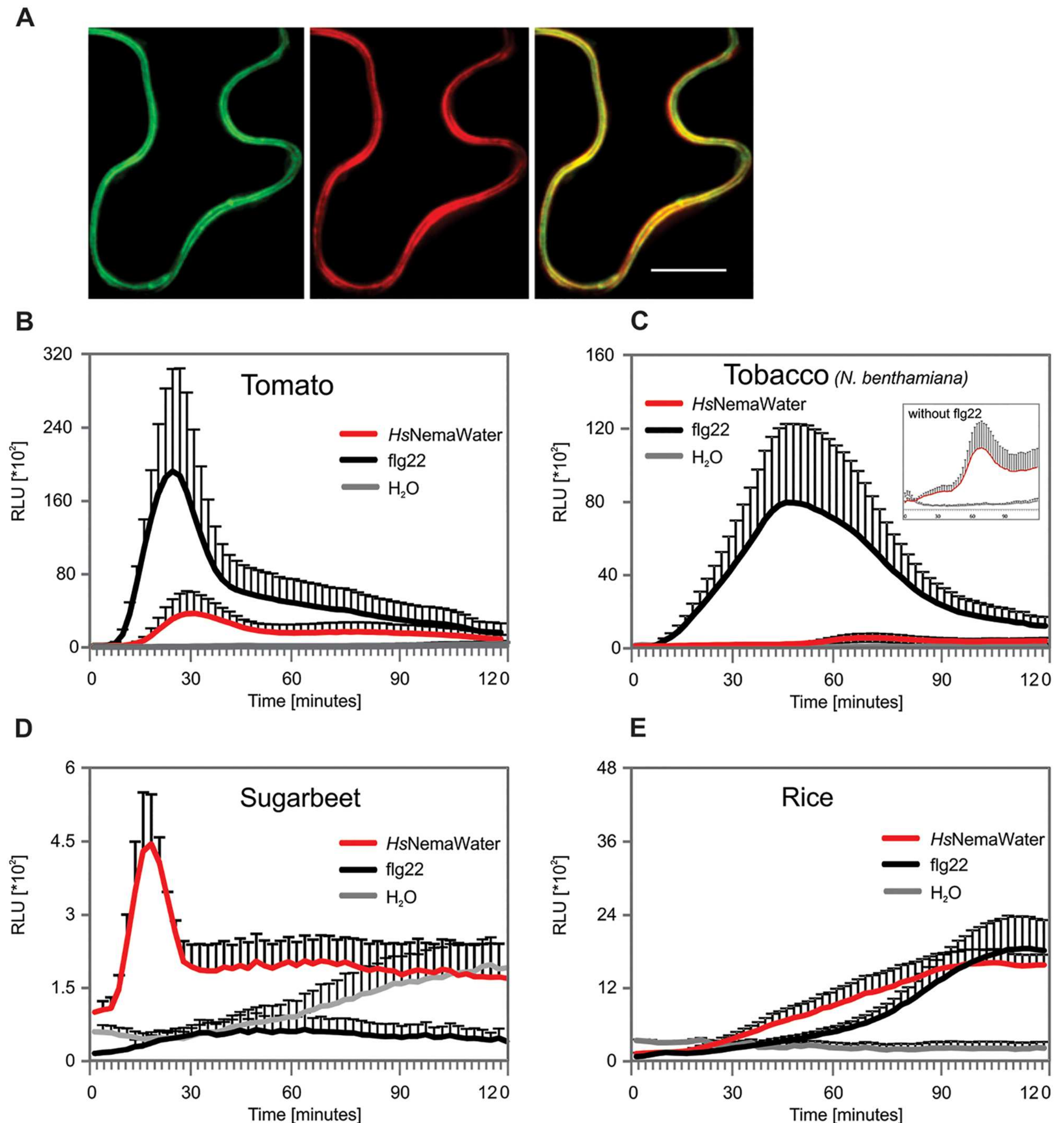

Fig 6. NILR1 is localised in plasma membrane. (A) Confocal microscopy of epidermis of *Nicotianna benthamiana* transiently expressing *35S:NILR1-GFP* and plasma membrane marker *35S:PIP2A-mCherry*. Scale, 50 μm. (B-E) Leaf discs from tomato (B), *N. benthamiana* (C), sugarbeet (D) and rice plants were treated with water, *Hs*NemaWater or flg22 and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for three technical replicates. Experiment was repeated three times with same results. RLU, relative light units.

monocotyledonous rice (*Oryza sativa*). We detected a strong ROS burst in sugar beet and tomato (Fig 6B and 6C), the magnitude of ROS burst was delayed and reduced in *N. benthamiana* (Fig 6D). In comparison to dicotyledonous, experiments with monocotyledonous rice showed that NemaWater induce a ROS burst, which was above the water control (Fig 6E). However, this burst was strongly delayed and was not consistent across several experiments.

A further exploration of publicly available Arabidopsis expression data through the eFP browser [33] revealed that *NILR1* is only moderately expressed in sepals and in senescent leaves under controlled growth conditions. However, *NILR1* expression is upregulated in response to biotic stresses such as *Botrytis cinerea*, *Phytophthora infestans* and non-adapted *Pseudomonas syringae* strains (Fig P and Q in S1 Text). Also NILR1 shows a low basal expression in various root tissues but displays a relatively high expression in endodermis, pericycle and stele [34]. The overall structure of NILR1 and its similarity to BRI1 supports its role as a surface-localised receptor that is involved in the perception of extracellular signals.

Discussion

In comparison to other pathosystems, not much is known about the importance of PTI in host defense against nematodes. In fact, no PRR involved in nematode perception has thus far been characterized. Additionally, so far only ascarosides have been recently shown to act as NAMPs. On the other hand, a number of nematode resistance genes (*R-genes*) either at the cell surface or inside cells have been characterised [22, 23]. In the present study, we provide insights into the molecular events associated with the basal resistance of plants to nematodes. We demonstrate that PTI-like responses are activated upon nematode infection and that they contribute significantly to basal resistance against nematodes.

The observation that cyst nematode infection induces the activation of a number of JA biosynthesis and signalling genes during migratory stages is supported with biochemical measurements showing an elevated amount of JA in Arabidopsis roots 24 hours after nematode infection [24]. In contrast to JA there was no strong activation of SA signalling in our transcriptome data during migratory stages. Nevertheless, a slight increase in some SA biosynthesis and signalling genes was observed. Intriguingly, plants that are deficient in different aspects of SA-signalling and biosynthesis have been shown to be more susceptible to cyst nematode infection [35]. These observations raise the question as to whether JA activation in roots upon nematode infection is only because of wounding during migration. Remarkably, we observed the same pattern of JA activation in roots upon treatment with *Hs*NemaWater indicating that JA activation is an important component of defense responses that are activated upon nematode recognition and is not only correlated to wounding. This hypothesis contradicts the general view that SA plays a more prominent role against biotrophs while JA/ET appears to be more important in resistance against necrotrophic pathogens and herbivorous insects [36–38]. This view, however, is mainly based on observations with leaf pathogens, whereas only limited information is available on the role of plant hormones in defense against root pathogens [39]. It may be that JA plays a more dominant role in the plant-pathogen interactions in roots. This hypothesis is supported by experiments on rice plants that indicated a key role for JA during interaction with root-knot nematodes [40]. Unlike the migratory phase, a number of studies addressing changes in gene expression during the sedentary phase of cyst and root-knot nematodes infection revealed a strong suppression of host defence responses [4–6]. Based on data from the current study and previous literature, we concluded that nematode invasion activates PTI responses, which are suppressed during later stages of nutrient acquisition and feeding site development. Indeed, an increasing number of nematode effectors involved in suppression of PTI have been characterised during last few years [8, 10, 18, 22, 23].

We observed that NemaWater treatment triggers responses, including ROS burst, immune gene expression and seedling growth inhibition that are characteristic of PTI. In addition, plants treated with NemaWater were more resistant to nematodes compared with water-treated control plants. On the basis of these data we propose that NemaWater contains elicitor/s that is/are perceived by plant surface-localised receptors leading to activation of PTI.

The fact that NemaWater derived from two different nematode species induces similar responses suggests that the elicitor component/s is/are conserved among different nematode species. Although the identity of the elicitor in NemaWater remains unknown, it is likely to be a heat-sensitive protein since treatment with heat as well as with Proteinase K strongly reduced its activity. Nevertheless, the residual growth inhibition in spite of addition of Proteinase K in NemaWater hints towards the possibility of an additional non-proteinaceous NAMP in NemaWater. However, it is also plausible that the residual growth inhibition is caused by Proteinase K itself. This view is supported by our data (Fig 4A) and some previous studies where a slight ROS burst was observed upon Proteinase K treatment alone [27].

NemaWater-induced responses are dependent on BAK1, which has been shown to act as a co-receptor for LRR-type PRRs, which typically detect proteinaceous ligands [12, 15, 17]. Even though we hypothesise that the NemaWater-derived elicitor/s is/are perceived by a surface-localized receptor, the possibility remains that such elicitor/s may not come into contact with host plants during infection. However, the fact that NemaWater was produced by incubating the nematodes without any further treatment strongly supports the idea that the elicitor is naturally secreted into the environment. It is also possible that the treatment of seedlings with NemaWater leads to the release of plant endogenous elicitors (DAMPs), which are again sensed by plants leading to the activation of PTI responses. However, since diluting NemaWater reduced only the magnitude but did not slow down the kinetics of ROS burst and thus makes it unlikely that a NemaWater induced DAMP is responsible for activation of PTI responses. Regardless of the origin of elicitor, it is clear that induction of PTI responses involves a component of NemaWater (therefore a NAMP) and is not only due to direct mechanical wounding by nematodes.

Loss of *NILR1* expression enhances the susceptibility of plants to nematodes suggesting that it is involved in the recognition of nematode-associated patterns. We propose that NILR1 is a PRR (or a component of a PRR complex) that recognises a NAMP leading to the activation of PTI responses. This hypothesis is supported by experiments showing that *nilr1-1* is defective in the ROS burst as well as in seedling growth inhibition upon NemaWater treatment compared with Col-0. Notably, *nilr1-1 and nilr1-2* did not respond differently to flg22 as compared with Col-0. On the other hand, *bak1-5* was defective in PTI activation in response to both flg22 and NemaWater indicating a BAK1-mediated role for NILR1 in nematode recognition. In comparison to *nilr1 (nilr1-1*, *nilr1-2)*, *nilr2-1* did not show any change in susceptibility to neither cyst nor to root-knot nematodes compared to Col-0. Similarly, there was no change in seedling growth inhibition as compared with Col-0. Nevertheless, activation of ROS burst upon NemaWater treatment was decreased in *nilr2-1* as compared with Col-0. This seemingly contradictory observation raises the question as to whether NILR2 also plays a role in perception of nematodes. A possible explanation could be that knocking out NILR2 may alter receptor complex formation and function, which selectively influence downstream signalling pathways without substantially influencing plant susceptibility to nematodes. This hypothesis also predicts that distinct signalling pathways that are activated during nematode perception may lead to diverse signalling outputs independently from each other. In fact, a recent study suggests activation of BAK1-dependent and BAK1-independent PTI pathways in response to RKN infection [19].

In conclusion, the identification of NILR1 as an LRR-RLK required for NemaWater-induced immune responses and basal resistance to nematodes is a major step forward in understanding of the molecular mechanisms underlying plant-nematode interactions. Moreover, the wide distribution of NILR1 among monocot and dicot plants is different from the majority of currently known PRRs and provides a unique opportunity for manipulation. However, sequence similarity does not necessarily indicate similar functions. It is therefore plausible that some of these homologues represent BRI1 or similar receptors and appeared in our analysis due to close similarity between NILR1 and BRI1. In fact, absence of a consistent ROS burst in rice plants upon NemaWater treatment hints that rice plants may not encode a functional NILR1. However, it is also possible that production of ROS burst upon treatment with NemaWater in some plant species such as rice requires further optimisation. A more detailed study would be needed to investigate this aspect.

Future work will focus on the purification and identification of elicitor/s present in NemaWater that are recognised in an NILR1-dependent manner. Further, conservation and function of NILR1 in various crop plants will be investigated. This will not only help in increasing our understanding of induced immune responses, but also provide potential opportunities to breed or engineer durable resistance against nematodes.

Materials and methods

Plant growth and nematode infection

Arabidopsis thaliana seeds were sterilized with 0.6% sodium hypochlorite and grown in Petri dishes containing agar medium supplemented with modified Knop's nutrient medium under the previously described conditions [41, 42]. The infection assays with cyst nematodes were performed as previously described [41]. Briefly, 60–70 J2s of *H. schachtii* were inoculated to the surface of an agar Knop medium containing 12-days-old plants under sterile conditions. For each experiment, 15–20 plants were used per genotype and experiments were repeated at least three times independently. The number of females per plant was counted at 14 days after inoculation (dai). For each experiment, 15–20 plants were used per genotype, and experiments were repeated at least three times independently.

For infection assays with root-knot nematodes, approximately 100 J2s of *M. incognita* were inoculated to the surface of agar MS-Gelrite medium containing 12-day-old plants and number of galls was counted at 21 dpi. *M. incognita* was propagated on greenhouse cultures of tomato (*Solanum lycopersicum cv. Moneymaker*) plants. Galls on roots of tomato were cut into smaller pieces of approximately 1 cm, crushed, and incubated for 3 min in 1.5% $NaOCl_2$. Subsequently, the suspension was passed through a series of sieves to separate nematode eggs from root pieces. Eggs were collected in a 25 μm sieve. For surface sterilisation, eggs were incubated in a 10% $NaOCl_2$ for 3 minutes and washed with abundant sterile water. The clean egg suspension was further washed with 150 μL Nystatin (10,000 U/ mL) and 2mL gentamycin sulphate (22.5 mg/mL) in a total volume of 30 mL. The suspension was stored at RT in darkness. Freshly hatched J2s were rinsed in water, incubated for 20 minutes in 0.5% (w/v) streptomycin-penicillin and 0.1% (w/v) ampicillin-gentamycin solution and for 3 minutes in 0.1% (v/v) chlorhexidine and washed three times with liberal amounts of sterile autoclaved water. For each experiment, 15–20 plants were used per genotype, and experiments were repeated at least three times independently.

Gene expression analysis at the nematode migratory stage

Ten hours after inoculation with *H. schachtii*, small root segments containing nematodes with moving stylets were marked under the binocular. Movement of stylet indicates the migration

phase of nematodes. The infected area around nematode head was then dissected. Corresponding root segments from uninfected plants were used as a control. RNA was extracted using a Nucleospin RNA extraction kit (Macherey-Nagel, Durren, Germany) according to the manufacturer's instructions. The quality and quantity of RNA was analysed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) respectively. The cDNA synthesis was performed with NuGEN's Applause 3'Amp System (NuGEN, San Carlos, CA, USA) according to the manufacturers' instructions. NuGEN's Encore Biotin Module (NuGEN) was used to fragment cDNA. Hybridization, washing and scanning were performed according to the Affymetrix 30 GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). Three chips each were hybridized with control and infected samples, with each microarray representing an independent biological replicate. The primary data analysis was performed with the Affymetrix Expression Console v1 software using the MAS5 algorithm.

NemaWater production and gene expression analysis upon NemaWater treatment

Approximately 300 brown cysts were collected from nematode stock culture, which was maintained on mustard roots under sterile conditions. These cysts were incubated in 3 mM $ZnCl_2$ in funnels (hatching chambers) to induce hatching. Before collection of J2s, the hatching chamber was checked for microbial contamination. After seven days, J2s were collected in a falcon tube containing double distilled autoclave water. The mixture of nematode in $ZnCl_2$ was spinned at 800 rpm for 3 min and supernatant was discarded. Afterwards, 1 ml of 0.05% $HgCl_2$ was added and nematodes were incubated in it for 3 min to surface-sterilize them. $HgCl_2$ was then removed and autoclaved double distilled water was added in excess (approximately 30 ml). The J2s were left in water for three min to wash them and remove $HgCl_2$. After 3 min, nematodes were spinned down at 800 rpm for 3min and the entire washing step was repeated three times.

Approximately 40,000 sterile J2s *of H. schachtii* were incubated in 2 ml dd H_2O for 24 hours at room temperature with continuous shaking. Afterwards, the nematode-water mixture was briefly centrifuged at 800 rpm for 2 minutes. The supernatant was removed to a new Eppendorf tube and was labelled as NemaWater. All steps of NemaWater production were performed under sterile conditions. Twelve-days-old Arabidopsis plants grown in Knop medium, as described above, were removed from agar plates and incubated in NemaWater for one hour each. Whole roots from 10 plants were cut and frozen in liquid nitrogen. Arabidopsis roots treated only with dd H_2O were used as a control. Three biological replicates were performed. RNA was extracted, amplified and hybridised to perform a microarray analysis, as described above. Three chips for each were hybridised for a control and for NemaWater treated samples, with each microarray representing an independent biological replicate.

Statistical analysis of microarray data

Affymetrix.CDF and.CEL files were loaded into the Windows GUI program RMAExpress (http://rmaexpress.bmbolstad.com/) for background correction, normalisation (quantile) and summarisation (median polish). After normalisation, the computed robust multichip average (RMA) expression values were exported as a log scale to a text file. Probe set annotations were performed by downloading Affymetrix mapping files matching array element identifiers to AGI loci from ARBC (http://www.arabidopsis.org). All genes that were more than 1.5 fold differentially regulated (t-test; $P < 0.05$) were pre-selected for further analysis using False discover rate at 5%.

Validation of microarray chip data upon NemaWater treatment

To validate the microarray expression data, 11 up- and two down-regulated genes were randomly selected. The samples were collected in the same manner as the microarrays analysis for NemaWater. RNA was extracted using a Nucleospin RNA Xs (Macherey- Nagel, Germany) kit according to the manufacturer's instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life technologies cat.no. 4368814), according to the manufacturer's instructions. The transcript abundance of targeted genes was analysed using the Stepone Plus Real-Time PCR System (Applied Biosystems, USA). Each sample contained 10 μL of Fast SYBR Green qPCR Master Mix with uracil-DNA, glycosylase, and 6-carboxy-x-rhodamine (Invitrogen), 2 mM $MgCl_2$, 0.5 μL of forward and 0.5 μL of reverse primers (10 μM), 2 μL of complementary DNA (cDNA) and water in 20 μL of total reaction volume. Samples were analysed in three technical replicates. To serve as an internal control, 18S genes were used. Relative expression was calculated as described previously [43], by which the expression of the target gene was normalized to 18S to calculate fold change. All primer sequences are listed in S6 Data.

Genotyping and expression analysis of knock-out mutants

Single T-DNA inserted knockout mutants for selected genes (AT1G74360: *nilr1-1*, SAIL_859_H01, *nilr1-2*, GK-179E06; AT1G53430: *nilr2-1*, SALK129312C) were ordered from relevant stock centre. The homozygosity of mutants was confirmed via PCR using primers given in S6 Data. The homozygous mutants were confirmed to be completely absent from expression through RT-PCR with primers given in S6 Data.

Oxidative burst assay

The production of an ROS burst was evaluated using a modified protocol adapted from previous work [27]. Small root segments (approx. 0.5 cm) were cut from 12-days-old plants and floated in ddH_2O for 12 hours. Afterwards, the root segments were transferred to a well in a 96-well plate containing 15 μl of 20 μg/ml horseradish peroxidase and 35 μl of 0.01M 8-Amino-5-chloro-2,3-dihydro-7-phenyl-pyrido[3,4-d] pyridazine sodium salt (L-012, Wako Chemicals). Next, 50 μl of either 1 μM flg22 or NemaWater was added to the individual wells. The experiments were performed in four technical replicates, and ddH_2O was used as a negative control. Light emission was measured as relative light units in a 96-well luminometer (Mithras LB 940; Berthold Technologies) over 120 minutes and analysed using instrument software and Microsoft Office Excel. For experiments with Proteinase K, 100 μl of Proteinase K was added to 1 ml of NemaWater or flg22, and the mixture was incubated at 37˚C for 4 hours. For heat treatment, samples were incubated at 90˚C for 30 min. ddH_2O was used as a negative control. The experiments were performed in three technical replicates and independently repeated multiple times as indicated in figure legends.

Growth inhibition assay

Arabidopsis plants were grown in Knop medium, as described above. Five-days-old plants were transferred to a well in a 6-well plate containing a liquid MS medium supplemented with either 1 ml of 1 μM flg22 or NemaWater. ddH_2O was used as a negative control. Fresh weight and length of the roots were measured 7 days after they were transferred to MS medium. The experiments were performed in three technical replicates and independently repeated multiple times as indicated in figure legends.

In silico structural analysis and localization of NILR1

The amino acid sequence for ECD of NILR1 was used to blast against all land plants sequences resulting in 318 hits across kingdom. Representative sequences from 44 unique species were used to generate a multiple alignment file. A Gblock function was used to refine alignment, and a maximum-likelihood analysis was performed with the PHYML software [44]. A non-parametric approximate likelihood ratio test was used for branch support as an alternative to usual bootstrapping procedure [45].

ECD sequence of NILR1 was used to search the SWISS-MODEL template library (SMTL version 2016-03-23, PDB release 2016-03-18) with Blast and HHBlits for evolutionary related matching structures matching [46–48]. NILR1 match best with BRASSINOSTEROID INSENSITIVE 1 (BRI1) and the PDB file from SWISS-MODEL was used to view 3-dimensional structures with NCBI Cn3D [49].

Coding region of NILR1 was amplified without stop codon using gateway forward and reverse primers as given in S6 Data. The amplified fragment was cloned into pDONR207 using BP clonase (Invitrogen) according to manufacturer's instructions. The resultant pENTRY vector (pENTRY/NILR1) was then used to clone NILR1 into the destination vector pMDC83:CGFP [50] using LR clonase (Invitrogen) according to manufacturer's instructions. The expression vector (35S:NILR1-GFP) was transformed into *Agrobacterium* strain GV3101 and co-infiltrated together with a plasma membrane mCherry marker *35S:PIP2A-mCherry* [51] into epidermis of 6-week old *Nicotianna benthamiana* leaves [52]. The GFP and mCherry signal was detected using a confocal microscope (Zeiss CLSM 710).

Supporting information

S1 Text. (A) GO categories preferentially upregulated during migratory stages of nematode infection. **(B)** Inhibition of root growth upon NemaWater treatment. 5-day-old Col-0 seedlings were incubated in water, *Hs*NemaWater or flg22 for seven days. Fresh weight of root was measured at 12 days after germination. Data were analyszed using *t-test*. Asterisk represent significant difference to water-treated control root segments ($P<0.05$). Hs, *Heterodera schachtii*. **(C)** GO categories preferentially upregulated upon NemaWater treatment. **(D)** An illustration of our method for cyst nematode counting. Each petridish is screened at 14 dpi under the binocular microscope and each female nematode is marked (represented by dots) to calculate rate of infection per plant. **(E)** NemaWater treatment growth inhibition was reduced strongly in *bak1-5*. 5-day-old Col-0 and *bak1-5* seedlings were incubated in water, NemaWater, or flg22 for seven days. Fresh weight of the root was measured at 12 days after germination. Data were analyzed using single-factor ANOVA and Dunnet's post hoc test ($P<0.05$). Columns sharing same letter are not statistically different. **(F)** Genotyping for NILR1 and NILR2 mutants. Genomic DNA of Col-0 or knockout lines (*nilr1-1*, *nilr2-1*) was PCR amplified using primers given in S6 Data. The presence or absence of intact wild-type allele is shown. **(G)** RT-PCR for presence or absence of gene expression in Col-0 or knockout mutants. RNA from Col-0 or knockout lines (*nilr1-1*, *nilr2-1*) was extracted to synthesize single stranded cDNA. The presence or absence of expression is shown using primers given in S6 Data. **(H)** Knock-out *nilr1* enhances susceptibility to nematodes. Root segments from Col-0, and *nilr1-1* plants were treated with water, flg22 or NemaWater from *M. incognita* (*Mi*NemaWater) and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for twelve biological replicates. **(I)** NemaWater-induced growth inhibition was reduced strongly in *nilr1-1*. 5-day-old Col-0, *nilr1-1and nilr2-1* seedlings were incubated in water, NemaWater, or flg22 for seven days. Fresh weight of the root was measured at 12 days after germination. Data were analyzed using single-factor ANOVA and Dunnet's post hoc test ($P<0.05$). Columns sharing

same letter are not statistically different. **(J)** Expression analysis of for *nil1-2* mutants. RT-PCR for presence or absence of gene expression in Col-0 or knockout mutants. RNA from Col-0 or knockout line (*nilr1*-2) was extracted to synthesize single stranded cDNA. The presence or absence of expression is shown using primers given in S6 Data. **(K)** Knock-out *nilr1-2* enhances susceptibility to nematodes. Average number of female nematodes per plants in Col-0 and *nilr1-2*. Bars represent mean ± SE for six biological replicates. **(L)** Knock-out *nilr1-2* enhances susceptibility to nematodes. Root segments from Col-0 and *nilr1-2* plants were treated with water, flg22 or NemaWater from *M. incognita* (*Mi*NemaWater) and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for three technical replicates. Experiment was repeated three times with similar results. **(M)** NILR1 encodes a LRR receptor kinase. Primary structure of the NILR1 divided into signal peptide; N-terminal containing a pair of cysteine residues (underlined); the LRR domain with LRR consensus residues in grey; the island domain containing a cysteine cluster with the pattern of Cx2Cx16C; the transmembrane domain; and the Ser/Thr kinase domain. **(N)** A putative structural model for ECD of NILR1. The model was built using BRI1 as template. Conserved and similar residues between BRI1 and NILR1 are highlighted as red or blue respectively. Grey color represents additional residues. **(O)** Conservation of NILR1 in land plants. A phylogram tree generated from maximum-likelihood trees construction method based on alignment of sequence spanning NILR1's ECD. The number next to each branch (in brown) indicates a measure of support. The number varies between 0 and 1 where 1 represent maximum. **(P)** Expression of NILR1 during development stages of plants. As revealed by eFP browser. **(Q)** Expression of NILR1 under different biotic stress conditions as revealed by eFP browser [34].
(PDF)

S1 Data. Arabidopsis genes differentially regulated *(FDR* <0.05; Fold change >1.5).during migratory stages of nematode infection. Root segments from uninfected roots were used as control. Values indictae fold change compared with control.
(XLSX)

S2 Data. Expression data for a selection of Jasmonic Acid- (JA), Ethylene- (ET) and Salicylic Acid genes (SA)-related biosynthesis, signaling and marker genes with fold changes obtained from microarrays analysis representing migratory stages of nematode infection. Values indictae fold change compared with control. Values in green are significantly different *(FDR* <0.05; Fold change >1.5).
(XLSX)

S3 Data. Arabidopsis genes differentially regulated *(FDR* <0.05; Fold change >1.5) upon *Hs*NemaWater treatment. Root segments from uninfected roots were used as control. Values indictae fold change compared with control.
(XLSX)

S4 Data. A set of commonly upregulated genes between two microarrays (S1 and S3 Data).
(XLSX)

S5 Data. All RLKs and RLPs differentially commonly upregulated between two microarrays (S1 and S3 Data).
(XLSX)

S6 Data. Primer sequences used in this study.
(DOCX)

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Chapter 3

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A proteinaceous molecule from plant parasitic nematodes activates defense responses in Arabidopsis

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Abstract

Plants defend themselves against invading pathogen through recognition of conserved molecular pattern (PAMPs) by plasma membrane localized pattern recognition receptors. The recognition of these molecular signatures activates responses as PAMP-triggered immunity. The output as a result of PTI activation includes the generation of reactive oxygen species, defense gene expression and production of antimicrobial compounds. The role of PTI during plant pathogen interaction has been well documented for many microbes of pathological importance. However little is known about PTI in nematode plant interaction till now. Here we showed that a proteinaceous molecule from plant parasitic nematode activates PTI-like response including ROS-burst, seedling growth inhibition and defense gene expression in Arabidopsis. Moreover, pre-treatment of seedling with NemaWater protein fraction induce resistance against cyst nematode *H. schachtii*. NemaWater fraction analysis by SDS-polyacrylamide and Tandem liquid-chromatography mass-spectrometry revealed the presence of nematode proteins component. Nematode surface coat proteins were also represented in the data which could be assumed at least one or more of them are involved in activation of defense. This study shows that plants are able to recognize nematode through an unknown conserved protein molecule. Understanding of this pattern will help in genetically modifying crops for resistance against plant parasitic nematodes and in doing so improve crop yield.

Keywords: Nematode, defense, protein molecules, PTI, Arabidopsis

Introduction

Plants are sessile and cannot escape pathogen and other environmental interference.Therefore, they evolved sophisticated mechanisms for protection against those biotic and abiotic influence. During initial contact, plant recognized conserved pathogen associated molecular pattern by cell surface pattern recognition receptors leading to the socalled pathogen associated molecular pattern- triggered immunity (Jones & Dangl, 2006; Zipfel, 2008). PAMP-triggered immunity (PTI) has been well studied for many phytopathogens including bacteria (Gómez-Gómez & Boller, 2002; Nürnberger *et al*., 2004; Kunze *et al*., 2004), fungi (Kaku *et al*., 2006; Miya *et al*., 2007), oomycete (Kanzaki *et al*., 2008), and insect pest (Howe & Jander, 2008). However, the importance of PTI during plant nematode interaction is still at an infant stage. Ascarosides are signaling molecules that are widely conserved among different types of nematodes (Choe *et al*., 2012). Ascaroside 18 (*Ascr#18*) is highly abundant in plant parasitic nematodes and has recently been shown to beperceived by plants in nanomolar and picomolar concentration (Manosalva *et al*., 2015). Nevertheless, molecular machinery including the receptor involved in *Ascr#18* recognition remains unknown. PAMPs are diverse and vary in different forms. A 22-amino acid peptide sequence from flagellin (flg22) and elongation factor Tu (elf18) are among themost widely studied PAMPs that activate PTI responses in plants (Gómez-Gómez & Boller, 2002; Kunze *et al*., 2004). Similarly, the role of chitin (polysaccharide) from fungal cell wall is well studied as a PAMP with the receptor well characterized (Kaku *et al*., 2006; Miya *et al*., 2007). As evidenced by numerous research findings, plant receptor-like kinase of leucine-rich repeat (LRR) class perceived proteinaceous molecules forming a heterodimer with BRASSINOSTEROID INSENSITIVE 1-Associated Receptor Kinase 1 (BAK1) as co-receptors (Chinchilla *et al*., 2007; Heese *et al*., 2007). Reports showed that silencing of BAK1 orthologues *SISERK3A* and or *SISERK3B* in tomato (*Solanum lycopersicum*), result in increased susceptibility of plant to root-knot nematodes (Peng *et al*., 2014). Nematode infection in Arabidopsis triggers PTI responses in a BAK1-dependent and also independent manner showing that *bak1-5* mutant were more susceptible to root-knot in comparison to control (Teixeira *et al*., 2016). In our previous work, we reported the activation of PTI-like responses in Arabidopsis by a nematode aqueous *diffusate* termed NemaWater in a manner depending on BAK1 (Mendy *et al*., 2017). Similar to root-knot nematodes, *bak1-5*, was also found to be hypersusceptible to both cyst (*Heterodera schachtii*) and root knot nematode (*Meloidogyne incognita*) confirming the role

of BAK1 gene in basal defense against plant parasitic nematode. Our finding also further indicates that a proteinaceouse molecule present in NemaWater is/are involved in activating plant defense responses. This was confirmed by experiments performed after treatment of NemaWater with proteinase K and also with heating, which reduces the ROS burst and seedling growth inhibition phenotype observed in untreated samples (Mendy *et al.,* 2017). A leucine-rich repeat receptor like kinase referred to as NILR1 (Nematode induce LRR-receptor 1) has been shown to be specifically expressed upon treatment with NemaWater in Arabidopsis plants (Mendy *et al*., 2017). However, the ligand(s) that induces NILR1 expression is still elusive. In this work, we used column chromatography, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and protein Mass-spec analysis method to identify all proteins present in NemaWater and tried to functionally characterize them for nematode associated molecular pattern (NAMPs) screening.

Results

NemaWater concentrated protein induce PTI responses in Arabidopsis

We previously reported the induction of PTI-like responses such as ROS burst, seedling growth inhibition, defense gene expression and induce resistance to nematodes in plants upon NemaWater treatment (Mendy *et al*., 2017). To fractionate and identify protein available in NemaWater, we used vivaspin column and concentrated proteins present in NemaWater for further analysis. The concentrated protein label as (Fraction) and flow through (Filtrate) was then used to treat Arabidopsis seedlings and ROS burst was measured (see method for detail). Flg22 and ddH_2O were used as positive and negative controls respectively. Treatment with 10 kDa fraction of *Hs*NemaWater protein as well as unfractionated *Hs*NemaWater and flg22 induces ROS-burst both in Arabidopsis shoot and root samples (**Fig.1a, b**). Interestingly, we also observed a slight ROS burst upon treatment of Arabidopsis with flow through (filtrate). Nevertheless the amount of ROS produced by fractionated protein was much higher than filtrate. Next, we tested *bak1-5* mutant for activation of ROS-burst upon protein (fraction or filtrate) treatment. Compared to Col-0 plant treated with *Hs*NemaWater protein fraction, there was a significant reduction in ROS burst in *bak1-5* mutant **Fig.1d**.

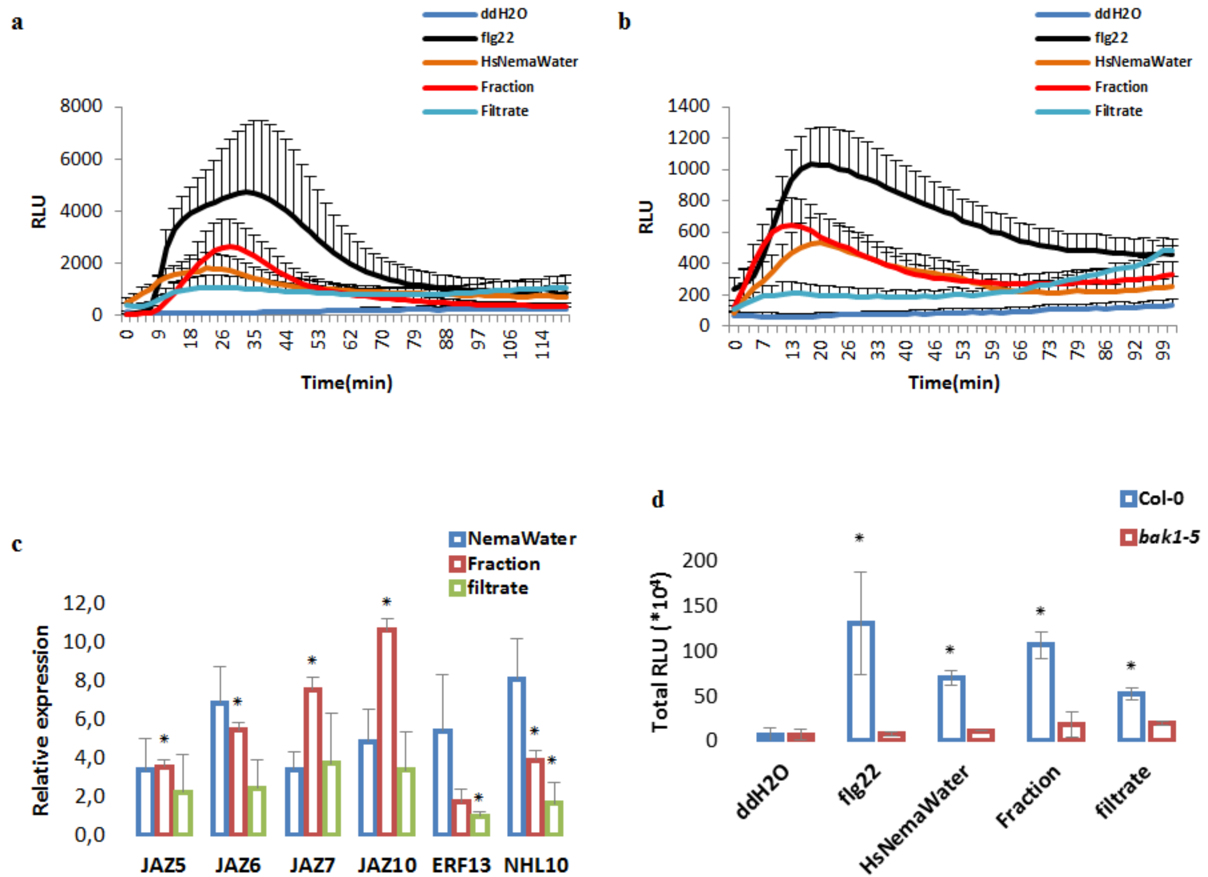

Figure 1: NemaWater concentration proteins induce PTI responses in Arabidopsis. **a)** Leaf discs from Col-0 plant were treated with *Hs*NemaWater or *Hs*NemaWater protein fraction, filtrate, or flg22 and ROS burst was measured using L-012 based system from 0 - 120 min. **b)** Root segments from Col-0 plant were treated with *Hs*NemaWater or *Hs*NemaWater protein fraction, filtrate, or flg22 and ROS burst was measured using L-012 based system from 0 - 120 min. Bars represent mean ± SE for three technical and biological replicates respectively. RLU - Relative Light Unit (s). **c)** Gene expression analysis after treatment with *Hs*NemaWater concentrated protein. Col-0 seedlings were incubated in *Hs*NemaWater, *Hs*NemaWater protein fraction or filtrate for 1hr. Total RNA was extracted and cDNA synthesize and used as a template for gene expression analysis using gene specific primers. Bar represent mean ± SD for three technical and three biological replicates. **d)** Col-0 and *bak1-5* plants root segments were treated with *Hs*NemaWater, *Hs*NemaWater fraction, filtrate and flg22 and ROS-burst was measured using L-012 based method between 0-120 min, ddH$_2$O was used as negative control. Bar represent mean ± SE for four technical and two biological replicates.

NemaWater treatment of seeding induces PTI gene expression (Mendy *et al.,* 2017); we therefore wanted to test whether we observe a similar trend by treating plants with fractionated protein. To do so, 12 day old seedlings were incubated in 1mL protein fraction (10kDa) or in filtrate. After 1 hour, the roots were separated from the shoot; RNA was extracted and cDNA was synthesized. Gene expression analysis was performed for marker genes (JAZ6, CYP81F2, ERF13, JAZ10), which have been shown to be induced by NemaWater treatment. Excitingly, we observed a significant increase in transcript of all tested marker genes **Fig.1c**, confirming our hypothesis that plants are able to recognize an unknown proteinaceous molecule in NemaWater. To test whether treating plants with fractionated protein also leads to similar seedling growth inhibition as observed for NemaWater, we treated 5 day old Arabidopsis seedling with *Hs*NemaWater, protein fraction, filtrate, and flg22 or water control. The results showed that *Hs*NemaWater protein fraction causes seedling growth inhibition to a similar extent as observed in *Hs*NemaWater treated plants **Fig. 2a-c** (Mendy *et al*., 2017). Nevertheless, we also observed a significant growth inhibition for samples treated with NemaWater filtrate. The phenomena underlying the cause of seedling growth inhibition during prolong treatment with peptide elicitors is poorly understood, but one common explanation is that plants reallocate resources to immunity during pathogen attack thereby compromising growth (Kunze *et al*., 2004, Mendy *et al*., 2017). Next, we treated plants with protein fraction and incubated them for 48 hours prior to infection with juveniles of cyst nematodes. Numbers of adult female and male nematodes were counted at 13 day post infection (dpi). Result showed that pre-treatment with both *Hs*NemaWater and also *Hs*NemaWater protein fraction led to a reduction in nematode development **Fig. 2d**.

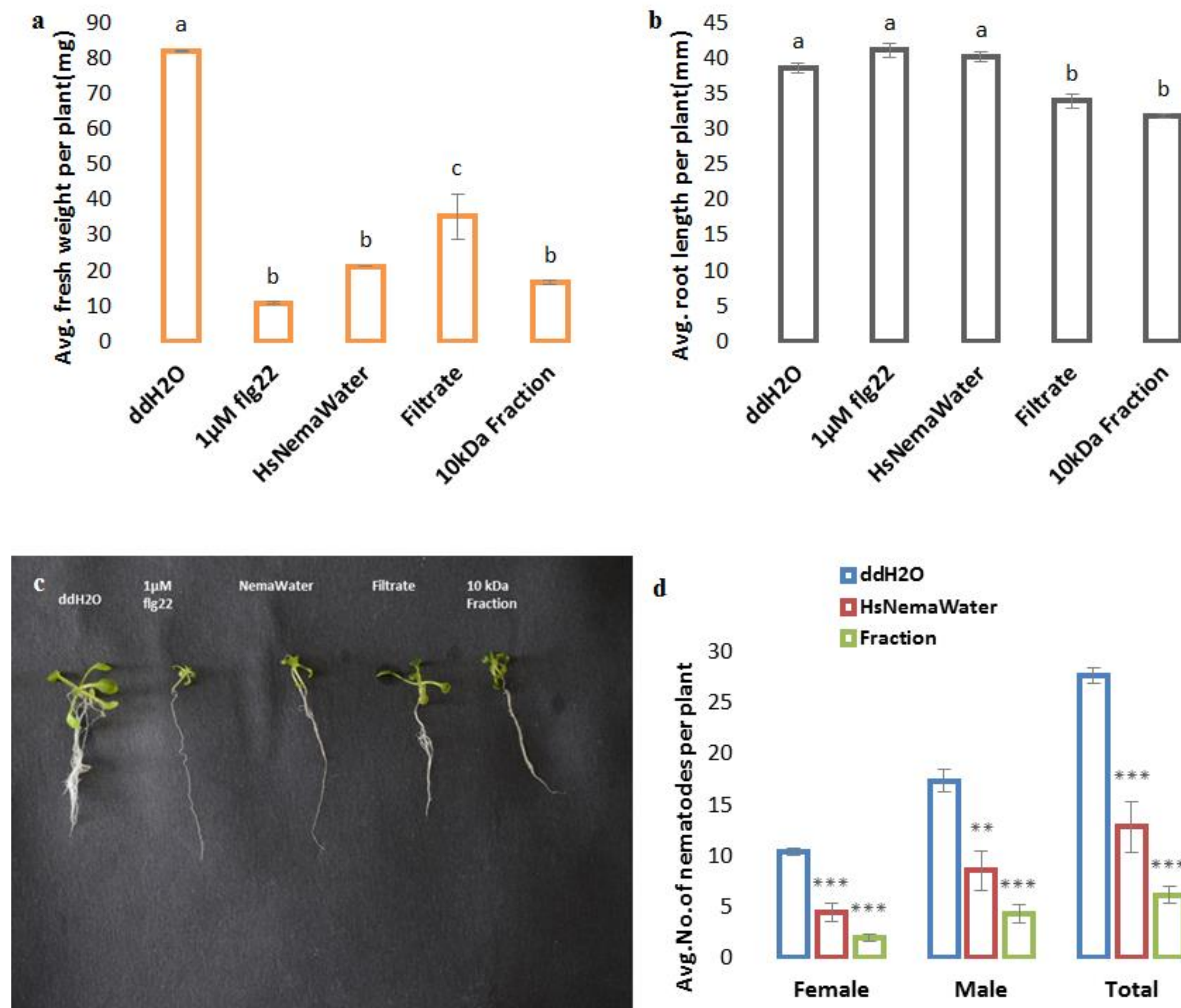

Figure 2: **Seedling growth inhibition and induce resistance**. **(a-c)** Five day old Arabidopsis Col-0 seedlings were incubated in water, *Hs*NemaWater, protein fraction, filtrate or flg22 for seven days. Fresh weight was measured at 12 days after germination. Data were analyzed using One-way ANNOVA and Fishers LSD test ($P<0.05$). Those columns sharing same letter are not statistically different from each other. **d)** Roots of Col-0 12 day old plants were treated with water, *Hs*NemaWater or protein fraction prior to infection with ~ 60 second stage juveniles. Female were counted at 14 dpi, bars represent mean ±SE for three independent biological replication.

Protein gel electrophoresis revealed the abundance of protein in NemaWater

Next, we made a Trichloroacetic acid (TCA) precipitation of *Hs*NemaWater and resultant pellets were analyzed through Mass Spectrometry for the presence of nematode proteins in it. We found

that more than 200 proteins were present in the *Hs*NemaWater, many of them with known biological functions **Fig.3a**.

To further evaluate the proteinaceous molecule in NemaWater causing PTI responses, we used spin column to concentrate protein in *Hs*NemaWater (see material and methods for more detail). The concentrated protein was acetone precipitated and loaded on 10% acrylamide gel for protein analysis. After silver staining and Coomassie blue staining solutions (**Fig.3b & Fig.3c),** we observed abundance of stained protein band in both 10kDa and 50kDa protein fraction. For sample analysis, we used gel stained in Coomassie blue by excising protein bands. Due to difficulty in separating individual bands in 50kDa column samples (**Fig.3b)**, no sample was included for Tandem liquid-chromatography mass-spectrometry (LC-MS/MS) analysis.

Mass-spectrometry analysis revealed the presence of nematode cuticle proteins in *Hs*NemaWater

To identify potential nematode molecular patters that cause PTI responses, we perform a LC-MS/MS analysis of 13 highly intensively stained protein bands **Fig.3c**. The 13 protein bands were analyzed individually to elucidate the available peptides or amino acids. The LC-MS/MS data were blasted against *Globodera pallida* genome (Cotton *et al.,* 2014), which is closest available genome sequence of cyst nematode since *H. schachtii* complete genome sequences was not available. More than 400 nematode proteins were found in NemaWater **Fig. 3d**. More proteins were found in protein gel band number 2 (Nema-02) when looking at individual protein band data **Fig. 3e.** Using WormBase parasite (http://parasite.wormbase.org/index.html), and NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), we annotated all the detected proteins to the respective protein coding gene. We found that more than 100 proteins have known biological functions with remaining more than 300 proteins were uncharacterized or no clear biologically know function described **Fig. 3d, S1.**

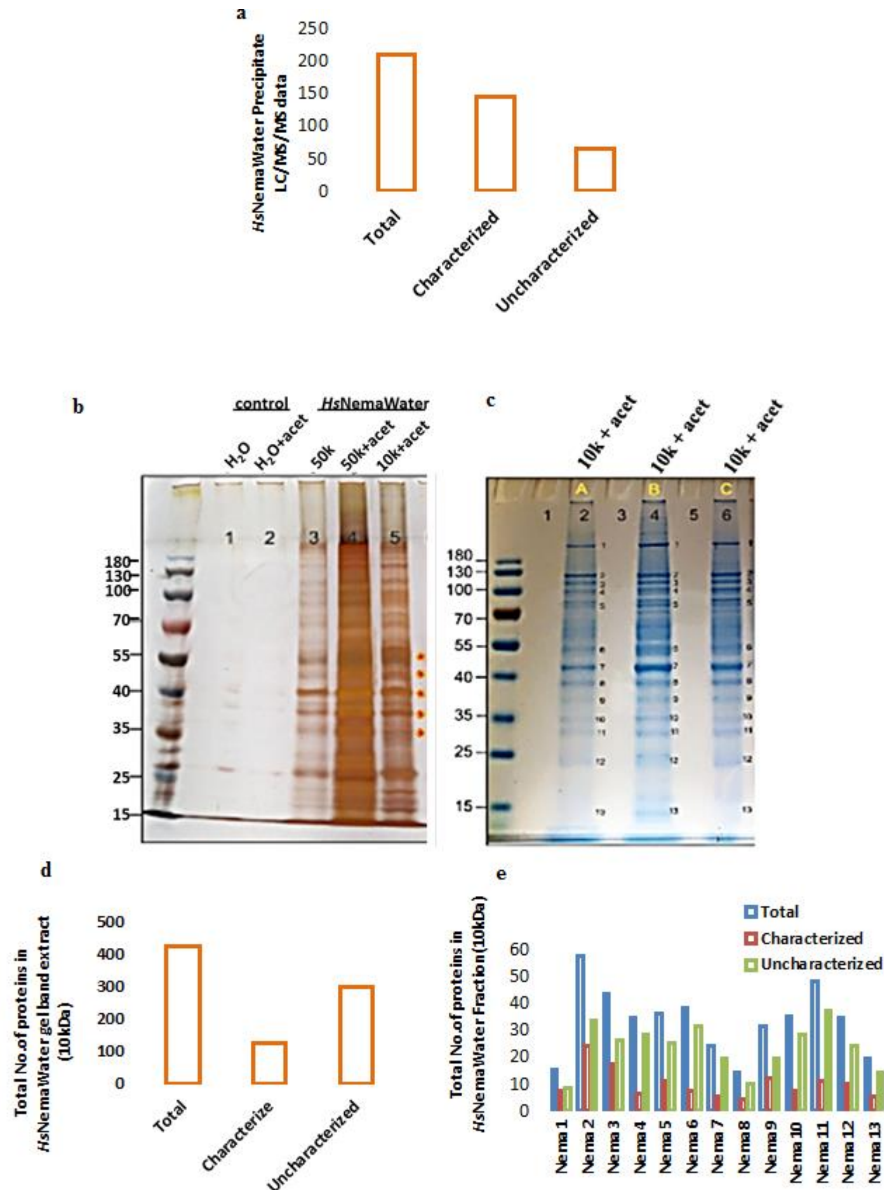

Figure 3: **Protein gel electrophoresis and LC-MS/MS analysis revealed the abundance of protein in NemaWater. a)** *Hs*NemaWater was TCA precipitated and the protein pellet analyze with LC/MS/MS for available peptides. **b)** Silver staining after NemaWater protein concentration, acetone precipitation and sodium dodecylsulfate gel electrophoresis. 1- ddH_2O control, 2- ddH_2O acetone precipitated also control, 3 - 50k vivaspin concentrated, 4- 50k vivaspin concentrated plus acetone precipitated, 5 - 10K vivaspin plus acetone precipitated, 6 - empty well. **c)** Coomassie blue staining after NemaWater protein concentration, precipitation and SDS-PAGE electrophoresis. 1- empty well, 2 - 10K plus acetone precipitated, 3- empty well, 4 - 10K plus acetone precipitated, 5 - empty well, 6 10K plus acetone precipitated. Protein label 1 to 13 represent those band excised from the gel for peptide analysis. **d)** Total number of protein in Mass-spec, the number of characterizes and uncharacterized proteins were also shown. **e)** Chart showing the total protein identified from each band. The bands were incised from the gel and analyze individually comparing to corresponding band from H_2O control.

Discussion

Nematodes are a continuous threat to global food security and an estimated 10% of yield is lost every year due to nematode damage (Sasser & Freckman, 1987). The mode of defense against nematode by plants has not been well understood. Plant recognizes invading pathogen through their conserved molecules which serves as molecular signature to activate a cascade of defense signals, thereby prevents further establishment and development of pathogens (Jones & Dangl, 2006). We previously reported the involvement of a novel proteinaceous molecule from a nematode aqueous solution in defense activation mediated by BAK1 in Arabidopsis (Mendy *et al*., 2017). The concentrated proteins were used to test for defense elicitation in Arabidopsis and also further analysis. As previously observed in NemaWater treated plants (Mendy *et al*., 2017), treatment with concentrated proteins (Fraction) causes ROS burst in both shoots and roots of *A. thaliana* **(Fig.1a, b**). The ROS burst in both root and shoot indicates that the responses for NAMPs are conserved in both plant tissues. This also raises the question whether plant can recognize all kind of plant parasitic nematodes in a similar manner. Further studies need to be done to answer that. Mutant plants of *bak1-5*were impaired in activation of ROS burst as observed in Col-0 when challenged with *Hs*NemaWater protein fraction. BAK1 has been known to play role as co-receptor for many plant PRRs especially those that recognize proteinaceous molecules (Liebrand *et al*., 2014; Macho & Zipfel, 2014; Albert *et al*., 2015). Previous studies also showed that *Hs*NemaWater defense activation in plant is mediated by BAK1 gene. This was confirmed by ROS burst and seedling growth inhibition with *bak1-5* mutant in which there was impaired immune responses as observed in Col-0 plants treated with *Hs*NemaWater (Mendy *et al*., 2017).

We tested the expression of defense related plant hormone genes upon treatment with protein fraction. We observed an increase in transcriptome especially, Jasmonic acid (JA) hormone genes 1hour after treatment with protein fraction (**Fig. 1c**). These genes were also seen in our microarray data to be increased in transcription during early phase of nematode infection and as well as upon treatment with *Hs*NemaWater (**Fig. S1)**. Together, these results confirm our previous finding that Arabidopsis is able to recognize some unknown protein molecules from plant parasitic nematodes and activate PTI. The finding further strengthens the important role played by plant hormones JA/ET pathway during nematode recognition and signaling (Nahar *et*

al., 2011; Mendy *et al.,* 2017). However, the late phase of nematode development is characterized by down-regulation of defense genes and defense related phytohormones (Ithal *et al.*, 2007[a, b]; Jammes *et al.*, 2005; Barcala *et al.*, 2010). This is due to the nematodes secreting effector proteins that suppress early defense responsive genes including those coding for hormones biosynthesis (Holbein *et al.*, 2016; Gardner *et al.*, 2015; Goverse *et al.*, 2014; Baum, 2012). This enables the nematode to effectively establish and successful development in the host plant, hence, leading to a compatible interaction.

To further characterize the protein molecules, we did a protein gel electrophoresis after acetone precipitation of protein fraction. A clear protein stained bands were observed ranging from as low as 15kDa to more than 180kDa confirming the presence of protein molecules in *Hs*NemaWater (**Fig. 3**) and also gave us clue of the different protein sizes. Nematode surface proteins such as cuticle collagen, cuticulin, surface enolase, tetraspanin with extracellular domain, heat shock proteins etc., were found to be present in the mixture of proteins from LC-MS/MS analysis. We assume that plants may have evolved to recognize nematode through one of their conserved surface proteins and activate basal defense responses mediated by BAK1 gene against them.

In animal parasitic nematodes, a number of reports have described extracellular vesicles (EV) trafficking and their involvement in parasites-host-interactions (Coakley *et al.*, 2015). Apart from the important role they serve in normal cell physiology, vesicles also transport molecules including excretory secretory proteins from pathogens to host (Coakley *et al.,* 2015; Marcilla *et al.*, 2012). Extracellular vesicles delivered in the host organism may carry molecules that helps the pathogen either escape or activate immune responses (Eichenberger *et al.*, 2018). The role of EV trafficking have not been described in host plant-nematode-interaction. In bacterial feeding free living nematode *Caenorhabditis elegans*, EV have been reported with predicted function in communication and mating behaviors (Liégeois *et al.*, 2006). Helminths worm parasite EV proteomes have revealed abundance of proteins involved in immune modulation such as 14-3-3 and Heat-shock protein family (Eichenberger *et al.*, 2018). The 14-3-3 protein has been shown to play vital role in modulating toll-like-receptors ability to initiate pro-inflammatory cytokine induction and also suppression of nitric oxides (NO) production by macrophages (Butt *et al.,* 2012). Hsp70 family proteins are molecular chaperones which can act as damage associated

molecular pattern (DAMPs) to activate inflammatory immune responses (Eichenberger *et al.*, 2018). These proteins, (Hsp), and other exosome associated proteins including enolase and aldolase were highly abundance in *Hs*NemaWater protein analysis data. However, the molecular mechanism by which these proteins are released into the host plant and their unique role in immune modulation is not well understood.

In conclusion, the analysis and identification of proteins in NemaWater is one step closer to finding potential NAMP involved in defense elicitation in plant. Identification of nematode NAMPS will serve as a molecular marker to screen for resistance mechanism in plants. Nematodes continue to be a potential threat to food security worldwide with little or no effective control mechanism in place. Therefore, it's important to understand the plant molecular aspects of pathogen recognition and immune activation pattern. Information of which will be important in breeding for durable resistance against nematode in the near future.

Material and Methods

NemaWater Preparation

NemaWater was prepared as described previously (Mendy *et al.,* 2017) by hatching of cysts in 3mM $ZnCL_2$ in a modified hatching chamber. The $ZnCl_2$ served as inducer of hatching for the cyst maintained on mustard plants. After several days of incubation (approximately seven days), the hatched second stage juvenile were collected and surface sterilized with 0.05% $HgCl_2$ for 3 min followed by washing to removed traces of $HgCl_2$ all of which was done under sterile conditions. Afterwards, the clean and sterile J2s (~ 40,000 J2s/2ml), were incubated on a shaker for 24 hours for NemaWater production. NemaWater was finally recovered by centrifugation at 800 rpm for 2 minutes and the supernatant transferred to a new Eppendorf tube and labeled as NemaWater.

NemaWater Protein Concentration with Vivaspin column method

Vivaspin®6 (Sartorius) sample concentrators were used to concentrate proteins from NemaWater based on sizes MWCO (molecular weight cut-off). Three different column were used (5000, 10000 and 50000 MWCO). The column contained two compartments, lower and upper chamber where the upper chamber contained concentrated protein (fraction) and the lower holds the flow

through (filtrate). Both compartments are separated by a semi permeable membrane. The protein was concentrated by application of centrifugation which force solvent through the membrane thereby leaving protein in the upper compartment. Concentrated proteins was recovered with pipette and used for analysis or stored at -80ºC for further use.

NemaWater Trichloroacetic acid (TCA) precipitation

The TCA protein precipitation was done according to (Link & LaBaer, 2011); Fresh TCA was prepared by adding 0.8g TCA in 0.7ml MilliQ water. 80% (w/v) TCA was added to the protein solution to bring the TCA concentration to 20%. The sample mixture was incubated on ice for 1.5 hrs, and at -20°C overnight. Afterwards the samples were spin at maximum speed in a microcentrifuge for 15 min / 4°C. The pellets were washed 3x with ice cold acetone; followed by spinning for 10min / 4°C. The pellets were allowed to air dry for 10 min at RT (caution not to dry too long!). The pellets were resuspended in 10µl buffer for Trypsin digestion.

NemaWater acetone precipitation

Ice cold acetone was used to precipitation protein. Four times volume (4x vol.) acetone was added to the sample and incubated at -20ºC for 1 hour. Afterwards, the samples were spinned down at 13000 rpm for 8 min to form a protein pellet. Acetone was poured out carefully without disturbing the protein pellet and the samples were further dried under a clean bench for few mins to remove remaining acetone. The protein pellet was dissolved with water and stored in -80ºC for further use.

SDS-PAGE gel electrophoresis

Precast 10% polyacrylamide gel was used for the SDS-PAGE electrophoresis. 15µl H_2O and 15 µl of GLB (gel loading dye) were added to the protein pellet and mix thoroughly until pellet completely dissolved. Afterwards the samples were incubated at 95ºC for 10min to denature the protein. After incubation, the samples were spinned at 13000rpm for 1min followed by loading on gel. For control sample, ddH_2O treated in the same condition as NemaWater was used.

Staining of protein gel

Two methods were applied to stain protein gel after electrophoresis silver staining and Coomassie brilliant blue method.

Silver staining

Silver staining method was use to visualize the protein band on a 1D gel after electrophoresis due to its high sensitive in detecting total protein by deposition of metallic silver on the surface of a gel where protein bands are present. This method was applied as previously described in Mortz *et al*., 2001. The 1D gel after electrophoresis was incubated in a fixer containing 40% ethanol, 10% acetic acid, and 50% H_2O for 1 hr. Afterwards, the gel was transferred to a new container with H_2O and incubated overnight with 2-3 times change of water to wash away traces of acetic acid. This also helps reduce background staining at the same time increase sensitivity. The following day, the gel was sensitized in 0.02% sodium thiosulfate for 1 min followed by washing in H_2O 3 times with 20 sec incubation for each wash. Afterwards, the gel was incubated in 4°C cold 0.1% silver nitrate solution containing 35% formaldehyde followed by washing in H_2O again for 3 times with 20 sec incubation time. The gel was developed in 3% sodium carbonate containing 0.05% formaldehyde and the process was terminated when staining was sufficiently done by incubating the gel in 5% acetic acid for 5 min. For storage the gel was place in 1% acetic acid solution and stored in 4°C. Samples from silver staining method were not use in LC-MS/MS analysis. This method was only applied for protein visualization process.

Coomassie blue Staining

To visualize and obtain samples for mass spectrometer analysis, the SDS-PAGE gel bands were stained with SimplyBlue ™ SafeStain (Invitrogen) Coomassie staining solution. SimplyBlue ™ SafeStain is easy to use, with less harm to users and environment. All staining procedures were performed according to the manufacturer's instruction, unless stated otherwise (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/simplyblue_man.pdf). Gel was rinsed in deionized water 3 times with 5 min incubation between the changes of water. This helps to remove SDS and salts from the buffer which can interfere with the dye binding to the proteins. In a new container with gel, 20mL of SimplyBlue ™ SafeStain with 2mL of 20% NaCl (w/v) was added and incubated with gentle shaking overnight. The staining solution was removed and the

gel washed 5 times in 100 mL of water with replacement every 1 hour. Gel picture was taken after wards Fig.2a. To collect samples for protein analysis, highly visibly stained bands were label 1-13 and the label band were incised carefully and place in an Eppendorf 0.5mL Safe-Lock protein LoBind Microcentrifuge Tubes Cat No. 0224331064. Control samples were collected from H_2O loaded wells corresponding to the same size as NemaWater samples.

Mass-spectrometer protein analysis

Protein bands were excised from polyacrylamide gel and placed in a low protein binding tube. The tubes were then label and send for LC-MS/MS based protein analysis.

ROS measurement

Reactive oxygen species measurements were done based on Mendy *et al.,* 2017. Small root segments were cut from 12 days old Arabidopsis plants and incubate in ddH_2O overnight. Prior to experiment, the root segments were transferred into a 96 well plate containing 15 µl of 20 µg/ml horseradish peroxidase and 35 µl 0.1M 8-Amino-5-chloro-2,3-dihydro-7-phenyl-pyrido[3,4-d] pyridazine sodium salt (L-012, Wako Chemicals). Next, 50 µl of either 1 µM flg22 or concentrated NemaWater protein was added to the individual wells and ddH_2O was used as control. Photon was measured as relative light unit (RLU) in a 96-wel luminometer (TECAN Infinite ® 200 PRO) over 120 mins and data analysis done with the instrument software and Microsoft Office Excel.

Gene expression analysis upon treatment with concentrated protein from NemaWater

Twelve-days-old Arabidopsis plants grown in Knop medium were incubated in concentrated *Hs*NemaWater protein for one hour. Afterwards the whole roots were cut and frozen in liquid nitrogen. Arabidopsis roots treated only with ddH_2O were used as a control with the experiment replicated biologically three times. RNA was extracted from the samples using a NucleoSpin RNA Xs (Macherey-Nagel, Germany) kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life technologies cat.no. 4368814), according to the manufacturer's instructions. The transcript abundance of targeted genes was analyzed using the StepOnePlus™ Real-Time PCR System (Applied BioSystems, USA). Each sample contained 10 µL of Fast

SYBR Green qPCR Master Mix with uracil-DNA, glycosylase, and 6-carboxy-x-rhodamine (Invitrogen), 2mM $MgCl_2$, 0.5 µL of forward and 0.5 µL of reverse primers with both primers having 10µM concentration, 1µL of complementary DNA (cDNA) and H_2O was added making a 20 µL of total reaction volume. Samples were analyzed in three technical replicates with the 18S genes used as internal control. The expression of targeted genes was normalized to the internal control (18S) in order to calculate relative expression as described in Pfaffl, 2001.

Seedling growth inhibition assay

Modified knop media was used to grow Arabidopsis plants as previously described. Five day old seedlings were transferred into a 6 well plate containing liquid MS medium supplemented with either 1 ml of *Hs*NemaWater, *Hs*NemaWater protein fraction, 1 µM flg22 or water control. The plants fresh weight was taken after 7 days of growth inside peptide supplemented medium. Root length was also measured. The experiments were conducted in five technical replicates and three biological replicates.

Induce resistance in Arabidopsis

Ten day old Arabidopsis seedling growing on knop media in 6 well plates were treated with 1 mL of *Hs*NemaWater, *Hs*NemaWater protein fraction, or water control and incubated for 48 hours prior to infection with ~ 60 sterile second stage juveniles of *H. schachtii*. Nematode adult female and males were counted at 13 dpi.

Statistically analysis

Data analysis were done with SigmaPlot 12 version, using One-way ANNOVA and Fisher LSD test ($P<0.05$) for pair-wise comparisons. For qRT-PCR ΔCT values were analyzed as previously recommended (Livak & Schmittgen, 2001)

Supplementary data

Table S1: Arabidopsis defense related hormones genes differentially regulated *(FDR*<0.05; Fold change >1.5) during migratory stages of nematode infection and also upon plant treatment with *Hs*NemaWater. Root segments from uninfected roots were used as control. Values indicate fold change compared with control.

ATG locus	*Gene Symbol*	*Microarray data*				
		Migratory	**p-Value**	**NemaWater**	**p-Value**	**Gene Title**
At1g72450	JAZ6	3,40	0,0117	4,29	0,0002	JAZ6 (JASMONATE-ZIM-DOMAIN PROTEIN 6)
At5g57220	CYP81F2	16,66	0,0180	7,80	0,0111	CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 2", CYP81F2
At2g44840	ERF13	5,90	0,0153	6,04	0,0035	ERF13 (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13)
At5g13220	JAZ10	12,54	0,0006	3,90	0,0121	JAZ10 (JASMONATE-ZIM-DOMAIN PROTEIN 10)
At1g17380	JAZ5	6,48	0,0171	3,06	0,0038	JAZ5 (JASMONATE-ZIM-DOMAIN PROTEIN 5)
At2g34600	JAZ7	1,74	0,2522	3,10	0,0071	JAZ7 (JASMONATE-ZIM-DOMAIN PROTEIN 7)
At5g64900	PROPEP1	3,17	0,0004	3,63	0,0019	PROPEP1 (Elicitor peptide 1 precursor)
At2g35980	NHL10	13,17	0,0001	7,14	0,0185	ARABIDOPSIS NDR1/HIN1-LIKE 10

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Chapter 4

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Damage-associated responses of the host contribute to defence against cyst nematodes but not root-knot nematodes

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RESEARCH PAPER

Damage-associated responses of the host contribute to defence against cyst nematodes but not root-knot nematodes

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Abstract

When nematodes invade and subsequently migrate within plant roots, they generate cell wall fragments (in the form of oligogalacturonides; OGs) that can act as damage-associated molecular patterns and activate host defence responses. However, the molecular mechanisms mediating damage responses in plant–nematode interactions remain unexplored. Here, we characterized the role of a group of cell wall receptor proteins in Arabidopsis, designated as polygalacturonase-inhibiting proteins (PGIPs), during infection with the cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita*. PGIPs are encoded by a family of two genes in Arabidopsis, and are involved in the formation of active OG elicitors. Our results show that *PGIP* gene expression is strongly induced in response to cyst nematode invasion of roots. Analyses of loss-of-function mutants and overexpression lines revealed that *PGIP1* expression attenuates infection of host roots by cyst nematodes, but not root-knot nematodes. The PGIP1-mediated attenuation of cyst nematode infection involves the activation of plant camalexin and indole-glucosinolate pathways. These combined results provide new insights into the molecular mechanisms underlying plant damage perception and response pathways during infection by cyst and root-knot nematodes, and establishes the function of *PGIP* in plant resistance to cyst nematodes.

Key words: Damage-associated molecular patterns (DAMPs), glucosinolate, nematode, oligogalacturonide (OG), pattern-triggered immunity (PTI), plant-parasitic nematodes, polygalacturonase (PG), polygalacturonase-inhibiting protein (PGIP).

Introduction

Plant-parasitic nematodes attack almost all major crops throughout the world, causing damage that has been estimated at >US$100 billion per year (Nicol *et al.*, 2011). The ~4100 known species of plant-parasitic nematodes (Decraemer and Hunt, 2006) display a wide variety of parasitic strategies, including simple migratory endoparasites that live in soil and feed on different tissue layers, and more complex migratory endoparasites that move continuously as they feed, thereby causing extensive necrosis of the infected tissues. However, the most complex and economically important is a group of sedentary endoparasites that includes cyst nematodes (CNs; *Globodera* spp. and *Heterodera* spp.) and

root-knot nematodes (RKNs; *Meloidogyne* spp.). Infective-stage CN and RKN juveniles (J2) invade the plant root near the tip and move through different tissue layers to reach the vascular cylinder. Once inside the root, RKN J2s move intercellularly, whereas CN J2s move intracellularly, causing more damage to the host tissues. After reaching the vascular cylinder, CNs induce the formation of a syncytium, whereas RKNs induce the formation of 5–7 giant cells. Both the syncytium and giant cells are hypermetabolic sink tissues, and serve as the sole source of nutrients for growing nematodes throughout their entire life cycle (Kyndt *et al.*, 2013; Siddique and Grundler, 2015). In the case of RKNs, the development of giant cells is accompanied by hypertrophy and hyperplasia of neighbouring tissues, leading to the formation of typical knot-like galls in roots.

The first barrier encountered by nematodes during root invasion is the cell wall. Nematodes utilize two strategies to penetrate the plant cell wall: a stylet is used to pierce through the wall, and an array of cell wall-degrading enzymes is secreted to disrupt wall rigidity, including pectate lyase (de Boer *et al.*, 2002; Vanholme *et al.*, 2007), endo-β-1, 4-glucanase (Smant *et al.*, 1998; de Boer *et al.*, 1999), and polygalacturonase (PG) (Jaubert *et al.*, 2002). PGs are key enzymes that cleave the α1–4 linkage between the D-galacturonic acid residues of homogalacturonan (Kalunke *et al.*, 2015; Rahman and Joslyn, 1953*b*; Themmen *et al.*, 1982). PGs are well characterized in fungi, bacteria, and insects, and their action on the outer plant cell wall is essential for further wall degradation by other wall-degrading enzymes (Rahman and Joslyn, 1953*a, b*; Kester and Visser, 1990). Several fungi secrete PGs, including *Aspergillus flavus* (Whitehead *et al.*, 1995), *Botrytis cinerea* (Cabanne and Doneche, 2002; Favaron *et al.*, 1992), *Aspergillus niger* (Maldonado and de Saad, 1998), *Claviceps purpurea* (Oeser *et al.*, 2002), and *Sclerotinia sclerotiorum* (Reymond-Cotton *et al.*, 1996). A number of bacteria also produce PGs, including *Agrobacterium tumefaciens* (Rodriguezpalenzuela *et al.*, 1991), *Ralstonia solanacearum* (Huang and Allen, 2000), and *Bacillus polymyxa* (Nagel and Vaughn, 1961). Similarly, the salivary glands of some insect species that feed on plants produce PGs, which help them feed on host tissues (Strong and Kruitwagen, 1968; Laurema *et al.*, 1985; Celorio-Mancera *et al.*, 2008, 2009). As stated above, nematodes also secrete PGs. In fact, the first PG of animal origin was isolated from the RKN *Meloidogyne incognita*, where it has been suggested to have a role in parasitism (Jaubert *et al.*, 2002). In addition, the transcriptome of the beet cyst nematode (BCN), *Heterodera schachtii*, was recently described to encode a PG (Fosu-Nyarko *et al.*, 2016).

Plant cell walls can inhibit microbial PG activity via a leucine-rich repeat defence protein called PG-inhibiting protein (PGIP), which attenuates pectin degradation. The crystal structure of PGIP contains a central leucine-rich repeat domain with 10 imperfect repeating units, each derived from 24 amino acid residues. Most leucine-rich repeat proteins have one β-sheet connected with a helix on the convex side or β-turns (Di Matteo *et al.*, 2003). In contrast, the leucine-rich repeat domain in PGIP is organized to form two β-sheets; sheet B1 occupies the concave inner side of the molecule and contains amino acid residues that are crucial for interactions with PGs (Di Matteo *et al.*, 2003). The association of PGIP with PG inhibits PG-mediated cell wall degradation and generates oligogalacturonides (OGs) with elicitor activity (Bishop *et al.*, 1981; Hahn *et al.*, 1981; Nothnagel *et al.*, 1983; Benedetti *et al.*, 2015). These OGs have a degree of polymerization between 10 and 15 (Cote and Hahn, 1994), and they activate defence responses such as the reactive oxygen species (ROS) burst (Galletti *et al.*, 2008), callose deposition (Bellincampi *et al.*, 2000), phytoalexins (Davis *et al.*, 1986), and nitric oxide (Rasul *et al.*, 2012).

The importance of PGIPs in nematode infection is supported by a study in pea (*Pisum sativum* L.) where *PsPGIP1* has been shown to be differentially expressed in susceptible and resistant genotypes in response to *Heterodera goettingiana* infection (Veronico *et al.*, 2011). *In situ* hybridization analysis confirmed that *PsPGIP1* is localized specifically in the syncytium of a resistant pea genotype, suggesting that *PsPGIP1* disrupts syncytium development inside the host root (Veronico *et al.*, 2011). Further progress in this field requires a detailed analysis of the roles of PG, PGIP, and OG in plant–nematode interactions (Holbein *et al.*, 2016). Here, we investigate the role of PGIPs in Arabidopsis during infection with the BCN *H. schachtii* and the RKN *M. incognita*. We found that *PGIP1*-mediated defence responses form an important component of host basal resistance to CNs but not to RKNs.

Materials and methods

Plant growth conditions and nematode infection assays

Arabidopsis plants were grown in either Knop medium (for BCN infection) or Murashige and Skoog (MS) medium (for RKN infection) as described previously (Siddique *et al.*, 2015). The T-DNA insertion mutants were ordered from the Nottingham stock centre (*pgip1-1*, SALK_001662.33.10.x. *pgip1-2*, GK-092G09-012001, *pgip2-1*, and GK-717A02-025309). Salk lines were genotyped (Supplementary Fig. S1 at *JXB* online) using primers listed in Supplementary Table S1. GK lines were screened for homozygosity through sulfadiazine resistance. The homozygous T-DNA insertion mutants were checked for lack of expression (Supplementary Fig. S2) using the primers listed in Supplementary Table S1. Twelve-day-old plants were infected with surface-sterilized 60–80 J2 individuals of BCN or RKN (*M. incognita*). For BCN, the average number of males and average number of females was counted at 12 days post-inoculation (dpi) (Siddique *et al.*, 2015). For RKN, the average number of galls was determined at 21 dpi. All infection assays for BCN and RKN were repeated a minimum of three times and each experiment consisted of 15–20 individual plants. The average area of syncytia and average female area were measured at 14 dpi as described previously (Siddique *et al.*, 2015). Approximately 30 syncytia and associated nematodes were measured for each experiment, and each experiment was repeated three times. To determine the average area of galls, ~30 galls were outlined and measured for each experiment, and each experiment was repeated three times

Cloning and transformation of promoter::GUS lines

Promoter regions upstream of the start codons of *PGIP1* (1214 bp) and *PGIP2* (483 bp) as previously described by Ferrari *et al.*

(2003) were amplified from genomic DNA using primers given in Supplementary Table S1 and cloned in a Gateway cloning vector, pDONR 207 (Invitrogen), according to the manufacturer's instructions. The verified fragments were fused with the *β-glucuronidase* (*GUS*) gene in the expression vector pMDC162 (Curtis and Grossniklaus, 2003). These promoter::GUS constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 for the transformation of 4- to 6-week-old Arabidopsis plants by the floral dip method (Clough and Bent, 1998). After drying of plants, seeds (T_0) were harvested and sterilized before growing on Knop medium supplemented with 25 µg ml^{-1} hygromycin. Three independent homozygous plants were selected for further analysis. Homozygous lines were grown in Knop medium and infected with nematodes to analyse the GUS expression in a time-course analysis. The infected or uninfected roots were incubated with X-gluc for 12–14 h at 37 °C. After overnight incubation, the reaction was stopped and samples were washed with 70% ethanol. Staining was carried out at different time points for *H. schachtii* (1, 3, 5, and 10 dpi) and *M. incognita* (1, 3, 7, and 15 dpi). The stained syncytia and galls were photographed with a Leica DM4000 inverted microscope equipped with LAS software (Leica Microsystems) and fitted with an Olympus C-5050 digital camera.

Quantitative RT–PCR

Arabidopsis plants were grown and infected with nematodes as described above. Root segments containing the infection zone were cut, and total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Contaminating DNA was digested with DNase1 using a DNA-*free*™ DNA Removal Kit (Ambion) and the RNA was used to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosynthesis, Darmstadt, Germany) following the manufacturer's instructions. Quantitative reverse transcription–PCR (qRT–PCR) was performed with the StepOne Plus Real-Time PCR System (Applied Biosystems) using the primers given in Supplementary Table S1. Each sample contained 10 µl of Fast SYBR Green qPCR Master Mix (Invitrogen), 2 mM $MgCl_2$, 0.5 µl each of forward and reverse primers (10 µM), 2 µl of cDNA, and water in a 20 µl total reaction volume. *UBQ5* and *β-tubulin* was used as an endogenous control except for assays involving nematode feeding sites (galls and syncytia). For galls and syncytia, *18S* and *UBP22* were used as housekeeping genes as recommended previously (Hofmann and Grundler, 2007). cDNA was diluted 1:100 for 18S amplification. Data were analysed using Pfaffl's method (Pfaffl, 2001). Data shown are an average of three independent experiments. Each experiment consisted of three technical replicates. Primer sequences used for qRT–PCR analysis along with their respective efficiencies are listed in Supplementary Table S1.

Generation of overexpression and complementation lines

To overexpress *AtPGIP1* and *AtPGIP2*, full-length coding sequences of both genes were amplified from cDNA synthesized from RNA isolated from 12-day-old Arabidopsis plants. The primer pairs used to amplify the coding sequences from both genes are listed in Supplementary Table S1. The amplified PCR product was cloned into Gateway cloning vector pDONR207 (Invitrogen). The cloned fragments were verified through sequencing and transferred via Gateway recombination into the pMDC32 vector, where they were placed under the control of the double *Cauliflower mosaic virus* (CaMV) 35S promoter to engineer *AtPGIP1* and *AtPGIP2* overexpression. The verified constructs were introduced into *A. tumefaciens* strain GV3101, which was used for the transformation of 4- to 6-week-old Col-0 plants by the floral dip method (Clough and Bent, 1998). After drying of plants, seeds (T_0) were harvested and sterilized before being sown on Knop medium supplemented with 25 µg ml^{-1} hygromycin. Transformants were selected to produce homozygous plants. At least two independent homozygous lines with the highest up-regulation were selected for further studies. Complemented lines of *pgip1* mutants were obtained by cloning a wild-type copy of the *PGIP1* gene under the control of the CaMV *35S* promoter using the Gateway cloning system as described above. Two homozygous complemented lines carrying an insertion of the wild-type gene were used in this study.

Plant treatment with OGs

OGs with a degree of polymerization between 10 and 15 were purchased commercially (GAT114, Elicityl, France). Arabidopsis seeds were sterilized and grown in 6-well plates containing 5 ml of liquid Knop medium. After 9 d of germination, the medium was removed and 3 ml of fresh medium was added to the wells before adding 30 µl of OGs to a final concentration of 50 µg ml^{-1}. After 24 h of treatment, the plants were gently placed in semi-solid Knop medium and allowed to recover from any stress for a 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 and evaluated for infection after 12–14 dpi as described above.

Measurement of ROS

Apoplastic measurement of hydrogen peroxide in small root segments was carried out via a luminol-based detection method as previously described (Mendy *et al.*, 2017). Arabidopsis plants were grown in Knop medium for 2 weeks, after which uniform root pieces measuring ~0.2 cm were cut with a knife and placed in a 96-well plate with water for 24 h to reduce the wounding response. After overnight incubation, the water was removed and replaced with flg22 solution, and ROS was measured as described (Mendy *et al.*, 2017).

Statistical procedures

Data were statistically analysed using SigmaPlot 12, applying *t*-test ($P<0.05$) for pairwise comparisons. For qPCR, statistical procedures were applied to ΔCT values as recommended previously (Livak and Schmittgen., 2001).

Results

PGIP1 *and* PGIP2 *are induced by nematode infection*

Arabidopsis plants contain a family of two *PGIP* genes designated as *PGIP1* and *PGIP2*. To assess the regulation of *PGIP* genes during different stages of nematode infection, we evaluated the expression of these genes in published transcriptomic data (Jammes *et al.*, 2005; Szakasits *et al.*, 2009; Barcala *et al.*, 2010; Mendy *et al.*, 2017). These analyses revealed that *PGIP1* expression increased during migratory (10 h post-inoculation, hpi) and sedentary stages of BCN infection with *H. schachtii* (Supplementary Table S2). In contrast, there were no significant differences in *PGIP1* and *PGIP2* expression levels in microarrays of root segments containing giant cells or galls infected with the RKN *M. javanica or M. incognita* (Jammes *et al.*, 2005; Barcala *et al.*, 2010; Cabrera *et al.*, 2014). However, a recent next-generation sequencing-based transcriptome profiling of Arabidopsis found that expression of both *PGIP1* and *PGIP2* is significantly up-regulated in galls (3, 5, and 7 dpi) induced by the RKN *M. incognita* (Yamaguchi *et al.*, 2017).

We validated these microarray data using Arabidopsis plants that were grown *in vitro* and infected with BCNs or

RKNs. RNA was extracted and analysed for the expression of *PGIP1* and *PGIP2* via qRT–PCR. For BCNs, infected root segments were sampled at 10 hpi (migratory stage; ~0.2 cm around the nematode head) or 10 dpi (sedentary stage). The results confirmed that *PGIP1* expression increases during the migratory stage at 10 hpi upon BCN infection (Fig. 1a), but we were unable to confirm *PGIP1* up-regulation during the sedentary stage at 10 dpi (Fig. 1b). For RKN, root segments were collected at 24 hpi (root tips; migratory stage), 7 dpi (sedentary stage), or 15 dpi (sedentary stage). We found no change in expression for *PGIP1* and *PGIP2* at the migratory stage with RKN (Fig. 1c), but the expression of both was increased during the sedentary stages at 7 dpi and 15 dpi (Fig. 1d, e).

To determine the spatiotemporal expression patterns of *PGIP* genes during plant–nematode interactions, we transformed Arabidopsis with *PGIP1*::*GUS* or *PGIP2::GUS* constructs and generated 3–5 independent homozygous lines. Although *PGIP1* and *PGIP2* are induced by wounding in leaves, their expression patterns in roots have not been determined. Therefore, we wounded the roots of 10-day-old plants and performed GUS staining 1 h after wounding. We observed specific and strong GUS staining indicating *PGIP1* and *PGIP2* expression at and around the wounding sites (Fig. 2a). Next, we performed a time-course analysis of *PGIP* expression during BCN infection using the *PGIP* promoter::GUS fusions. The majority of root infection zones exhibited strong GUS staining at 1, 3, and 5 dpi, and no GUS staining was observed in uninfected root segments. The GUS staining intensity declined considerably at 10 dpi for both *PGIP1* and *PGIP2* (Fig. 2a). Next, we analysed the spatiotemporal expression patterns of *PGIP1::GUS* and *PGIP2::GUS* in response to infection with the RKN. No GUS staining was observed at 1 dpi for both *PGIP1* and *PGIP2*. In contrast, GUS-specific staining was observed at 3 dpi onward in galls induced by *M. incognita* (Fig. 2b). Taken together, we concluded that gene expression for both *PGIP1* and *PGIP2* is strongly induced during migratory stages of BCN infection but not during RKN migration.

PGIP-mediated signalling is involved in cyst nematode infection

To explore the role of PGIPs in nematode infection, we characterized loss-of-function T-DNA insertion mutants for *PGIP1* (*pgip1-1* and *pgip1-2*) and *PGIP2* (*pgip2-1*)

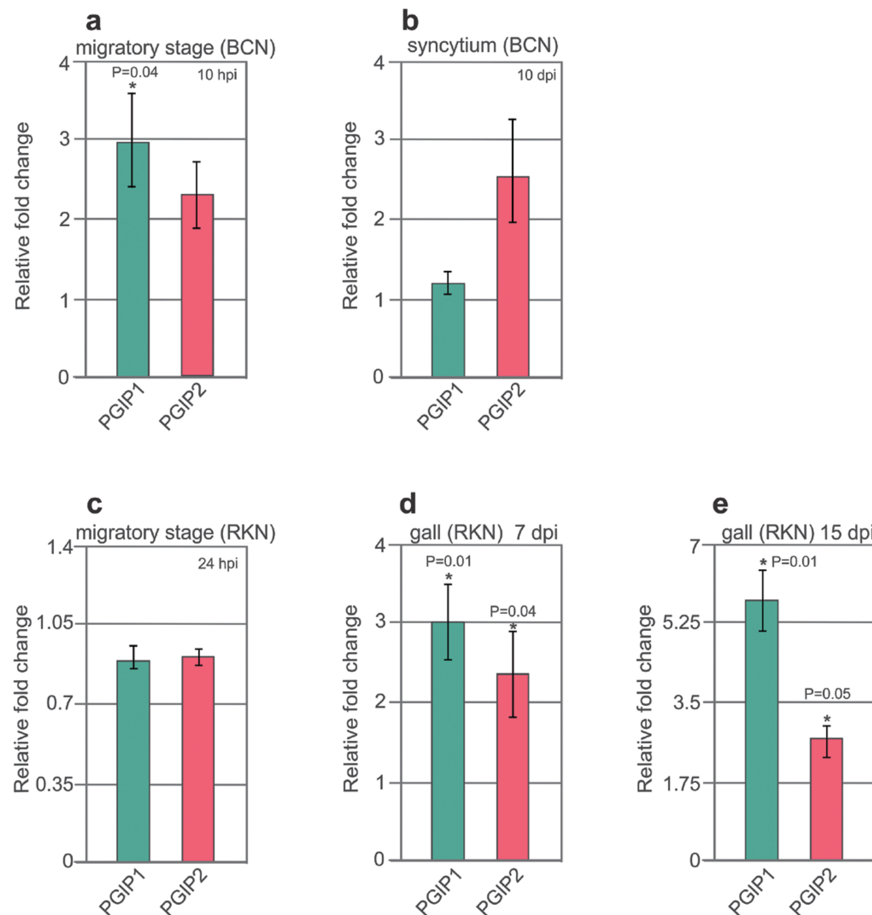

Fig. 1. *PGIP* genes are activated in Arabidopsis upon nematode infection. Validation of changes in *PGIP* gene expression upon nematode infection via qRT–PCR. The values represent relative fold change in response to nematode infection with the value in uninfected control root set to 1. (a, c) *UBQ5* and β-*tubulin* were used as housekeeping genes to normalize the data. (b, d, e) *18S* and *UBP22* were used as housekeeping genes to normalize the data. (a–e) Data bars represent the mean ± SE for three independent experiments. Data were analysed using *t*-test ($P<0.05$). Asterisks represent statistically significant differences from uninfected control root.

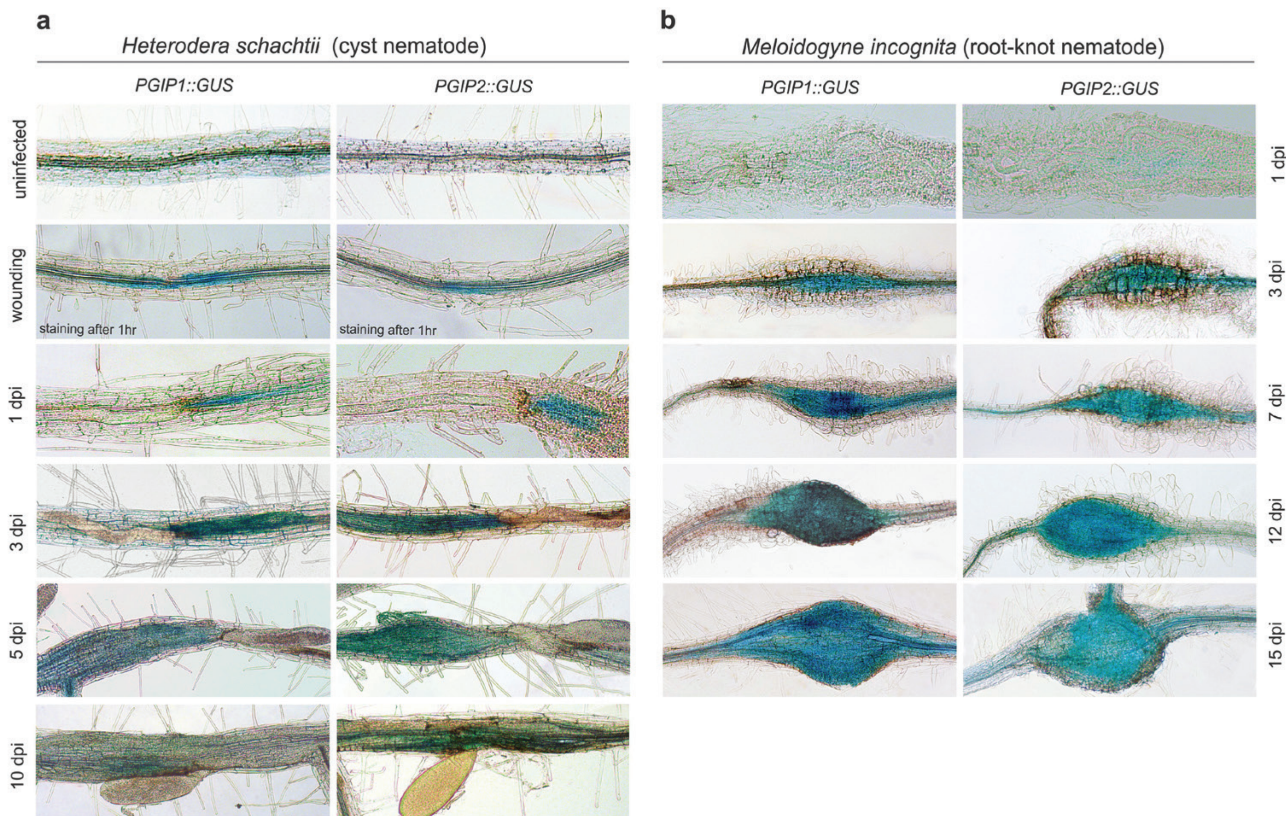

Fig. 2. Activation of PGIP::GUS expression in Arabidopsis roots upon CN and RKN infection. (a) Expression of *PGIP1::GUS* and *PGIP2::GUS* in Arabidopsis roots upon wounding or *H. schachtii* infection at 1, 3, 5, and 10 dpi, respectively. Scale bar=200 µm. (b) Expression of *PGIP1::GUS* and *PGIP2::GUS* in Arabidopsis roots upon *M. incognita* infection at 1, 3, 7, 12, and 15 dpi, respectively. Scale bar=200 µm.

(Supplementary Figs S1, S2). Plants were grown *in vitro* for 12 d and then infected with J2s of BCN or RKN. For BCN, we counted the numbers of nematode females and males at 12 dpi, and the average syncytium size and average size of nematode females at 14 dpi. For RKN, we counted the number of galls and average area of galls at 21 dpi. After BCN infection, we observed a significant increase in the average number of females in both *PGIP1* mutants (*pgip1-1* and *pgip1-2*) compared with the Col-0 control (Fig. 3a; Supplementary Fig. S3a). Moreover, we also observed a significant increase in average syncytium size in *pgip1-1* and *pgip1-2* infected with BCN, but did not observe any significant differences in average female size (Fig. 3b, c; Supplementary Fig. S3b, c). However, our data did not show any significant differences in average number of females, average female size, or average syncytium size in *pgip2-1* infected with BCN, but we did observe a significant reduction in the average number of males compared with the Col-0 control (Supplementary Fig. S4a–c). After RKN infection, we did not observe any changes in the average gall number or size in all tested lines (Fig. 3d–g). These combined results suggest that *PGIP1* knockout leads to hypersusceptibility of plants to CNs but not to RKNs. To confirm this differential susceptibility further, we transformed *pgip1-1* mutants with the *35S::PGIP1* overexpression construct and analysed the homozygous transgenic plants using nematode infection assays. The number of females of BCNs in transgenic plants did not differ from that of Col-0. However, one of the lines showed a significant increase in the number of males as well as the total number of nematodes (Supplementary Fig. S5a–d).

PGIP1 *overexpression and OG treatment reduce susceptibility to cyst nematode infection but not root-knot nematode infection*

As loss-of-function *PGIP1* mutants were hypersusceptible to CN infection, we hypothesized that *PGIP1* overexpression might mitigate plant susceptibility to nematode infection. We produced transgenic plants expressing *PGIP1* or *PGIP2* under the control of 35S promoters (*35S*::*PGIP1* and *35S*::*PGIP2*), performed qRT–PCR analysis of the resultant lines, and selected three homozygous lines (L2, L9, and L10) that displayed the highest *PGIP* expression levels for further experiments (Fig. 4a). We did not observe any significant phenotypic differences in the transgenic lines and the Col-0 controls. Then, 12-day-old transgenic (L2, L9, and L10) and Col-0 plants were infected with J2s of *H. schachtii*, and the results were evaluated at 14 dpi. The number of females and total number of nematodes per plant were significantly reduced for L9 and L10 compared with Col-0, but neither of these parameters differed for L2 (Fig. 4b). The average syncytium size significantly declined in all three transgenic lines compared with Col-0, but there were no significant differences in the sizes of female nematodes (Fig. 4c, d). In contrast, no significant differences were observed for any parameters in any lines overexpressing *PGIP2* (Supplementary Fig.

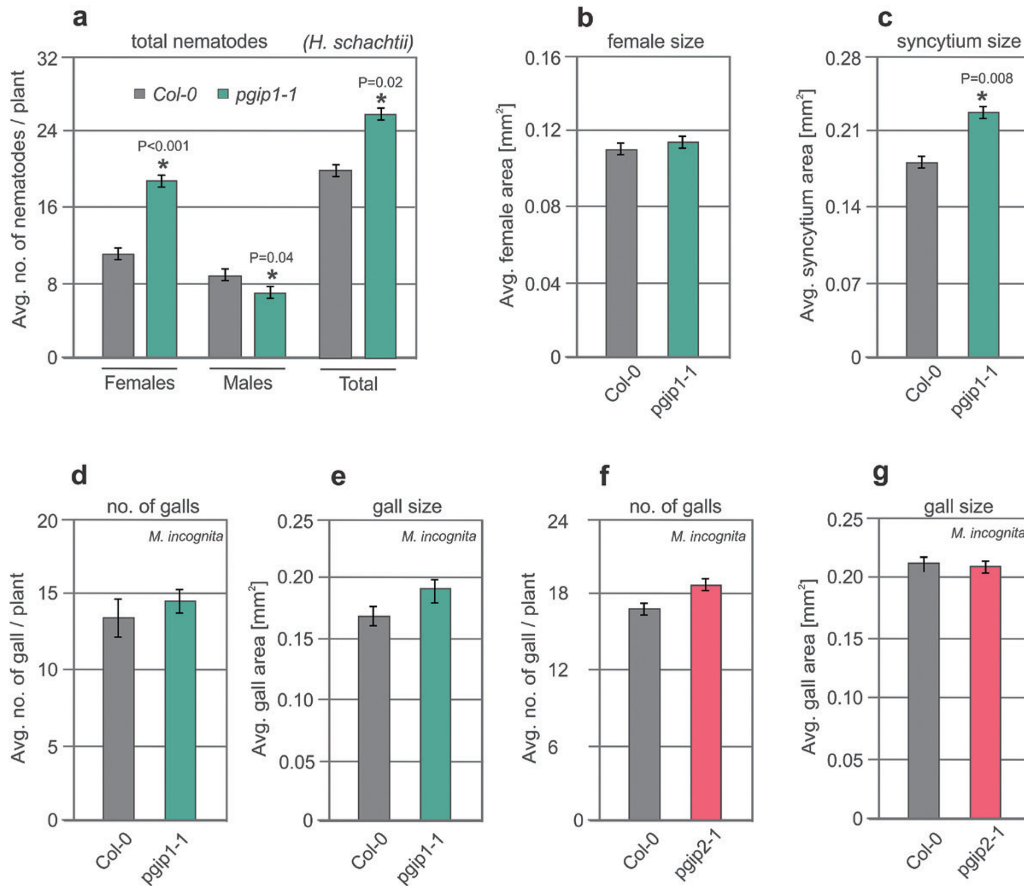

Fig. 3. CN and RKN infection assays in PGIP1 and PGIP2 receptor mutant plants. (a) Average number of females and males per plant present in Col-0 *pgip1-1* mutant lines at 12 dpi. (b, c) Average sizes of female nematodes (b) and plant syncytia (c) in Col-0 and *pgip1-1* mutant lines at 14 dpi. (d, f) Average number of galls per plant present in Col-0, *pgip1-1* (d), and *pgip2-1* (f) mutant lines at 21 dpi. (e, g) Average area of galls per plant present in Col-0, *pgip1-1* (e), and *pgip2-1* (g) mutant lines at 21 dpi. (a–g) Bars represent the mean ± SE for three independent experiments. Data were analysed using *t*-test (P<0.05).

S6a–d). Overexpression of *PGIP1* or *PGIP2* also did not affect the average gall number or size induced by RKN infection (Supplementary Fig. S7). These data suggest that overexpression of *PGIP1* leads to reduced susceptibility of plants to CNs but not to RKNs.

PGIP promotes the formation of OGs, which in turn activate host defence responses to restrict pathogen development. To evaluate whether OGs have a similar role in plant–nematode interactions, we treated the Col-0 plants with OGs and infected them with BCN. The number of females and the sizes of syncytium and females were significantly lower in plants treated with OGs than in water-treated (mock) control plants (SupplementaryFig. S8a–c), suggesting that OG-induced host defence responses are able to restrict infection of nematodes.

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

Apoplastic ROS production is one of the hallmarks of pattern-triggered immunity (PTI) responses, which are activated after pathogen attack or elicitor treatment (Siddique *et al.*, 2014). To investigate whether PGIPs are involved in PTI responses and whether *pgip1* hypersusceptibility to nematode infection results from impaired ROS production, we quantitatively evaluated PTI responses by performing a luminol-based detection assay. Root segments from 2-week-old *pgip1-1* and *pgip2* mutant plants displayed the same level of ROS production in response to the immunogenic peptide flg22 as wild-type plants (Fig. 5a). These results indicate that elicitor-induced ROS production is independent of both *PGIP1* and *PGIP2*, suggesting that it plays no role in PGIP-mediated defence responses.

We hypothesized that the hypersusceptibility of *pgip1* mutants might be due to impaired expression of genes in defence-related pathways. Therefore, we assessed the expression of the following genes that are strongly up-regulated during the migratory stage of infection as determined in our recent microarray data (Mendy *et al.*, 2017): *JAZ8* (Chini *et al.*, 2007), which is involved in jasmonic acid signalling; *NPR2*, a salicylic acid marker gene (Canet *et al.*, 2010); *PROPEP1*, a member of the PROPEP family that is induced in response to wounding (Huffaker *et al.*, 2006); and three genes involved in the synthesis of camalexin and indole-glucosinolate, including *CYP81F2* [encodes a cytochrome P450 involved in indol-3-yl-methyl glucosinolate catabolism (Clay *et al.*, 2009)], *CYP71B15* [PAD3, catalyses the final step in camalexin biosynthesis (Zhou *et al.*, 1999; Schuhegger *et al.*, 2006)], and *CYP71A12* [dehydrates indole-3-acetaldoxime

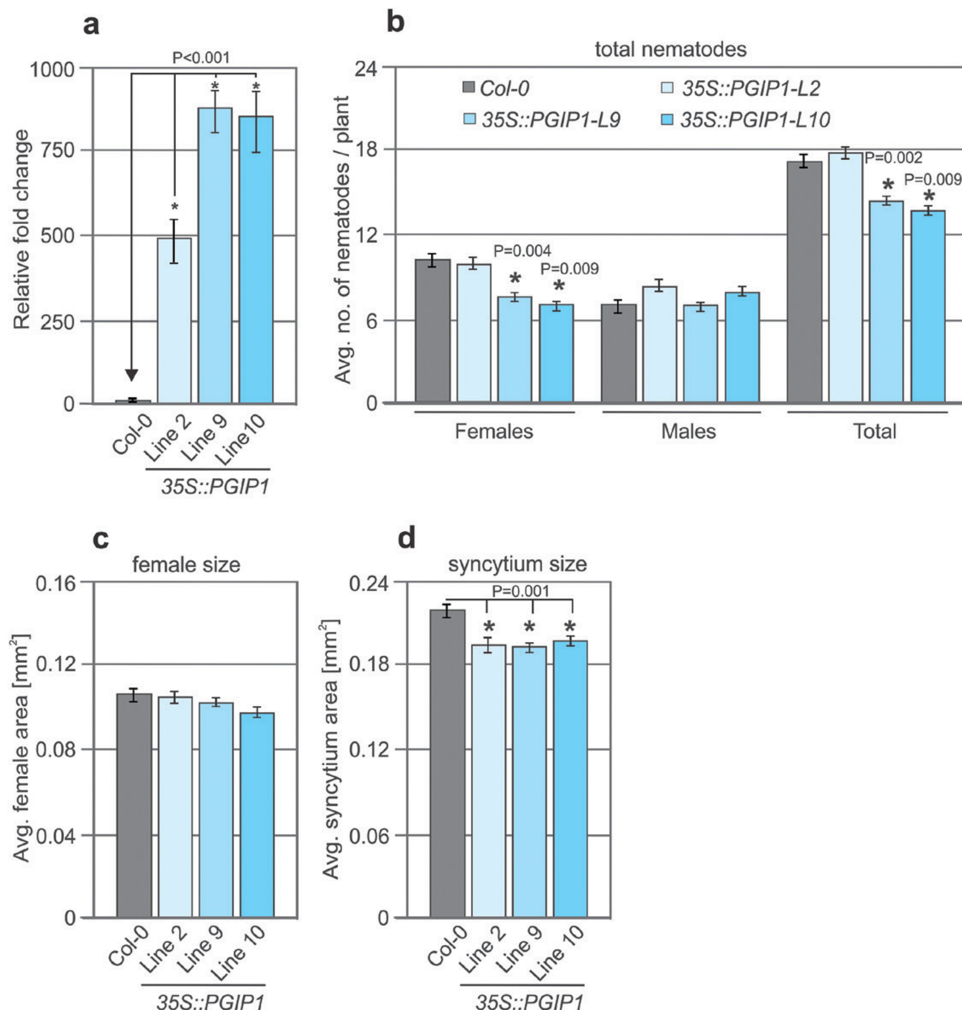

Fig. 4. Nematode infection assays in *PGIP1* overexpression lines. (a) Three independent homozygous lines (L2, L9, and L10) overexpressing *PGIP1* (*35S::PGIP1*) were selected and analysed for changes in transcript abundance of *PGIP1*. The values represent relative fold change with the value in Col-0 plants set to 1. *UBQ5* and and β-*tubulin* were used as housekeeping genes to normalize the data. (b) Average number of females and males per plant present in Col-0 and *PGIP1* overexpression lines at 12 dpi. (c, d) Average sizes of female nematodes (c) and plant syncytia (d) in Col-0 and *PGIP1* overexpression lines at 14 dpi. (a–d) Bars represent the mean ± SE for three independent experiments. Data were analysed using Student's *t*-test (P<0.05). Asterisks represent statistically significant differences from the corresponding Col-0.

(IAOx) to indole-3-acetonitrile (IAN) (Millet *et al.*, 2010)]. The results from qRT–PCR analyses showed no significant changes in the expression of all tested genes between *PGIP* mutants and Col-0 in uninfected roots. Next, we sampled roots at 10 hpi (migratory stage of nematode infection) and used these samples for qRT–PCR analysis. There were no changes in the expression of *JAZ8*, *PROPEP1*, or *NPR2* in *pgip1-1* or *pgip2-1* compared with Col-0 (Fig. 5b–d). In contrast, the normal up-regulation of genes involved in indole-3-glucosinolate and camalexin biosynthesis (*CYP81F2*, *CYP71A12*, and *PAD3*) was significantly impaired in *pgip1-1* compared with Col-0 (Fig. 5e–g). These results indicate that *pgip1-1* susceptibility to nematode infection results from impaired induction of camalexin and indole-3-glucosinolate biosynthesis pathways. To confirm these results, we used a double mutant *cyp79b2/b3*, which is strongly impaired in indole-glucosinolate and camalexin biosynthesis and accumulation (Zhao *et al.*, 2002; Kliebenstein *et al.*, 2005). The *cyp79b2/b3* plants were grown for 12 d *in vitro*, inoculated with cyst nematodes, and the numbers of males and females were counted. The number of females increased significantly in *cyp79b2/b3* compared with Col-0 (Fig. 6a). However, we did not observe any significant differences in the average sizes of females and syncytia in *cyp79b2/b3* and Col-0 (Fig. 6b, c). Taken together, these results suggested that BCN migration within roots induced camalexin and indole-glucosinolate biosynthesis pathways in a PGIP1-dependent manner, which restricted the number of nematodes.

Discussion

In the present study, we established a molecular framework for PGIP regulation and downstream signalling in Arabidopsis during CN and RKN parasitism. We first analysed the expression of *PGIP1* and *PGIP2* in response to BCN and RKN infection and found commonalities, but also differences between two nematode species. We found that expression of both *PGIP1* and *PGIP2* is induced during migratory

stages of BCN infection. This expression was localized to the infection zone close to the head of nematodes, suggesting that the induction is highly specific to infection. In contrast to BCN, RKN migration inside the roots did not induce *PGIP* expression at 1 dpi (migratory stage), unravelling what may be a key difference in *PGIP* regulation between the two nematode species. Previously, Ferrari *et al.* 2003 showed that expression of *PGIP1* and *PGIP2* is induced by wounding in leaves and we also observed a highly specific activation of *PGIP* gene expression in roots upon wounding. Therefore, the difference in *PGIP* expression during migratory stages is likely to be due to a difference in the migration style of CNs versus RKNs. Whereas RKNs migrate intercellularly and cause little damage, CNs migrate intracellularly and cause severe damage to root cells (Wyss and Zunke, 1986; Wyss *et al.*, 1992). The hypothesis that RKN do not cause damage during their migration inside the root is also in line with a recent study showing that Arabidopsis lines with altered damage perception do not show any change in susceptibility to RKN (Teixeira *et al.*, 2016).

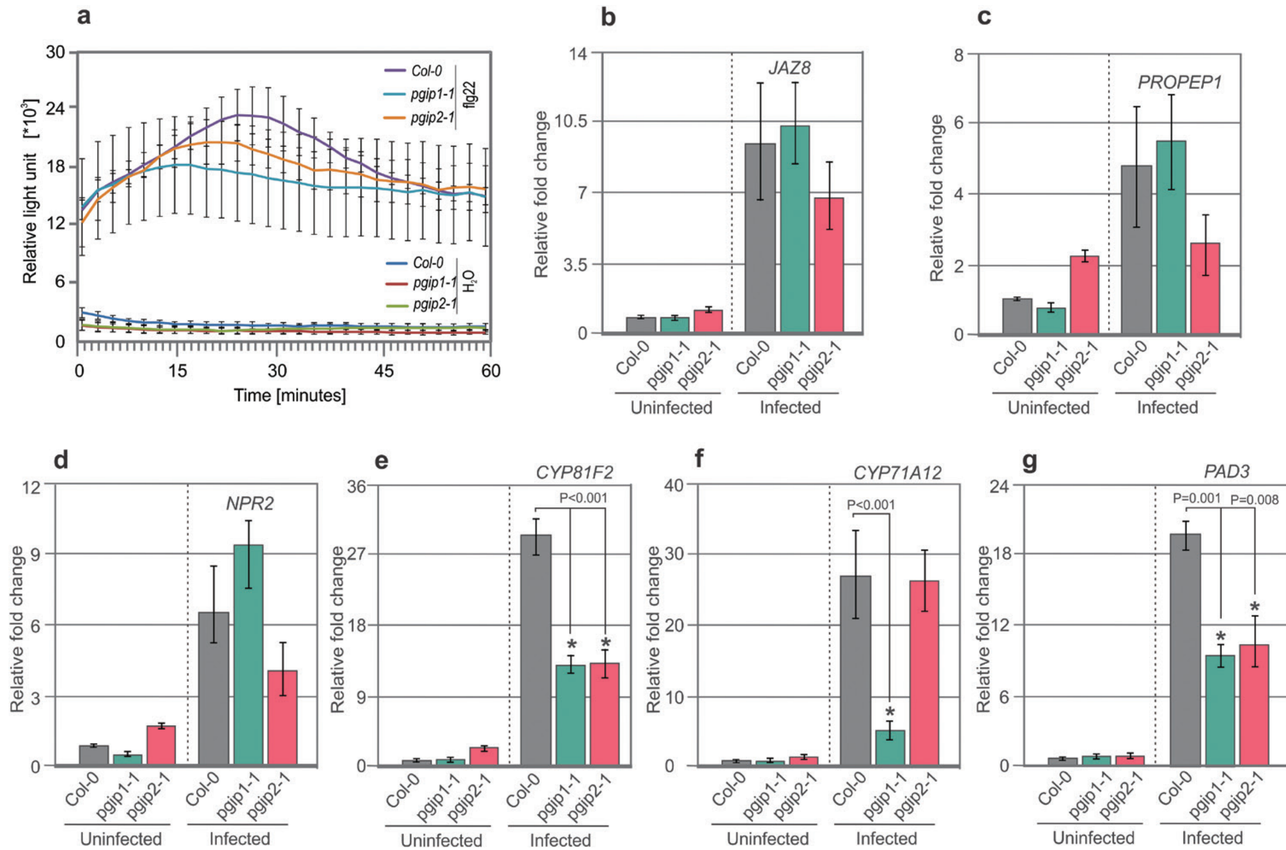

Fig. 5. ROS production and gene expression analysis on root segments. (a) Root segments from Col-0, *pgip1-1*, and *pgip2-1* plants were treated with water or flg22, and ROS burst was measured using an L-012-based assay from 0 to 60 min. (b–g) Infected and uninfected root segments (~0.2 cm) from Col-0, *pgip1-1*, and *pgip2-1* plants were cut and gene expression was measured. For uninfected roots, data represent relative expression of the indicated genes with the value in Col-0 plants set to 1. For infected roots, data represent relative expression of the indicated genes with the value in uninfected roots set to 1. Bars represent the mean ±SE for three independent experiments. Data were analysed using Student's *t*-test (P<0.05). Asterisks represent statistically significant difference from the corresponding Col-0.

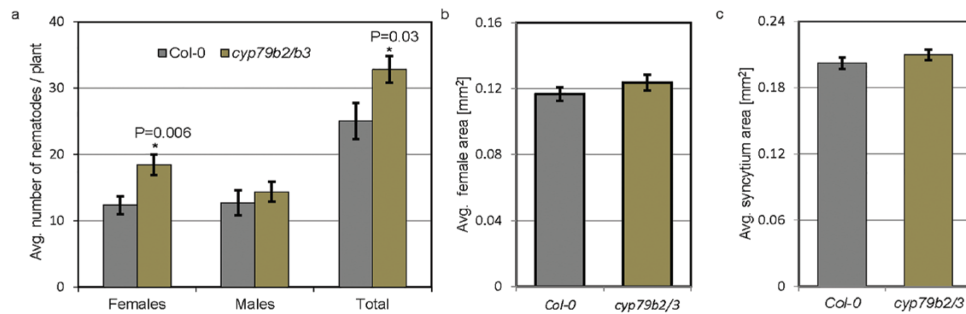

Fig. 6. Cyst nematode infection assays in *cyp79b2/b3* lines. (a) Average number of females and males per plant present in Col-0 and *cyp79b2/b3* lines at 12 dpi. (b, c) Average sizes of female nematodes (b) and plant syncytia (c) in Col-0 and *cyp79b2/b3* lines. Data were analysed using Student's *t*-test (P<0.05). Asterisks represent statistically significant differences from the corresponding Col-0.

The RKN *M. incognita* encodes a PG (MI-PG-1) that is secreted into the host tissue to weaken the plant cell wall during nematode penetration and intercellular migration (Jaubert *et al.*, 2002). However, our experiments to identify a functional PG in CN have proven unsuccessful. These observations raise the question of whether PG secretion by nematodes (if any) has a role in activation of *PGIP* expression during nematode infection of plant roots. We did not observe any *PGIP* expression during the migratory stage of RKN infection and CNs do not appear to encode a PG. Therefore, we postulate that *PGIP* induction during nematode infection is independent of nematode-derived PGs, at least during the migration stage. This hypothesis is consistent with observations that MI-PG-1 is an exo-PG, which are not usually inhibited by PGIPs (Jaubert *et al.*, 2002; Schacht *et al.*, 2011).

OG-mediated resistance to the necrotrophic fungal pathogen *Botrytis cinerea* is independent of salicylic acid, jasmonic acid, and ethylene, but requires PAD3, which catalyses the final step in camalexin biosynthesis (Ferrari *et al.*, 2007). Here, we found that knocking out or overexpressing *PGIP1* significantly increased or decreased, respectively, the susceptibility of plants to CN. Further, pre-treatment of plants with OGs led to a significant reduction in nematode infection. Based on these data, we propose that activation of *PGIP* in response to CN infection promotes the formation of active OG elicitor, which in turn activates the expression of genes involved in indole-glucosinolate and camalexin biosynthesis. Indeed, we found that up-regulation of three key indole-glucosinolate and camalexin biosynthesis genes (*CYP71A12*, *CYP71B15/PAD3*, and *CYP81F2*) in response to BCN infection was impaired in *pgip* mutants (especially in *pgip1*) compared with Col-0 control plants. Conversely, the double mutant *cyp79b2/b3*, which is deficient in camalexin and indole-glucosinolate production, displays enhanced susceptibility to CN (Zhao *et al.*, 2002; Kliebenstein *et al.*, 2005). The relevance of camalexin in CN infection is further evidenced by the fact that loss-of-function *pad3* mutants are more susceptible to the BCN (Ali *et al.*, 2013). The impaired up-regulation of camalexin and indole-glucosinolate genes is only partial in *pgip* mutants, which is probably due to the functional redundancy in this gene family. It is also plausible that these genes are regulated in both a PGIP-dependent and a PGIP-independent manner during CN parasitism. RKN invasion of the Arabidopsis root has been shown to induce *PAD3* expression during migratory stages of infection. In addition, mutants that are impaired in indole-glucosinolate or camalexin biosynthesis are hypersusceptible to RKN (Teixeira *et al.*, 2016). These previous observations, together with the fact that we did not observe any *PGIP* expression during early stages of infection, suggest that camalexin and indole-glucosinolate biosynthesis is regulated in a PGIP-independent manner during plant–RKN interactions.

The consistent expression of *PGIP* genes in syncytia and giant cells during biotrophic stages of parasitism suggests that these genes may have a role in nematode parasitism other than activation of PTI-like defence responses. PGIPs have been shown to interact with partially or completely de-esterified homogalacturonan (HG) in pectin, and protect it from the hydrolysing activity of plant or pathogen PGs (Spadoni *et al.*, 2006). Thus, the PGIP expression level probably reflects a contribution to the mechanical properties of the cell wall related to growth and development. Previous studies showed that HG in the cell walls of younger syncytia (5 dpi) is highly de-esterified compared with that of older syncytia (15 dpi). In contrast, highly methylesterified HG was abundant in the cell wall of younger (7 dpi) and older (14 dpi) giant cells (Davies *et al.*, 2012; Wieczorek *et al.*, 2014). Although the syncytium and giant cells perform the same function, they have different ontogenies, which might underlie the differences in methylesterification of younger feeding sites associated with CNs or RKNs.

The syncytium expands through dissolution of the cell wall and fusion of root cells. During cell wall expansion, the wall is locally degraded and modified, which ultimately leads to local wall strengthening and thickening (Siddique *et al.*, 2012; Wieczorek *et al.*, 2014). In contrast, giant cells grow via repeated nuclear division without cytokinesis. Therefore, extensive de-esterification of the cell wall at 5 dpi may facilitate wall degradation and promote syncytium expansion. Conversely, a higher level of methylesterification in older feeding sites of both CNs and RKNs may provide 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). Based on these observations, we hypothesize that the high *PGIP* expression in younger syncytia at 5 dpi plays a role in regulation of local cell wall degradation by allowing PGIPs to bind directly PGs (of plant or nematode origin) and HG, protecting the cell wall from further degradation. This hypothesis is consistent with our observations that *PGIP1* knockout or overexpression significantly increases or reduces, respectively, the average size of the syncytium. Cell wall degradation slows down as the syncytium expands and reaches its maximum size, which was accompanied by a reduction in *PGIP* expression levels. In contrast, *PGIP1* was consistently and highly expressed in galls/giant cells throughout the sedentary stages of nematode development, which may protect the cell walls from enzymatic degradation by blocking de-esterified HG. However, no significant phenotypic differences were observed for RKN infection in any of the lines we tested, possibly due to functional redundancy within the *PGIP* gene family.

In conclusion, this study identified the molecular mechanism underlying PGIP-mediated damage-associated responses during CN and RKN parasitism of plants. We showed that differential regulation of *PGIP* genes occurs during CN and RKN invasion of roots, probably associated with differences in nematode migration and feeding habits. We also determined that PGIP regulates camalexin and indole-glucosinolate biosynthetic pathways in an infection-specific manner. These results provide new insights into the functional mechanisms underlying nematode parasitism. Clarifying further details of damage-associated pathways in plant–nematode interactions may lead to novel control measures for this important plant parasite.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Genotyping of SALK T-DNA insertion line.

Fig. S2. RT–PCR analysis of gene expression profiles in Col-0 and knockout mutants.

Fig. S3. Cyst nematode infection assays in *PGIP1* (*pgip1-2*) mutant lines.

Fig. S4. Cyst nematode infection assays in *PGIP2-1* mutant lines.

Fig. S5. Cyst nematode infection assays in complementation lines for *PGIP1* (*35S::PGIP1/pgip1-1*) mutants.

Fig. S6. Cyst nematode infection assays in *PGIP2* overexpression lines.

Fig. S7. Root-knot nematode infection assays in *PGIP1* and *PGIP2* overexpression lines.

Fig. S8. Cyst nematode infection assays upon OG treatment.

Table S1. Primer sequences used in this work.

Table S2. Overview of *PGIP1* and *PGIP2* expression patterns in published transcriptomic data.

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Chapter 5

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The role of PEPR receptors in damage responses during plant-nematode interaction

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The role of PEPR receptors in damage responses during plant-nematode interaction

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Abstract

Nematode causes cell wall damages during root invasion and migration into the plant cell. The damage result to the production of cell wall component like oligogalacturionides and small peptides, which can activate damage associated immune responses similar to PTI in plants. The role of plant peptides in defense responses during plant nematode interaction has not been well studied. Previous reports showed that PEPR receptors do not play vital role in plant responses to root-knot nematodes. In this work, we studied Arabidopsis peptide receptors (AtPEPRs) and their role in defense responses against beet cyst nematode *Heterodera schachtii*. Our result showed a high susceptibility of *pepr1/2* double mutant to *H. schachtii*. We did not observe any significant differences in the number of galls formed as compared to Col-0 wildtype infected with *Meloidogyne incognita.* We also tested the Arabidopsis extracellular ATP receptor *DORN1* (Does not Respond to Nucleotides) single mutant against both cyst and root knot nematode. Although there was an increase in female sizes of the cyst nematode compared to wild type, we did not observe any significant difference in total number of nematodes. Our result showed the likely involvement of PEPR receptors in mediating defense responses against cyst nematodes, probably due to the high damage caused by these nematodes during their migration within the cells.

Keywords: Damage responses, Arabidopsis, PEPRs, cyst nematodes, root-knot, DORN1

Introduction

All multicellular organisms have evolved ways to defend themselves against biotic and abiotic factors as well as responding to cellular tissue damages. Apart from recognition of conserved microbial/pathogen associated molecular patterns (MAMPs/PAMPs) by cell surface pattern recognition receptors (PRRs), plant innate immunity can also be activated by release of endogenous molecules into the extracellular space due to membrane damage. Such endogenous molecules are referred to as Damage-Associated Molecular patterns (DAMPs) (Lotze *et al*., 2007; Bianchi *et al*., 2007; Tang *et al*., 2012). Whereas MAMPs/PAMPs are conserved in microorganism and are recognized by the host as 'non-self', DAMPs are host-derived (Matzinger, 1994). DAMPs were first described in animals (Seong & Matzinger, 2004), and the High Mobility Group BOX 1 (HMGB1) is among the first and best characterized animal DAMPS (Lotze & Tracey, 2005; Schiraldi *et al*., 2012). Only a few DAMPs have been described in plants compared to animals. The best studied plant DAMPs belong to the class of polypeptides (peptides) produced from larger proteins and includes systemin recognized by a 160-kDa systemin cell-surface receptor (Pearce *et al*., 1991; Constabel *et al.,* 1998), plant elicitor peptides (Peps) recognized by a leucine-rich repeat (LRR) peptide receptor PEPR1 & PEPR2 with BAK1 as co-receptor (Pearce *et al.,* 2001; Huffaker *et al*., 2006; Yamaguchi *et al*., 2010). The recognition of these endogenous molecules helps in amplifying innate immune responses (Huffaker *et al*., 2007). Peptide receptors are well characterized in Arabidopsis (Huffaker *et al*., 2006; Krol *et al*., 2010), and their homologues described in other crop species such as the maize *ZmPep1* involved in regulating defense responses against fungal infection (Huffaker *et al*., 2011). However, the role of Pep-receptors during plant nematode interaction is not well understood. During plant invasion, nematode uses the stylet to puncture, rupture and make openings in the cell (Grundler *et al*., 1994). As a result, cellular components released into the extracellular space of the cell, which could then be recognized as DAMP (Haegeman *et al*., 2011; Mitchum *et al*., 2013). The recognition of DAMP leads to activation of downstream immune responses similar to PAMP recognition (Boller & Felix, 2009; Heil & Land, 2014). Previous studies showed the strong induction of wound-response pathways due to cell damage caused by migratory nematode in rice (Kyndt *et al* 2012a and 2012b). In our previous work, we reported the involvement of polygalacturonase-inhibiting proteins (PGIPs) in mitigating infection of host root responses to cyst nematode by activating plant phytohormones camalexin and indole-glucosinolate pathways (Shah *et al*., 2017), highlighting the important role of damage

responses and signaling during plant nematode interaction. In this study, we characterized the role of PEPR receptors in responses to cyst and root-knot nematodes. We found that PEPRs facilitate immune responses to *H. schachtii*. However we did not observed any significant deference in the infection of *pepr1/2* double mutant with the root-knot nematode *M. incognita*. These observations support previous report by Teixeira *et al*. 2016 that PEPR1/2 does not mediate defense responses to root-knot nematodes.

Results

Arabidopsis PEPR1/2 mediates defense responses to cyst nematode (*H. schachtii*) but not root-knot nematode (*M. incognita*).

Arabidopsis PEPR1 is a receptor kinase with extracellular leucine rich repeat domain and functions as a receptor for AtPeps. AtPep1, a 23-amino acids precursor peptide encoded by C-terminal of PROPEP1 gene is a DAMP which activates immune responses in *A. thaliana* (Huffaker *et al*., 2006; Boller & Felix, 2009). The genome of Arabidopsis encodes a close homologue of PEPR1receptor named PEPR 2 which has 76% amino acid similarity (Ryan *et al*., 2007; Yamaguchi *et al*., 2010; Krol *et al*., 2010; Yamaguchi & Huffaker, 2011). Nevertheless, PEPR1 and PEPR2 have different preferences for AtPeps (Yamaguchi *et al*., 2010). Both PEPR1 and PEPR2 were induce upon wounding as well as treatment with pathogen PAMPs and are reported to be essential in enhance resistance against bacteria pathogen *Pseudomonas syringae* pv tomato DC3000 (Yamaguchi *et al*., 2010). Nematode penetration causes damage to root tissues and depending on the species the damage varies significantly (Grundler *et al*., 1997; Wyss *et al*., 1992; Wyss & Grundler, 1992). To evaluate the important of damage responses during nematode infection, we used double mutant of the already well characterized DAMP receptors PEPR1 and PEPR2 (krol *et al*., 2010), to study the infection of cyst nematode *H. schachtii* and root-knot *M. incognita*. We observed a significant increased number of adult nematode upon infection with cyst nematode *H. schachtii* (**Fig.1a**). However, we did not observe any significant difference in average size of female nematodes and also in average size of nematode-induced syncytium (**Fig. 1b**). We also tested the already characterized ATP-receptor DORN1 (Choi *et al.,* 2014; Tanaka *et al*., 2014; Teixeira *et al*., 2016), and observed a significant difference in the number of adult female nematode (**Fig. 1a**).

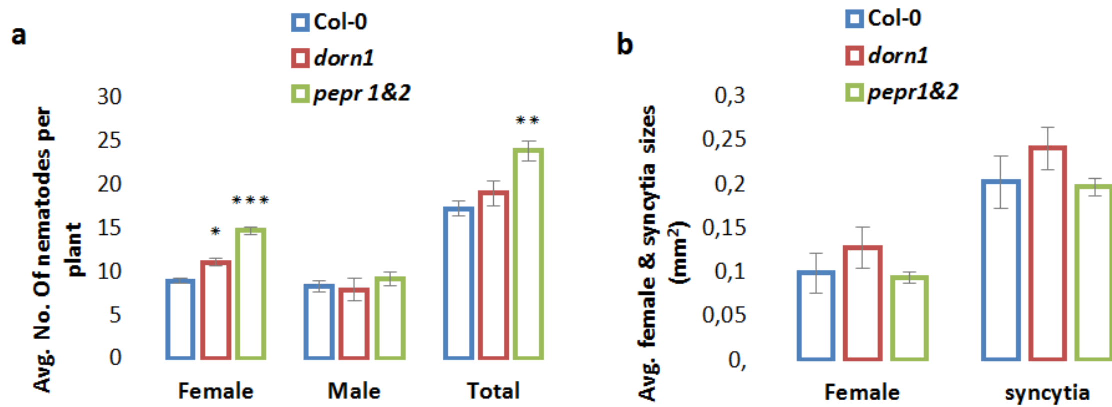

Figure 1: **Nematode (*H. schachtii*) infection assay with *pepr1/2* double mutant and *dorn1* single mutant compare to Col-0 wildtype. a)** Twelve day old Arabidopsis plants were infected with ~ 60-70 sterile second stage juvinile of *H. schachtii*. Adult female and males were counted at 13 day post infection (dpi). **b)** Female and feeding site were measure at 14 dpi. Bars represent mean ± SE for three biological replicates. Data analysis was performed using OneWay ANOVA and Dunnet test (P<0.05).

We did not see any significant difference in susceptibility with root-knot nematode *M. incognita* in *pepr1/2* double mutant and also in *DORN1* single mutant (**Fig.2a**). We measured the gall sizes and also found no differences between *pepr1/2*, and *dorn1* in comparison to Col-0 (**Fig.2b**).

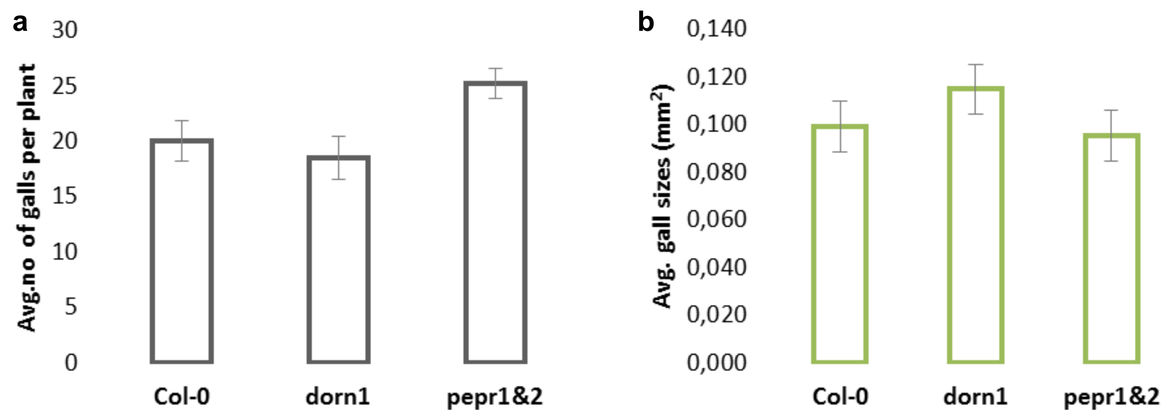

Figure 2: **Nematode (*M. incognita*) infection assay with *pepr1/2* double mutant and *dorn1* single mutant compare to Col-0 wildtype a)** Twelve day old Arabidopsis plants grown on MS-medium containing gelrite agar were infected with ~ 90-100 sterile second stage juvenile of *M. incognita*. Number of galls were count at 21 dpi.**b)** Feeding site measurement (galls) was done at 22 dpi. Bars represent mean ± SE for three biological replicates. Data analysis was performed using OneWay ANOVA and Dunnet test (P<0.05)

PEPRs gene are induced during nematode infection and upon treatment with *Hs*NemaWater

From our microarray data analysis, we observed an increased in transcription of both PEPR1 and PEPR2 gene during migratory stage of nematode infection (10 hours post infection) and also after treatment with *Hs*NemaWater **Fig. 3a** (Mendy *et al.,* 2017). The Precursor of peptide 1 in Arabidopsis (PROPEP1, PROPEP2, and PROPEP3), were also upregulated in both nematode migratory stage and upon treatment with *Hs*NemaWater **Fig. 3a**. **Table S1** (Mendy *et al*., 2017). However, DORN1 gene was significantly down regulated in both migratory and *Hs*NemaWater data respectively **Fig. 3a**. The low expression of DORN1 gene during infection could be associated to the fact that *H. schachtii* is a root pathogen and DORN1 is shown to recognize extracellular ATP (eATP) leading to enhance resistance to mostly above ground pathogen e.g. *Pseudomonas syringae* (Chen *et al*., 2017; Balagué *et al*., 2017) and *Phytophthora infestans* (Bouwmeester *et al*., 2011; Bouwmeester *et al*., 2014). Teixeira *et al*., 2016 found no differences in number of J2s inside the roots of *dorn1-3* and *oxDORN1* in comparison with the wild - type and therefore suggesting that DORN1 does not play significant role in RKN perception and immunity in Arabidopsis.

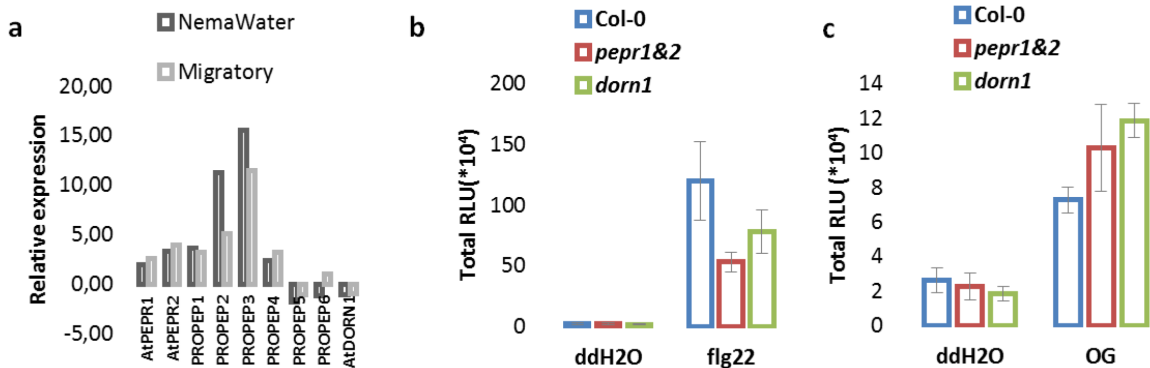

Figure 3: **PEPRs and PROPEPs gene expression analysis and ROS-burst measurement in Arabidopsis**. **a)** Microarray data analysis during nematode migratory stage and upon plant treatment with *Hs*NemaWater. Root segments from uninfected roots were used as control. Values indicate fold change compared with control. **b)** ROS-burst measurement with *pepr1/2*, *dorn1* and Col-0 treated with flg22, or water control. ROS-burst was measured using L-012 based assay from 0-120 min. **c)** ROS-burst measurement with *pepr1/2*, *dorn1* and Col-0 treated with, OG peptide or water control. ROS burst was measured using L-012 based assay from 0-120 min. Bars represent mean ± SE for four technical replicates. Experiment was repeated two times with same results. RLU, Relative light units.

PEPR1/2 and *DORN1* mutant are not impaired in ROS-burst responses triggered by flg22and OG

To check whether double mutant of *pepr1/2* and *dorn1* single mutant have compromised immune responses triggered by flg22 peptide and OG, we treated leaf disk of plants and did a ROS-burst measurement. We observed an increased ROS production in mutants treated with flg22 peptide **Fig.3b,** as well as with OG treated plants **Fig. 3c**. Oligogalacturonides (OGs) are oligomers of alpha-1,4-linked galacturonosyl released from plant cell walls upon partial degradation of homogalacturonan, or during cell wall damage (Ferrari *et al*., 2013). OGs are recognized by wall-associated kinases (WAKs) receptors and leads to accumulation of extracellular H_2O_2 (Galletti *et al*., 2008). *PEPR1/2* and *DORN1* mutant were not impaired in responses triggered by OGs meaning in the mutant background the WAK1 receptor maybe fully active and functional. It would be otherwise interesting to see how knockout of both WAK1 and PEPRs will have an effect on nematode development in plants.

Discussion

Plant cells recognize endogenous molecules leaked into the extracellular space of the cell as a result of damage. Whereas these molecules are not usually present in extracellular spaces in normal undamaged cells, exposure of DAMPs to the extracellular space during pathogen attack leads to recognition and activation of defense response similar to recognition of conserved (PAMP) molecules (Lotze *et al*., 2007). Several studies have reported the importance of DAMP responses during pathogen invasion with quiet few receptors identified to responses to specific kind of damage peptides. In Arabidopsis, LRR-RLKs *PEPR1* and *PEPR2* have been reported to respond to *Atpeps*, which is a 23-aa peptide AtPep1 derived from the C-terminus of a 92-aa precursor protein AtProPep1 are involved in defense responses against pathogen (Huffaker *et al*., 2006; Krol *et al*., 2010; Yamaguchi *et al*., 2010). Up to date, little is known about the role of DAMP responses during plant nematode interaction. Studies on *pepr1/2* mutant with root-knot nematode did not show any significant phenotypic differences in infection compared to control plants (Teixeira *et al*., 2016). However, Shah *et al*., 2017 reported that polygalacturonase-inhibiting proteins (PGIPs) gene family in Arabidopsis plays a significant role in defense responses against beet cyst nematode *H. schachtii*. Nevertheless, Polygalacturonase (PGs) from nematode that bind PGIP in Arabidopsis is not yet identified and it’s likely that plant responses to nematode infection could prompt the formation of active OGs, which then activates genes

involved in the biosynthesis of indole-glucosinolate and camalexin (Shah *et al.*, 2017). In this study, we tested the double mutant *pepr1/2* and the extracellular ATP receptor *dorn1* single mutant against cyst nematode *H. schachtii* infection. As previously reported by Teixeira *et al.*, 2016, we did not see any significant differences compared to wild type in both *pepr1/2* double mutant and *dorn1* with root-knot nematode**.** Root-knot nematodes penetrate plant cell close to the root tip and migrates intercellular between cells, thereby causing less damage (Holbein *et al.*, 2016). Lesser damage to cell could mean less cell wall fragments release. Genes encoding functional and endogenous cellulases and pectinases secreted from the subventral esophageal gland cells of plant parasitic nematodes, used to weaken and break down the plant cell wall have been reported (Smant *et al.*, 1998; Rosso *et al.*, 1999; Wang *et al.*, 1999; Gao *et al.*, 2004). Unfortunately, there are no reports on a plant receptor that recognized extracellular cell wall degradation products (e.g. glucose polymers cellodextrins and pectin fragments), derived from cell wall break down during nematode migration stage.

Contrary to root-knot nematode infection, we found a significant increase in average number of adult cyst nematode of *H. schachtii* in *pepr1/2* and in *dorn1* mutant in comparison to wildtype Col-0 **Fig. 1a**. Cyst nematodes unlike root-knot infective juveniles penetrate plant cells and migrate intracellularly into and through the root tissues (Wyss, 1992). During the course of migration, the nematode causes damage to cell wall, which may result in the possible release of small plant peptides as DAMPs. Nematode infection of plant is shown to triggered expression of defense genes including PEPR1 and PEPR2 but not DORN1 in Arabidopsis **Fig. 3a** (Mendy *et al.*, 2017). Consistently the precursors of peptide-1 in Arabidopsis (PROPEP1, PROPEP2, and PROPEP3) were upregulated in both nematode migratory stage and upon treatment with *Hs*NemaWater (Mendy *et al.*, 2017). This observation gave an impression that, either PEPR receptors recognizes peptides derived from cell damage or may rather play a role in amplifying the signal responses from recognition of nematode NAMPs by surface receptors and co-receptors (Yamaguchi *et al.*, 2010). The rapid production of reactive oxygen species (Mittler *et al.*, 2011; O´Brien *et al.*, 2012) and cytosolic Ca^{2+} burst (Monaghan *et al.*, 2015) are one of the early defense responses upon pathogen attack or elicitor treatment. Therefore, we investigate whether *PEPR1/2* and *DORN1* mutant have impaired ROS burst production observed in wildtype treated with flg22 peptide (Gómez-Gómez & Boller, 2000) and OGs damage peptide (Savatin *et al.*, 2014). We observed a strong ROS-burst in both *pepr1/2* and *dorn1* mutant similar to wildtype. These results indicate that the *pepr1/2* and *dorn1* mutant have active

receptors for flg22 and OGs. Moreover, knocking down these genes does not completely impair plant immunity. This study provides evidence that PEPR receptors play significant role in defense responses during cyst nematode infection. Increased susceptibility to cyst nematode and not root-knot nematode probably correlates with the fact that cyst nematode causes more cell damage compared to root-knot during early stages of infection. As cyst nematode continues to be a problem in crop production, understanding both exogenous and endogenous recognition pathway is crucial for engineering durable resistance cultivars.

Material and methods

Plant growth medium and nematode infection assay

Arabidopsis plants growth medium preparation and nematode infection assays were carried out as previously described (Siddique *et al*., 2015; Mendy *et al.,* 2017; Shah *et al.,* 2017). Homozygous double mutant of *PEPR1/2* were obtained from Belkhadir's lab. *DORN1* mutant were also obtain from Gary Stacey's lab. Plants were grown on modified knop medium for cyst nematode (*H. schachtii*) infection assay and Murashige & Skoog (MS) medium containing gelrite agar for root-knot (*M. incognita*) assay. Twelve day old seedlings were infected with ~60-70 live and sterile second stage juveniles (J2s) of *H. schachtii*. Adult female and male numbers were evaluated at 13 day-post infection (dpi) and the female and feeding site were measured at 14 dpi. For *M. incognita* infection assay, 12 day old plants grown on MS-medium were infected with ~90-100 live and sterile second stage J2s. The numbers of galls were evaluated at 21 dpi, and the gall sizes measured at 22 dpi. All experiments for both cyst and root-knot nematodes infections were repeated three times with ~25-30 individual plants. For size measurement, approximately 30 -35 syncytia and nematodes or galls were measured.

ROS-burst assay

Reactive Oxygen Species (ROS) burst measurement was done as previously described (Mendy *et al.,* 2017). Leaf discs were cut from 12 day old seedlings and incubated overnight in water. Afterwards, the water was replaced with 15µl of 20 µg/ml horseradish peroxidase and 35µl of 0.1M 8-Amino-5-chloro-2, 3-dihydro-7-phenyl-pyrido [3, 4-d] pyridazine sodium salt (L-012, Wako Chemicals) in a 96 well plate. Next, 50 µl of either 1 µM flg22 or, *Oligogalacturionides* (OGs) peptide was added to the individual wells. The experiments were performed in four technical replicates, and ddH$_2$O was used as a negative control. Light emission was measured as

relative light units over 120 minutes in a luminometer (TECAN Infinite® 200 PRO) and data were analyzed using instrument software and Microsoft Office Excel.

Statistical analysis

Data analysis were done using SigmaPlot 12 version, using OneWay ANNOVA and Fisher LSD ($P<0.05$) for pairwise comparisons

Supplementary material

Table S1: Microarray data analysis during nematode migratory stage and after plant treatment with Nemawater

Gene locus	Gene Name	Fold Change				Gene discription
		NemaWater	p-Value	Migratory	p-Value	
AT1G73080	AtPEPR1	1,91	0,0003	2,56	0,0003	PEP1 RECEPTOR 1
AT1G17750	AtPEPR2	3,34	0,0022	3,95	0,0014	PEP1 RECEPTOR 2
At5g64900	PROPEP1	3,63	0,0019	3,17	0,0004	ARABIDOPSIS THALIANA PEPTIDE 1, precursor of AtPep1
At5g64890	PROPEP2	11,27	0,0009	5,13	0,0002	PROPEP2 (Elicitorpeptide 2 precursor)
At5g64905	PROPEP3	15,53	0,0014	11,43	0,0047	PROPEP3 (Elicitorpeptide 3 precursor)
At5g09980	PROPEP4	2,40	0,0698	3,26	0,0307	PROPEP4 (Elicitorpeptide 4 precursor)
At5g09990	PROPEP5	-1,83	0,0897	-1,07	0,2853	PROPEP5 (Elicitorpeptide 5 precursor)
At2g22000	PROPEP6	-1,21	0,0371	1,04	0,3333	PROPEP6 (Elicitorpeptide 6 precursor)
AT5G60300	AtDORN1	-1,08	0,6119	-1,00	0,9105	DOES NOT RESPOND TO NUCLEOTIDES 1

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Chapter 6 General Discussion

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Plant-parasitic nematodes (PPN) are a major threat to world food security. Previous methods to control PPN have several setbacks due to environmental concerns about chemicals usage as control mechanisms (Tytgat *et al.*, 2000). Control methods involving host resistance have become the focus of many researches nowadays. Understanding the molecular basis by which plant recognize nematodes and activate immunity is the main core of this thesis.

Plants can defend themselves against invading pathogens through recognition of microbe/pathogen associated molecular pattern (MAMPs/PAMPs) by cell surface localized pattern recognition receptors (PRRs) leading to PAMP-Triggered immunity (PTI). Plant PRRs are either receptor-like kinase, which comprises a ligand-binding ectodomain, a transmembrane domain and an intracellular kinase domain, or a receptor-like protein, which do not have any known intracellular signaling domain (Macho & Zipfel, 2014). Plants PRRs are structurally similar to that of animals suggesting convergent evolution between plant and animal recognition and signaling system (Zipfel, 2008). Most receptors in defense signaling are specific to certain pathogens molecule but others may require a partner protein working as a co-receptor (Yadeta *et al.*, 2013). PAMPs are evolutionally conserved across a certain class of pathogens and are also evolutionarily distant from their host. Perception of these PAMPs triggers a set of responses, which can be used to monitor the recognition process. Examples for such responses include generation of reactive oxygen species (ROS), ion fluxes, accumulation of ethylene, as well as up-regulation of defence-related genes (Zipfel, 2009). Failure to detect the presence of a particular pathogen leads to establishment, development and diseases initiation in host plant. PAMPs and PRRs have been well characterized for several important pathogens including bacterial flagellin (Gómez-Gómez *et al.*, 1999; Felix *et al.*, 1999; Gómez-Gómez & Boller, 2002), elongation factor Tu (Kunze *et al.*, 2004; Zipfel *et al.*, 2006), and bacterial cell wall component peptidoglycan PGN (Girardin *et al.*, 2003; Gust *et al.*, 2007) are all been shown to induce plant immune responses. In fungal pathogens, chitin is a well studied PAMP and the receptors in both dicot and monocot have been described (Felix *et al.*, 1993; Boller, 1995; Nürnberger & Brunner, 2002). However, the role of PAMP-Triggered Immunity during plant nematode interaction is not well studied (Holbein *et al.*, 2016). Several studies have reported that plant respond to PPN infection by rapidly activating defense response pathways similar to those induced by other pathogens (Kyndt *et al.*, 2012; Vercauteren *et al.*, 2001). Nonetheless, the

molecular nature of the nematode derived peptides that elicit immune respond in plant remains unknown. Therefore, activation of PTI in plants upon nematode infection is the focus of this thesis.

Ascarosides pheromones are widely conserved among nematodes (Choe *et al*., 2012) and have been shown to activate PTI like responses in plant (Manosalva *et al*., 2015). Till date no PRR that recognize nematode associated molecular patterns (NAMPs) have been characterized although quiet a number of nematode resistance genes (R-genes) have been reported (Goverse & Smant, 2014; Lozano-Torres *et al.,* 2012). In the past few years, there are reports suggesting that nematode infection activate PTI responses in plants (Peng *et al*., 2014; Teixeira *et al*., 2016). In this study we found out that cyst nematode infection triggered the expression of early defense markers including JA/ET biosynthesis and signaling genes (Mendy *et al*., 2017). These data corroborate previous findings by Kammerhofer *et al.,* 2015, where they found an increase in level of JA in roots of Arabidopsis 24 hours post infection (hpi). To differentiate nematode-induced mechanical damage from PAMP recognition, we developed a robust PTI screening method based on aqueous *diffusate* of nematodes known as NemaWater. Treatment of plants with NemaWater produced from *H. schachtii* J2 activated early defense gene expression in a similar pattern as previously observed during the migratory stage of the nematode infection. This finding suggested that NemaWater contains elicitors that activate early defense responses against nematodes. These findings also contradict the general notion that JA/ET play role in response against necrotrophic and herbivore insects while SA is important against biotrophs (Howe & Jander, 2008; Glazebrook *et al*., 2003). Maybe that concept only works for leafy pathogens and differ in roots. SA biosynthesis genes were only slightly affected in our data in comparison to JA/ET even though Arabidopsis plants impaired in SA biosynthesis genes were previously showed to be more susceptible to cyst nematodes (Wubben *et al*., 2008).

Production of ROS (Mittler *et al*., 2011; O´Brien *et al*., 2012) and cytosolic Ca^{2+} burst (Monaghan *et al*., 2015) are one of the early defense responses upon pathogen attack or elicitor treatment. We showed that plant respond to NemaWater treatment by generating ROS. It was also interesting to see that NemaWater produced from both cyst and root-knot forming nematode cause ROS-burst in Arabidopsis (chapter 2). This further indicates that either the molecules involved in eliciting immunity are conserved in both species, or plant recognized both nematode species through a different pattern. It will be interesting to find whether plants can also detect conserved pattern in other nematodes apart from PPNs. Previous reports showed that

entomopathogenic nematodes (EPN) are also able to induce plant defense. This was proven by experiments, where it was observed that catalase activity in *A. thaliana* leaves was significantly higher in *Steinernema carpocapsae* infective juveniles (IJs) treated plants than in the control. Similarly, peroxidase activity was increased in host plants treated with *S. carpocapsae* IJs compared to control (Jagdale *et al*., 2009). PR1-gene was also induced upon infection with *S. carpocapsae* IJs in transgenic *A. thaliana* leaves through GUS (β-glucuronidase) activity assay (Jagdale *et al*., 2009). Looking at these findings together with our observation, it is possible that molecular elicitors contained in NemaWater may be conserved across many nematode genera. Although, the identity of the elicitor contained in NemaWater is still elusive. What we know is that the elicitor/s in question is proteinaceous in nature and probably heat sensitive. This was proven by treatment of NemaWater with Proteinase K enzymes and also heat treatment, in either case we observed a reduce PTI activity in plants different from control (non-proteinase k treated NemaWater) and ddH_2O treatment (Mendy *et al*., 2017). However, the possibility that plant recognizes other molecule types e.g. nematode cuticle associated surface carbohydrates, are not ruled out.

The plant receptor kinase BAK1/SERK3 has been reported to act as co-receptor for those PRRs of LRR-class that perceive proteinaceous ligands (Sun *et al*., 2013; Macho *et al*., 2014; Albert *et al*., 2015). We found that loss of function BAK1 was more susceptible to nematode infection and also impaired in immune responses triggered by NemaWater in wildtype Arabidopsis. This further indicates that NemaWater induce immune responses in plants is dependent on BAK1gene. BAK1 gene was also induced upon cyst nematode infection as well as NemaWater treatment (Mendy *et al*., 2017). Other works also reported the involvement of BAK1 in immune response against nematodes in crop plant as evidence by silencing of BAK1 orthologues *SISERK3A* and or *SISERK3B* in tomato (*Solanum lycopersicum*), resulted in enhanced susceptibility of plants to root-knot nematodes (Peng *et al*., 2014). Teixeira *et al*., 2016, also reported that nematode infection in Arabidopsis triggers PTI responses in a BAK-dependent and also independent manner as they observed *bak1-5* mutant were more susceptible to root-knot in comparison to control. These findings together indicate that plants are able to recognize nematodes through a receptor that interact with BAK1 as well as through other receptors that do not necessarily interact directly with BAK1. Bioassays to identify BAK1 interactors during PTI responses to nematodes are a very important step close to finding the receptor that binds ligands.

Considering that BAK1 acts as co-receptor for LRR class of PRRs, we found a LRR-RLK that was specifically induced upon nematode infection. The Arabidopsis NEMATODE-INDUCED LRR-RLK1 (NILR1) is closely related to LRR-RLK BRI1, belonging to the subfamily X of LRR-RLKs (Matsushima *et al.*, 2012), encodes a serine/threonine kinase and localized in the plasma membrane, therefore, manifesting typical characteristic of a PRR. Functional characterization of *nilr1* mutant showed a high susceptibility to cyst and root-knot nematodes and also impaired ROS-burst (Mendy *et al.*, 2017). NILR1 gene is well conserved in land plants and orthologues are found in both dicotyledonous as well as in monocotyledonous plant species.

To further identify and characterize the elicitor/s present in NemaWater that are recognized in a NILR1-dependent manner, we used column chromatography fractionation methods. A number of nematode proteins were found in our NemaWater proteomic data. How all the proteins found in NemaWater are released after nematode incubation in water is not known. The epicuticle of nematodes is covered by a thin layer recognized by electron microscope as a fuzzy coating termed 'surface coat' (´Wright, 1987; Bird & Bird, 1991). The surface coat of nematodes contains various proteins, lipids and carbohydrates either as individual component or as glycoproteins, glycolipids or lipoproteins (Spiegel & McClure, 1995). The parasitic nematode cuticle and surface coat proteins represents the first site of contact with the host's immune responses (Kennedy & Harnett, (Eds.), 2013). Several research have demonstrated that protein molecule on the surface coat are secreted or released into the environment by *Meloidogyne spp* (Lin & McClure, 1996). In pre-parasitic juveniles of *M. incognita*, surface coat proteins are found to be released when J2s were incubated in water for 20 hours (Lin & McClure, 1996) indicating that surface coat proteins may be loosely attached on the nematode and therefore incubation can cause their release into the water. At this stage it is very difficult to predict the actual protein from NemaWater involved in elicitation of immune response in plant.

Interestingly, some nematodes surface coat associated proteins identified in NemaWater are also found to be conserved in animal parasitic nematodes (APN) and serve crucial role in immune modulation. For example, enolase a metalloenzyme that catalyzes the conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid in the glycolytic pathway has been shown to act as surface antigen. Experiment with recombinant enolase protein from *Clonorchis sinensis* is showed to induce a mix of Immunoglobulin1/2a (IgG1/IgG2a) immune responses in Sprague-Dawley rats when administrated subcutaneously (Wang *et al.*, 2014). This suggest that *C. sinensis* enolase (*Cs*enolase) can be used as a potential vaccine candidate to control the worm

parasite. Multifunctional enzyme enolase has also been reported to act as a cell surface receptor in host-pathogen interactions as well as binding to plasminogen (Wang *et al*., 2011). Studies have shown that enolase from *Streptococcus sobrinus* have the potential of acting as an immunomodulatory protein against dental caries the chronic human infection (Dinis *et al*., 2009). They found that rats immunized with recombinant enolase (rEnolase) developed an increased level of salivary Immunoglobulin A/G (IgA and IgG) antibodies specific to the protein and the scores of dentin caries decrease (Dinis *et al*., 2009). These findings indicate that enolase has immunogenic properties and the ability to modulate immune responses in animals. Enolase was highly abundant in our NemaWater protein analysis data.

Consistent with the role of enolase in immune modulation in animals we hypothesize that it may play a role in plant basal immune activation. Further studies are necessary to examine the immunogenic nature of plant parasitic nematodes enolase and determine its potential in eliciting plant immune responses. Other important immunogenic protein identify in NemaWater protein analysis includes heat shock proteins (HSPs), also reported to act as immune modulators in cancer cells by forming a complex with peptides release as a result of protein degradation in associated cancer cells (Udono & Srivastava, 1993). Heat shock proteins (e.g. Hsp70), were reported in animal parasitic nematodes to be secreted in the host cell by means of extracellular vesicle trafficking (Eichenberger *et al*., 2018). Extracellular vesicles transport secretory proteins to the host which may play important role in host immune signaling (Coakley *et al.,* 2015; Eichenberger *et al*., 2018). The role of extracellular vesicle trafficking in animal parasitic worm and animal-host-interactions has been well studied. Free living nematode *C. elegans* EV secretions may play important function in communication and mating behavior (Liégeois *et al*., 2006). The role of EV trafficking have not been described in host plant-nematode-interactions. It could well be that some of the nematode proteins identified in NemaWater protein analysis are secreted through extracellular vesicle trafficking.

The protein analysis and identification of nematode associated surface proteins is one step to identify potential NAMPs. We assumed that at least one or more of the immunogenic surface proteins identified in NemaWater is recognized by receptor(s) in plants leading to PTI. We also hypothesized that at least one of the plant receptor that recognizes nematode NAMPs require BAK1/SECRK3 as a co-receptor with others independence of BAK1.

We also studied the mechanism of damage responses in plant during nematode infection. Aside from the recognition of pathogen associated molecular pattern by surface receptors, plants also recognize endogenous molecules exposed to the extracellular space during cell damage (Lotze *et al*., 2007; Bianchi *et al*., 2007; Tang *et al*., 2012). These endogenous molecules/peptides activate plant immune responses similar to recognition of PAMPs (Boller & Felix, 2009; Heil & Land, 2014). Nematode invasion and intracellular migration causes massive cell damage (Grundler *et al*., 1994), as a result cellular component are released in extracellular spaces where they are recognized as danger signals (Haegeman *et al*., 2011; Mitchum *et al*., 2013). The role of DAMP responses during plant-nematode interaction is not well studied. We investigate how DAMP receptors are involved in activating immune responses against cyst and root knot nematodes. The plant cell wall serves as the first barrier encountered by nematodes during root invasion (Holbein *et al*., 2016). There are two main strategies the nematode applied to penetrate the plant cell wall. These strategies include the use of stylet to pierce through the wall, and also the secretion of cell wall-degrading enzymes which helps to disrupt wall rigidity e.g. pectate lyase (de Boer *et al.*, 2002; Vanholme *et al.*, 2007), endo-β-1, 4-glucanase (Smant *et al.*, 1998; de Boer *et al.*, 1999), and polygalacturonase (PG) (Jaubert *et al.*, 2002). PGs are key enzymes that cleave the α1-4 linkage between the D-galacturonic acid residues of homogalacturonan (Kalunke *et al.*, 2015; Rahman & Joslyn, 1953; Themmen *et al.*, 1982). PG was isolated in plant parasitic nematode *M. incognita* with a suggested role in facilitating parasitism (Jaubert *et al*., 2002). PG-inhibiting protein (PGIP), a leucine-rich repeat defence protein bind PGs resulting to the release of oligogalacturionides (OGs) capable of activating immune responses in plant (Benedetti *et al*., 2015). In pea plant (*Pisum sativum* L.), *PsPGIP1* has been shown to be differentially expressed in susceptible and resistant genotypes in response to *Heterodera goettingiana* infection and function in disrupting syncytium development inside the host root (Veronico *et al.*, 2011). In this work we found that *PGIP1* mediate defense responses against cyst nematode *H. schachtii* in Arabidopsis (Shah *et al.*, 2017). *PGIP1* mutant were shown to be susceptible to cyst nematode. The up-regulation of genes involved in indole-3-glucosinolate and camalexin biosynthesis (*CYP81F2*, *CYP71A12*, and *PAD3*) were significantly impaired in *pgip1-1* mutant plants which indicate that susceptibility of *pgip1-1* to nematode infection is as a result of impaired induction of camalexin and indole-3-glucosinolate biosynthesis pathways (Shah *et al*., 2017). To confirm these results, we used a double mutant *cyp79b2/b3*, which is strongly impaired in indole-glucosinolate and camalexin biosynthesis and accumulation and observed an increased number of nematodes infection. These suggest that cyst nematode during migration within the root

induced camalexin and indole-3-glucosinolate biosynthesis pathways in a PGIP- dependent manner thereby restricting nematode establishment and development. However, the *H. schachtii* PG that causes the expression of PGIP in Arabidopsis is yet to be identified. Future research in this area will focus on identifying molecular players involved in activating PGIP gene expression during plant cyst nematode interaction.

The role of peptide receptor *(PEPR1/2)*, and their involvement in defense responses against cyst and root-knot nematodes was also studied during the course of this thesis. PEPRs are LRR-RLKs reported to respond to a 23-aa peptide AtPep1 derived from the C-terminus of a 92-aa precursor protein AtProPep1 and together with BAK1 as co-receptor confers resistance to pathogens (Huffaker *et al*., 2006; Krol *et al*., 2010; Yamaguchi *et al*., 2010). Studies reported that knock-out mutant of *pepr1/2* did not show any significant differences in infection with root-knot nematode compared to control (Teixeira *et al*., 2016). We found that *pepr1/2* double mutant were susceptible to cyst nematode infection in Arabidopsis. Root-knot nematode, however, did not show significant changes as compared to control in our infection assay which correlates with previous finding by Teixeira *et al*., 2016. The reason for increase susceptibility of cyst nematode to *pepr1/2* mutant and not root-knot nematode could be associated to the fact that these two species of nematode have different penetration and migration pattern during initial infection. Cyst nematode infective juveniles penetrates and migrates through the plant root cells intracellularly causing severe damage (Wyss, 1992), whereas root-knot nematode migrate intercellularly between cells thereby reducing damage. Nevertheless spatio-temporal expression pattern for PEPR genes during cyst nematode and not root-knot nematode infection is not known. One possible explanation is that damage cause by cyst nematode could result in the release of small plant peptides which are then recognizes as DAMPs to activate defense responses. Previous studies showed that DAMPs signaling pathways can also be activated upon recognition of MAMPs/PAMPs by PRRs acting as immune amplifier (Yamaguchi *et al*., 2010). This could be one possible reason why DAMP related receptors were observed to be expressed upon plant treatment with *Hs*NemaWater as well as during the migratory stage of nematode infection (Mendy *et al*., 2017).

In conclusion, the identification of nematode surface proteins in NemaWater is a step towards finding NAMPs involved in immune elicitation in plants. Further work will focus on identification of the appropriate ligand in NemaWater that induced NILR1 immune responses and basal resistance to nematodes. We assumed there are more plant receptors apart from NILR1

involved in nematode NAMPs recognition. Receptors for ascarosides for example are still elusive. We also assumed plants may have evolved recognition pattern for nematode surface carbohydrate molecules. Lectin binding gene were significantly expressed in our microarray data which indicates that not only plants can recognize some unknown nematode surface proteins but also some unknown surface carbohydrates. The expression of both PAMP and DAMP receptor during cyst nematode infection indicate that plant deploys mechanism to recognize pathogen derived elicitors as well as host derived peptide. Understanding of both exogenous and endogenous recognition pathway is crucial for engineering durable resistance cultivars.

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