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Identification of plant recognition receptors for nematode derived molecular patterns

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Dedications

I would like to dedicate this work to my parents, Prof. Catherine W. Muthuri and Dr. Rebecca Ngumburu Karanja.

“Gathutha konagia mundu njira”

Na

“Cia uthoni ciambaga nguhi”

(Kikuyu proverbs)

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Abstract

Plant parasitic nematodes are pathogens of great economic importance causing major losses in various food crops world-wide. A reliable, effective and efficient control method is establishing resistant cultivars of which understanding plant defense against nematodes is the first step towards this solution. Plant defence relies on recognition of Pathogen-Associated Molecular Patterns (PAMPs) by surface-localised Pattern-Recognition Receptors (PRRs) prior to pathogen penetration. Upon PAMP perception, PRRs trigger intracellular signalling cascades leading to activation of PAMP-Triggered Immunity (PTI). PRRs perceiving a wide-range of PAMPs have now been characterized for various models of plant-pathogen interactions; however, even though Nematode derived PAMPs (NAMPS) such as ascarosides have been identified, none of their perceiving receptors have been characterized. Here we show that invasion of Arabidopsis roots by parasitic nematodes triggers PTI-like responses including an upregulation of defense related genes. Treating Arabidopsis roots with a nematode aqueous solution (NemaWater) similarly induced expression of defense genes. Among the upregulated genes were a number of plasma-membrane – localized Receptor-Like Kinases (RLKs) belonging to Leucine Rich Repeat (LRRs), Never In Mitosis A (NIMA) rElated Kinases (NEKs), Cysteine-Rich RLKs (CRKs) and Phytosulfokine Kinase (PSK) families. Nematode infection assays with candidate genes demonstrated that loss of NILR1 (for NEMATODE-INDUCED LRR-RLK 1) expression enhances the susceptibility of plants to a broad range of nematodes suggesting that NILR1 is a PRR that perceives a conserved nematode-derived NAMP. This finding is equally supported by experiments showing that *nilr1* is defective in ROS burst as well as in seedling growth inhibition upon NemaWater treatment compared with wild-type control. In addition, presence ROS burst by NemaWater on rice plants suggested triggering of PTI by a NILR1 homologue in rice.

We further showed AtNEK5 and NILR3 as potential NAMP receptors due to susceptibility of their knock out mutants to sedentary nematodes while two CRKs; CRK 19 and CRK10

portrayed roles in defense against nematodes in a species dependent manner. In addition, we demonstrated that the co-receptor BAK1 can be utilized to mine for potential receptors and signalling components involved in immunity against nematodes through successful BAK1-GFP pull down assay. The identification of NILR1 among others PRR perceiving NAMPs and successful baiting of BAK1 to pulldown nematode derived immunity components are major steps forward in understanding plant basal defense against nematodes. Consequently, these findings will not only increase knowledge into plant-nematode interaction but also pave way for further exploration of plant immunity studies. As a direct effect, the vital information from this study remains as a resource for molecular breeding of nematode resistant plants and a solution to yield loss due to nematode.

Zusammenfassung

Pflanzenparasitäre Nematoden sind Pathogene von großer ökonomischer Relevanz, da sie weltweit enorme Verluste in diversen Nutzpflanzensorten verursachen. Eine verlässliche, effektive und effiziente Regulierung ist die Verwendung resistenter Kultivare, wobei das Verständnis der Verteidigungsstrategien von Pflanzen gegen Nematoden ein erster Schritt zu dieser Lösung ist. Pflanzliche Verteidigungsstrategien beruhen auf der Erkennung sogenannter „Pathogen-Associated Molecular Patterns“ (PAMPs) durch „Pattern-Recognition Receptors“ (PRRs) bevor der Pathogen eindringt. Durch die Perzeption von PAMPs lösen PRRs intrazelluläre Signalkaskaden aus, die zur Aktivierung der PAMP-Triggered Immunity (PTI) führen. PRRs, die eine Viezahl von PAMPs erkennen, werden inzwischen in unterschiedlichen Modellen über die Planze-Pathogen Interaktionen beschrieben. Jedoch, obwohl nematodenbezogene PAMPs (NAMPs), wie zum Beispiel Ascaroside, identifiziert wurden, wurde bislang kein entsprechender Rezeptor charakterisiert. Hier zeigen wir, dass die Invasion von Arabidopsiswurzeln durch pflanzenparasitäre Nematoden PTI ähnliche Signale auslöst, einschließlich einer Hochregulation von Genen der Pflanzenabwehr. Die Behandlung von Arabidopsiswurzeln mit einer wässrigen Nematodenlösung (NemaWater) induziert auf eine ähnliche Weise die Expression von Abwehrgenen. Unter den hochregulierten Genen befinden sich eine Reihe „Receptor-Like Kinases“ (RLKs) der Plasmamembran, die zu den Familien der „Leucine Rich Repeat (LRRs), Never In Mitosis A (NIMA) rElated Kinases (NEKs), Cysteine-Rich RLKs (CRKs)“ und „Phytosulfokine Kinase“ (PSK) gehören. Nematoden-Infektionsstudien demonstrierten, dass der Verlust des Kandidatengens NILR1 (for NEMATODE-INDUCED LRR-RLK 1), die Anfälligkeit der Pflanzen gegenüber einer Reihe von Nematodenarten erhöht. Dieses Ergebnis legt nahe, dass es sich bei NILR1 um ein NAMP erkennendes PRR handelt. Gleichzeitig wird diese Annahme durch Experimente unterstützt, die zeigen, dass die transgene Pflanze *nilr1* eine beeinträchtigte ROS Ausschüttung sowie eine Hemmung

des Keimlingswachstums nach Behandlng mit NemaWater aufweist. Zusätzlich suggerierte die ROS Ausschüttung in Reispflanzen durch NemaWater Behandlung, dass in diesem Fall ein NILR1 Homolog PTI auslöst. Außerdem zeigten wir, dass es sich bei AtNEK5 und NILR3 um potentielle NAMP Rezeptoren handelt, da die entsprechenden Knockout-Mutanten anfälliger gegenüber sedentären Nematoden waren, während die Rollen von CRKs; CRK 19 und CRK10, in speziesabhängigen Abwehrmechanismen vermutet werden. Zusätzlich konnten wir demonstrieren, dass der Korezeptor BAK1 in einer „GFP pull down“ Analyse zur Suche nach potentiellen Rezeptoren und Signalkomponenten, involviert in Immunitätsmechanismen gegen Nematoden, verwendet werden kann. Die Identifizierung von NILR1 neben anderen PRR erkennenden NAMPs und das erfolgreiche Ködern von BAK1 zum Detektieren von nematodenbezogenen Immunitätskomponenten sind wichtige Schritte zu einem Verständnis der basalen Pflanzenabwehr gegen Nematoden. Folglich werden diese Erkenntnisse nicht nur das Wissen über die Pflanzen-Nematoden Interaktion bereichern, sondern auch den Weg ebnen für zukünftige Untersuchungen des Pflanzenimmunsystems. Als direkter Effekt stellt diese Studie eine Resource für molekulare Züchtung nematodenresistenter Pflanzen sowie eine Strategie zur Reduktion von Ernteausfällenn dar.

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Acronyms and abbreviations

DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
RNA	Ribonucleic acid
dsRNA	Double stranded ribonucleic acid
No.	Number
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
TAIR	Arabidopsis Information Resource
INRES	Institute of Crop Science and Resource Conservation

Chapter 1

INTRODUCTION

1.1 Nematodes

Nematodes are worm-like moulting animals believed to have been observed as early as during the ancient civilizations dating back to 450 B.C, by great minds like Hippocrates. They are described as long, thin and hair-like animals and the word nematode is derived from the greek word Nematoidea which is a combination of νῆμα (nêma, nêmatos) meaning thread and -eiδής (-eidēs) meaning species. During the 18th and 19th century, different types of nematode were continuously discovered and identified mainly based on observations and morphology. It is also during this time period that free-living and parasitic nature of nematodes was first described in both animals and plants. This paved way and greatly contributed to understanding basic nematode biology and hence taxonomic classification of nematodes. However, it is in the last century that nematodes have been well studied mainly due to realization of their economic importance, and as such Nematology; the study of nematode, has become a well advanced and integrated study in mainstream biology to date.

Nematodes inhabit all kinds of habitats except dry soils and are numerous in number with a prediction of one hundred million species in marine ecosystems alone. However, only about 26,646 nematode species cutting across different habitats have been described so far (Lambshead 1993; Hugot *et a*l., 2001). Regardless of their habitats, nematodes are structurally transparent consisting of three layers including ectoderm, mesoderm and endoderm (Kennedy and Harnett, 2001). They have muscles on their outer wall which is essential for their movement as well as moulting from the inner hypodermis. Their wavy-like movement is aided by the elasticity of their body since the spaces between the body wall and the body-long alimentary canal is filled with fluids (Lee *et al*., 2002).

As living organisms, nematodes have been classified as animals on the phylogenetic tree. The discovery of molecular-biology techniques in the past decades have made empirical analysis of the evolutionary history of the phylum nematoda possible, through a network stemming from kingdom animalia. The first molecular phylogenetic notation of Nematode was framed through ribosomal small units mapping and as a result, Nematoda is now claimed to be a sister phylum to Nematomorpha, both of which belong to taxa Ecdysozoa (Fig 1) (Blaxter *et al*., 1998; Schmidt-Rhaesa, 1997; Dunn *et al*., 2008). Even though these two phyla are quite similar, Nematomorpha species are described as horse hair worms due to hair-like structure on their body and have a parasitoid life cycle different from Nematoda species. Nematoda is comprised of three subclasses; Chromadoria, Enoplia and Dorylaimia (De Ley and Blaxter, 2004). Among these, chromadoria and Enoplia are mainly marine nematodes, and Dorylaimia are mainly plant parasites. Nematodes have been further classified based on their feeding behaviour, structural morphology and parasitism as Parasitic or free-living (Blaxter *et al*., 1998; Dorris *et al*., 1999). Majority of nematodes are free-living and feed on fungi, algae and bacteria.

A well-known free-living nematode is *Caenorhabditis elegans*. In 1963, Sydney Brenner discovered the potential role that coulc be played by *Caenorhabditis briggsae* in the expansion of his developmental and neurological studies to other field that were quite left out in mainstream science at the time (Brenner 1988, 2002). Later, it was discovered that *Caenorhabditis elegans* was the easy to grow, genetically modify and had the advantage of a short life cycle of the two genii. *C elegans* thus became a model organism who`s similarity in cellular and molecular processes to the rest of the metazoans, has become a point of reference for the rest of the Animalia kingdom (Felix, 2008). It was particularly a major breakthrough in animal and human science when *C elegans* became the first multi cellular organism whose complete cell lineage and entire connectome was described (The *C. elegans* Sequencing Consortium, 1998; Jarell, 2012). *C elegans* is currently utilized by more than 1000 laboratories across the globe (Bolker, 2012).

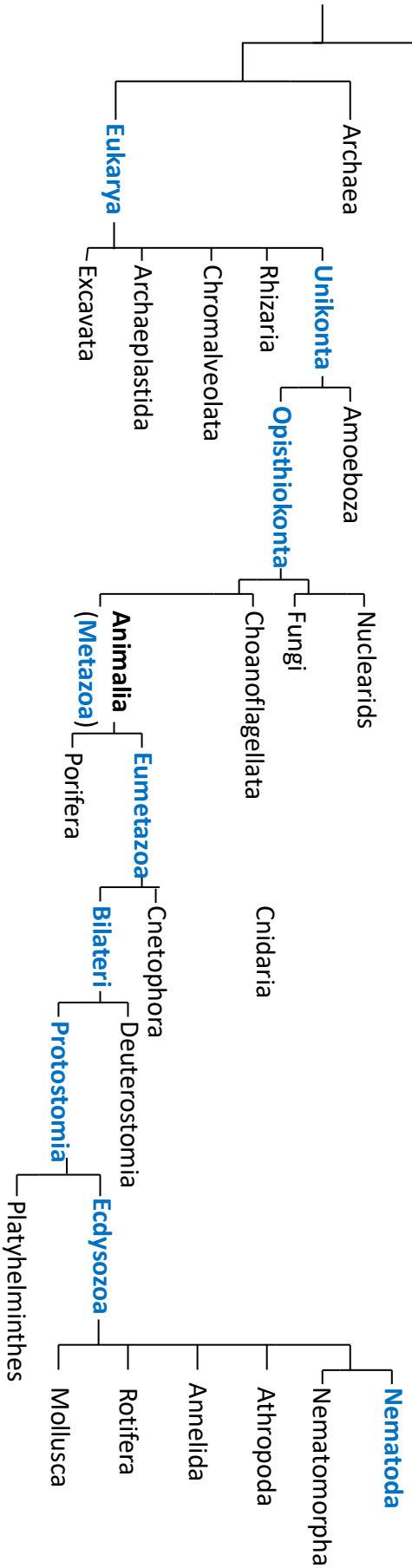
Bacteria
Archaea
Eukarya
Excavata
Archaeplastida
Chromalveolata
Rhizaria
Unikonta
Amoeboza
Opisthiokonta
Nuclearids
Fungi
Choanoflagellata
Animalia
(Metazoa)
Porifera
Eumetazoa
Cnetophora
Cnidaria
Bilateri
Deuterostomia
Protostomia
Ecdysozoa
Platyhelminthes
Mollusca
Rotifera
Annelida
Athropoda
Nematomorpha
Nematoda

Figure 1. **Nematode molecular phylogenetic framework.**

Nematodes, whether parasitic or free-living, have much in common in almost every stage of their biology. For example they switch to parasitic and free-living status within their life cycle mainly at the third juvenile or larval stage. However, parasitic nematodes major difference from free-living nematodes is their propensity for host interaction, an adaptation that has been evolutionary acquired due to need to counter host defence and competition for resources with the hosts (Maule, 2011). *Brugia malayi* (Chromadoria; Spiruromorpha) was the first parasitic nematode to have its whole genome sequenced. There are currently 19 publicly accessible nematode genomes of which the majority is parasitic (Ghedin *et al*., 2007). Nematode parasitism is mainly directed to animals and plants. Animal parasitic nematodes invade and parasitize animals; vertebrates and invertebrates including human beings, causing damage and in some cases death, for example the guinea worm (*Dracunculus medinensis*) and intestinal worm (*Ascaris lumbricoides*), which is believed live inside more than 1 billion human beings (Muller, 2011; Dold and Holland, 2011). They also include entomopathogenic nematodes which usually are utilized as biological pest controls (Dillman and Sternberg, 2012). Plant parasitic nematodes on the other hand account for 7% of the nematode phylum. They parasitize wide range of plant species for example the root-knot nematode (*Meloidogyne incognita*) (Decraemer and Hunt, 2006). However, regardless of their host choices, both animal and plant parasitic nematodes share some characteristics; the ability to locate and infect their host, to manipulate host for survival and nutrition and to suppress development at a critical stage in their life cycle.

1.1.1 Plant parasitic nematodes

These nematodes are also referred to as Phyto-parasitic nematodes. Phyto-parasitic nematodes are microscopic in size ranging from 0.25 mm to 3.0 mm. They possess a hollow needle-like mouth spear called a stylet which is the signature morphological characteristic traversing the whole plant parasitic nematode class. Phyto-parasitic nematodes occur in

distinctive stages; egg, juvenile and cyst. The juvenile stage is the main invasive one of all the stages.

In view of their life cycle, plant parasitic nematodes have a wide variety of interactions with their host. Depending on their life style, they are classified as endoparasitic or ectoparasitic and migratory or sedentary. Migratory ectoparasitic nematodes like the *Trichodorus spp*, the vector of Tobacco rattle virus, move within the soil and use roots tips and root hairs as a source of nutrients ephemerally (Decraemer and Geraert, 2006; Jones, 2013). Migratory endoparasitic nematodes on the other hand enter and move within the host while drawing nutrients from it; for example *Radopholus spp* (Fallas, 1996). The movement inside the plant not only cause extensive tissue damage but also increases chance of infection from other pathogens. Semi-endoparasitic nematodes have an initial migratory stage but enter the plant at one stage later in their parasitic cycle forming a feeding structure within the host; for example *Heterodera glycine*.

Decraemer and Hunt in 2006 reported the number of Phyto-parasitic nematodes species to be as high as 4100. In addition, a survey conducted in 2012 highlighted top 10 plant-parasitic nematodes in molecular plant pathology; Root-knot (Meloidoyne spp.), cyst (Heterodera and Globodera spp.), Root lesion (Pratylenchus spp.), Burrowing (Radopholus similis), Ditylenchus dipsaci, Pine wilt (Bursaphelenchus xylophilus), Xiphinema index, Nacobbus aberrans, Aphelenchoides besseyi, and Reniform (Rotylenchus reniformis) nematodes. Losses due to nematodes in agriculture globally are estimated at about 80 billion US dollars annually, and the figure is proposed to be higher considering the unreported cases from farmers in developing countries, who are unaware of symptoms of nematode attack (Nicol *et al.,* 2011). Management of these nematodes has for the past decades included cultural practices such as crop rotation, planting timing, flooding, and biological controls with antagonist and physical methods like solarisation and farrowing that have so far been effective. However most of these practices are only practical in small scale farming which is not sustainable if food production is to be increased (McDonald and Nicol,

2005). On the other hand, use of inorganic chemical pesticides such as nematicides and fumigants, has been on the lead as an effective method that actually eliminates the nematode. Unfortunately, they are expensive, harmful to the environment and the speed into which nematodes gain resistance renders them inefficient and ineffective in the long term. These negative impacts have prompted gradual withdrawal of pesticides such as methyl bromide which was banned by the European Union in 2010, hence further making the nematode problem more severe in absence of an immediate alternative (UNEP 2000; Kearns *et al*., 2014). In rue of this problem, an effective, stable, eco-friendly and long lasting solution to parasitic nematodes menace is required.

Scientists believe that a reliable and effective way of tackling the nematode problem is through introduction of nematode resistant and free transgenic plants. Recent successes in application of biotechnology tools and genetic advances are promising. Even though nematodes are obligate biotrophs and are difficult to culture *in virto* as research organism, and nematode at parasitic stages could live inside plant roots making the biochemical analysis of their secretions cumbersome, recent advances in molecular biology tools like RNA interference have made functional analysis of nematode genes possible. In addition, whole genome sequencing and ability of cyst and root-knot nematodes; most economically important nematodes, to infect *Arabidopsis thaliana*; a common model plant in plant pathology, have had great impact on nematode research (Sijmons *et al*., 1991; Opperman *et al*., 2008; Abad *et al.*, 2008; Kikuchi *et al*., 2011). As a direct result, plant nematode interactions studies have greatly advanced to cellular level with genetics to support it (Curtis *et al*., 2007; Jones, 2012). These studies form the basic understanding to which creation of nematode free transgenic plants can be achieved.

1.1.1.1 Cyst nematode *Heterodera schachtii*

Cyst nematodes are sedentary biotrophs which forms a “cyst”; a pear shaped reddish-brown dead body. Nematode cysts can survive under all kinds of environmental conditions for as

long as 20 years in the absence of a host (Wharton and Ramlov, 1995; Jones *et al*., 1998). Since the life of cyst nematode takes place in the soil, they are parasitized at egg, juvenile and or cyst stage by various microorganism, preys and insects (Kerry, 1988; Nordbring-Hertz *et al*., 2006; Khan and Kim, 2007). For example, nematophagus fungi have been reported to have nematicidal potential against nematodes. Mites on the other hand feed on them (Yang et al., 2011). Cyst nematodes belong to family Hoplolaimide in Order *Tylenchida*. They are also placed in subfamily *Heterodeninae* with 8 genera (*Heterodera*, *Globodera*, *Cactodera*, *Dolichodera*, *Punctodera*, *Paradolichodera*, *Vittatidera* and *Betulodera*) *and* 115 species (Turner and Evans, 1998; Turner and Subbotin, 2013). Even though the most economically damaging genera are *Heterodera* and *Globodera*, the former remains as the largest genus within this subfamily encompassing 82 species. The most damaging of the species include soybean (*Heterodera glycines*), potato (*Globodera pallida* and *G. rostochiensis*) and cereal (*H. avenae*, *H. filipjevi and H. latipons*) cyst nematode. However, a lot of information about cyst nematodes is drawn from research on the sugar beet (*Heterodera schachtii*), soybean and potato “golden” cyst nematodes (Bohlman, 2015). Cyst nematodes are the most invasive and specialized plant parasitic nematodes with a limited number of hosts. In addition, due to their specialized life style, their host range differs a lot between species within their class. In 1965, Steele reported *H. schachtii* to have the largest number of host plants; 218 species in 23 plant families with about 80% of them belonging to *Brassicaceae* and *Chenopodiaceae* families. Among these species, Sugar beet (*Beta vulgaris*) is the main host and most affected plant of economic importance. For example, it’s used to make the sugar hence its common name sugar beet cyst nematode. In addition, *Arabidopsis thaliana* was identify to be a host of *H. schachtii* and as a result, both the nematode and the plant have since been utilized as model system for plant-nematode interaction studies (Sijmons *et al*., 1991).

Morphologically, nematodes cysts are about 300-1700 µm long and 200-800 µm wide. Using the stylet, nematodes penetrate plant cells and allow entry or movement into or inside the

host. They have dorsal muscles and protractor muscles which moves the stylet forward while backward movement occurs by shear elasticity of the oesophagus. The head region also has the circumpharyngeal nerve ring from which, the dorsal and ventral nerves control sensory functions and movement especially when nematode is locating a host or a female to mate with. The stylet draws secretions from the dorsal gland and amphids which contain effectors that suppresses host defence. Amphids, the main chemo-sensors in *C. elegans* have neurons specialized in detection of a variety of stimuli. However, in plant parasitic nematodes, it remains just as a speculation that they play a role in sensing root leachates or diffusates during invasion (Bergmann, 2006; Sobczak, 1999).

The stylet, oesophagus, intestines, rectum and anus forms the digestive system. The oesophagus contains the metacorpus, procorpus and basal bulb. The key function of the metacorpus is to draw nutrients from the plant into the intestine and secretions from glands to the plant (Hewezi and Baum, 2013). Glands have glands cells, which contain secretory granules responsible for production of the effectors. Cyst nematodes have three main glands; one dorsal gland and two subventral glands. Subventral glands produce the cell-wall degrading enzymes for example cellulases, pectate lyases and expansins meaning they are highly active at the second juvenile stage which is the main infective stage (Davis *et al*., 2011; Chen *et al*., 2005; Vanholme *et al*., 2007). The dorsal oesophageal glands on the other hand are responsible for the production of effectors when juveniles are migrating inside the root and inducing the feeding site (Tytgat *et al*., 2002; Wyss, 1992).

The nematode`s body is covered with a cuticle which is moulted off by the hypodermis at every Juvenile stage. The cuticle is believed to contain proteins, lipids and carbohydrates which could play a role in host immunity through preventing recognition by the plants during nematode attack. For example, peroxidase has been reported on the surface of juveniles and could be a protectant from Reactive Oxygen Species (ROS) produced at nematode migratory stage during the nematode invasion (Eisenback, 1985; Waetzig, 1999; Robertson *et al*., 2000; Curtis, 2007). The cuticle allows exchange of gas and solutes and water

diffusion. Nematodes also have an inner pseudocoelom which is built along the longitudinal muscles and forms the hydroskeleton with which, together with the cuticle, maintain body shape and aid in locomotion by acting against internal turgor pressure.

Sexually, cyst nematodes are dimorphic. The males are slightly bigger than females and have spicules for mating. During mating, the protractor muscles moves the spicules forward from the cloaca into the female vulva to release sperms and retractor muscles backwards from the female. On the other hand, the female nematodes have double sets of genitalia, each having the ovary, the oviduct, uterus, and the spermatheca for holding sperms deposited in the vagina. Inside the female are eggs and each is have a three layered shell; the outer vitelline layer important for fertilization, the middle chitin layer, which contains chitin mircofibril core proteins that gives strength to the egg, and the semipermeable inner lipid layer that allows gases and ion movement (Burgwyn *et al*., 2003). While some eggs are laid in gelatinous matrix which hatches into J2s, others emerge directly from the cyst. Upon fertilization and complete maturation, the female harbours eggs in its body and its cuticle hardens and turns into a cyst. Cyst nematodes also have a tale region at its rear end where there are two phasmids.

Cyst nematodes are well adapted to their life cycle which begins in the soil with a cyst containing viable eggs and in presence of a host plant (Fig 3). Juveniles are held within the egg in a perivitelline fluid, which contains trehalose (Womersley and Smith, 1981). Trehalose restrict the movement of the second stage juveniles (J2) and thus for the nematode to be hatched out of the cyst, host factors for example glycinoeclepin A, α-solanine and α-chacoine, induces Calcium dependent reactions that increases permeability of the inner lipid layer permitting efflux of trehalose (Wesemael *et al*, 2006). At this point there is also influx of water into the egg which activates the J2 metabolically. Once J2s are mobile, they cut a slit in the egg shell mechanically using the stylet although Cotton *et al*., 2014 also suggested that chitinase might be involved in degradation of the middle chitin layer of the egg shell allowing J2s to exit from the cyst. The J2s finds the root point where to initiate invasion

based on physical-chemical gradients of CO_2, pH, redox potential and temperature (Wang *et al*., 2009). Usually, J2s invade the root at the elongation zone using the stylet and by the help of cell-wall degrading enzymes, to makes an entry hole (Chen *et al*., 2005; Vanholme *et al*., 2007). J2s then move on the outer layers of the root intracellularly through the cortex until they reach the vascular cylinder. Here they try out different cells until they find a suitable cambial or procambial cell which becomes the Initial Syncytial Cell (ISC) (Wyss and Zunke, 1986). For about 7hrs, J2 maintain its stylet in the ISC without movement awaiting plant responses. In the event there are defense responses for example callose disposition or protoplast disintegration, J2s escapes the ISC and finds another cell to form the ISC. (Golinowski *et al*., 1997; Sobczak *et al*., 1999). Once an ISC established in absence of plant defence responses, the stylet movement is restarted injecting secretions into the cytoplasm which induces formation of a feeding tube. It is through the feeding tube that nematodes draw nutrients of all kinds of metabolites and small proteins from the feeding site (Müller *et al*, 1981; Wyss, 1992; Böckenhoff and Grundler, 1994; Akker *et al*., 2014). At this stage, J2 whose dorsal gland is now enlarged to produce effectors injects these secretions which supresses host defense responses and reprogram developmental processes (Hewezi and Baum, 2013). In contrast, the activities of subventral glands are reduced and cell-wall degrading enzymes are now produced by the plant after the nematode-induced reprogramming. As a direct consequence, the cell wall of hundreds of neighbouring cells to the ISC disintegrates forming a multi-nucleate big cell which increases in size due to endoreduplication. This becomes the syncytium (De Almeida *et al*., 2013). In the syncytium, the vacuole disintegrates into small vacuoles suspended in the cytoplasm together with many plastids, mitochondria, ribosome and endoplasmic reticulum. The syncytium is the sole source of nutrients for the nematode which makes its metabolism to increase significantly (Szakasits *et al*., 2009). J2 remain immobile and moults to third stage juvenile (J3), a point at which the nematode start to internally differentiate into male or female. Both male and female depends on syncytium for nutrition even though females consume 29 times more food and have 10 times larger syncytium compared to the males.

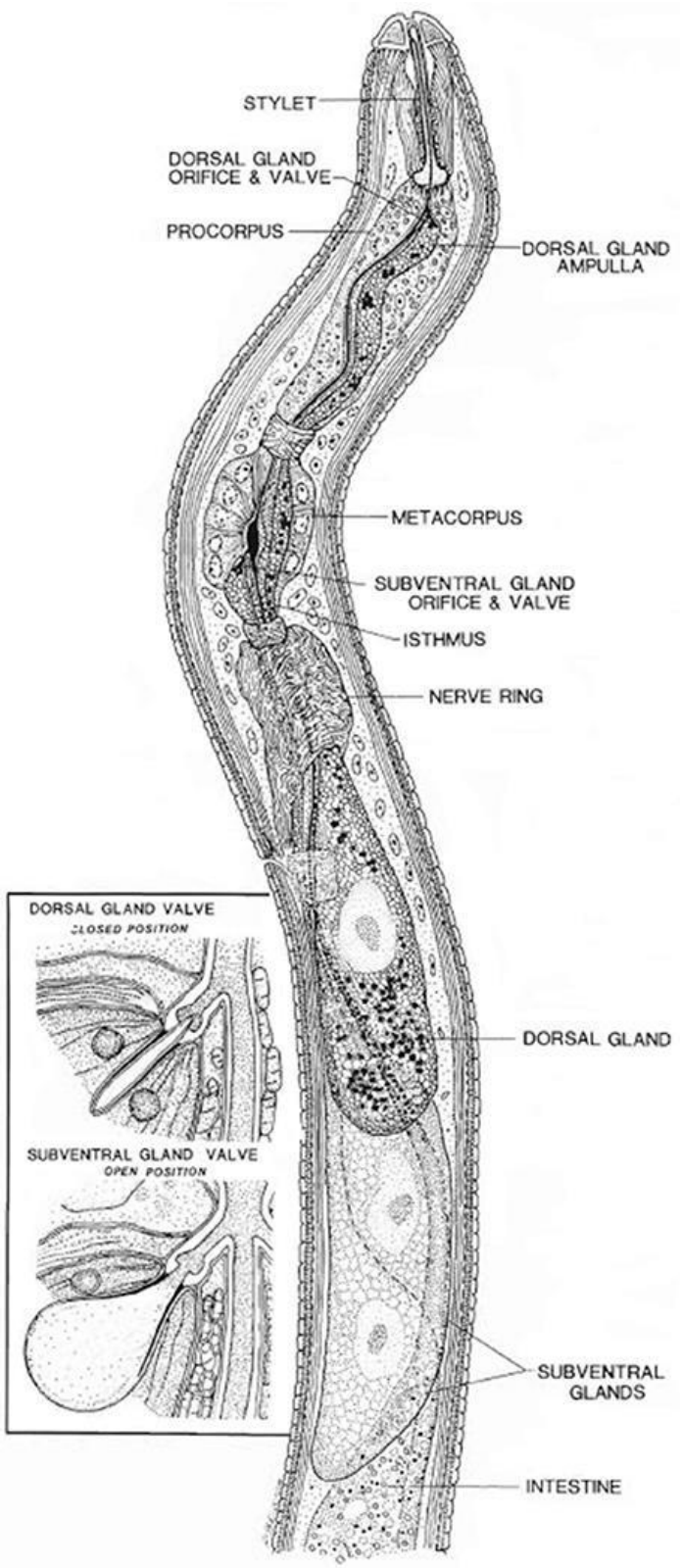

Figure 2. **Morphology of cyst nematode (Endo, 1984).**

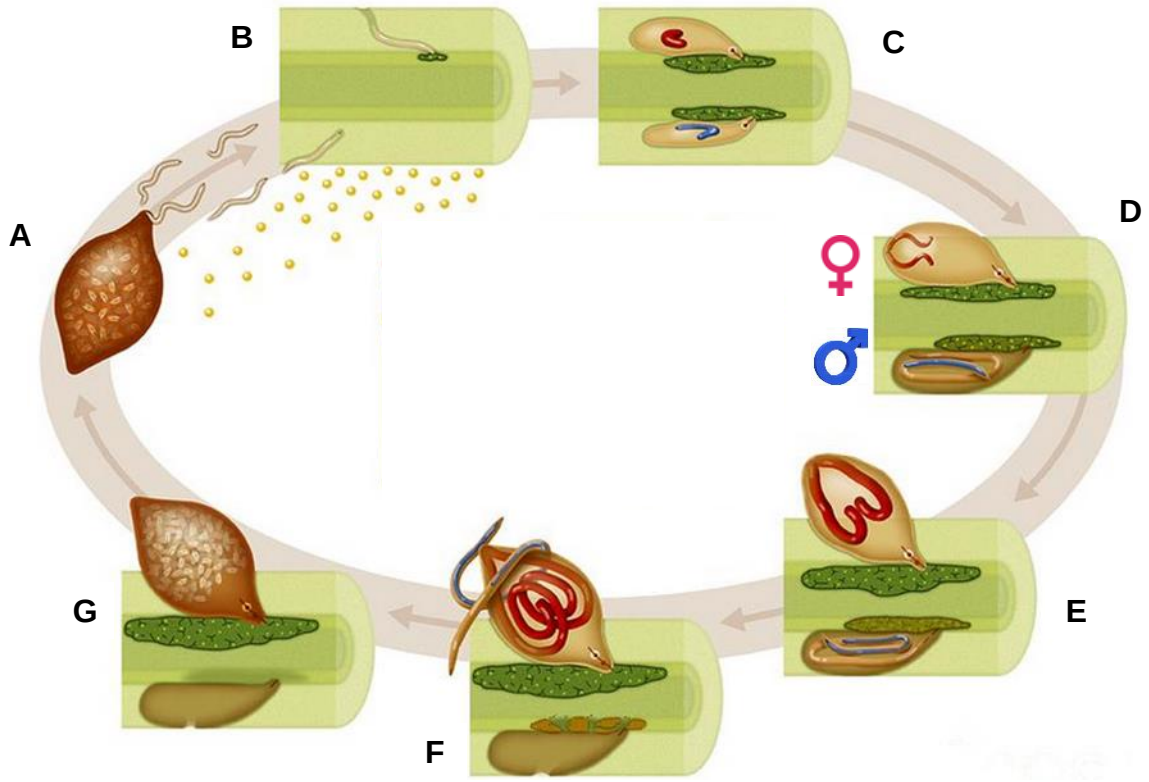

Figure 3. **Life cycle of cyst nematode.** The second stage Juveniles (J2s) emerges from the cyst, enter the plant at the elongation zone and move intercellularly until the vascular bundles (A). The J2s select one cell as an Initial Syncytium Cell (ISC) (B). The cell walls of the neighbouuring cells to the ISC dissolve and elarges the ISC into a single syncytium. The J2 molts into J3 ad start to differentiate as male and female. The nematode remains immobile (C). J3 moults to J4 (D). J4 differentiate completely as male and female where the male moves outside the plants and leaves its shell (E). The male moves in the soil search for a female to mate with for fertilization (F). The eggs develop inside the female body. The female dies and oxidizes to have a reddish brown colour which harbours the eggs. Within the eggs, the J1 develops to J2 which can infect the plant in the next cycle (G). (Modified from Art for science, 2015).

This is of course due to female reproduction role of producing hundreds of eggs which increases their body size as it gradually develops (Kerstan, 1969; Müller *et al*., 1981; Müller, 1985: Grundler *et al*., 1991). Male J3 stop feeding and its syncytium starts to degrade while its female counterpart enlarges. J3 from both of the genders then moults into fourth stage

juvenile (J4) of which the male develops within its cuticle. Depending on the environmental conditions and nutrients supply, most J4 undergoes a last moult into a completely differentiated female in abundance and or a male in adverse conditions. The male then become mobile again, shed off its shell and moves out of the root. The now mature female nematode produces sex pheromones which attracts the male to mate (Jaffe *et al*., 1989; Aumann *et al*., 1998). Upon fertilization, the eggs develop into single-celled embryos and the female further enlarges to form an ovoid shape. The embryo, still within the egg develops further into J1 and the female dies. Her cuticle hardens, oxidizes and become the reddish-brown cyst which carries and protects the eggs. A final moult occurs from the J1 to J2, ready to begin a new nematode cycle (Tylka *et al*., 1993; Niblack *et al*., 2006). Depending on temperatures, the life cycle of cyst nematode can take between 21 to 30 days to complete.

1.1.1.2 Root-knot nematodes

Root-knot nematodes share a lot of similarity to cyst nematodes and are mainly classified in genus Meloidogyne. Meloidogyne is of Greek origin, meaning "apple-shaped female". They were first reported in 1855 by Berkeley on cucumbers. There are approximately 100 Meloidogyne species described to date (Jones *et al*., 2013). The most widespread and economically important species are *M. incognita, M. javanica, M. arenaria, M. hapla, M. chitwoodi* and *M. graminicol*a. Root-knot nematodes are primarily tropical to sub-tropical organisms although *M. hapla* and *M. chitwoodi* are well adapted to temperate climates. Unlike cyst nematodes, they can parasitize any vascular plant and as such have a wide host range making them difficult to manage (Jones and Goto, 2011). General management methods apply to root-knot nematodes. Genes from tomato (*Mi*), prunus (*Ma* and *RMia*), carrot (*Mj*) and pepper (*Me*) have been reported to confer resistance to Meloidogne species. *Mi*-1 gene has gone further to be cloned successfully for commercial purposes, even though their success in the field is highly depended on temperature and *Mi* gene dosage (Jacquet *et al*., 2005).

Most of these nematodes are 400 to 2000 µm long. Generally, females of root-knot nematodes have a globose body, with a short neck containing their stylet, metacarpus, esophageal gland cells and distinct lips. The female cuticular morphological features of the perineum are used for perineal pattern analysis which is used in establishing differences among Meloidogyne species. The males on the other hand often have visible spicules for mating and a blunt-rounded tail. Many Meloidogyne species are parthenogenic or facultatively parthenogenic and as such, can reproduce without fertilization from a male. Generally, males are fewer and longer compared to females.

As of their life cycle, *Melodogyne spp* is quite similar to cyst nematodes. However, unlike cyst nematode, root-knot nematodes move within the plant intercellularly downwards to the elongation zone to escape the casparian strip after invasion in order to enter the vascular bundle. Their feeding site is actually a group of cells known as "giant-cells. Parasitized cells rapidly become multinucleate as nuclear division occurs in the absence of cell wall formation (uncoupled cytokinesis), resulting in bigger cells. Contrary to cyst nematodes´ syncytium, giant cells undergo continuous cycle of mitosis and their nuclei are irregular with large nucleoli. The giant-cells produce large amounts of proteins and also act as nutrient sinks, drawing plant nutrients such as carbohydrates into it. The root-knot nematode forms the feeding tube which acts as a sieve to filter cytosol as the nematode feeds. Esophageal gland cell secretions triggers increase in the production of plant growth regulators, demonstrated to play a role in increasing cell division and size. Cells neighbouring the giant-cells also become hypertrophied and divide rapidly, resulting in gall formation (Berg *et al*., 2008). Galls appear as knots in the roots and thus the name root knot nematodes. Inside the gall, the J2 becomes sessile by atrophy of the somatic musculature of their body excluding the head. The nematode moults three times after which its adult stage resumes feeding. Just like cyst nematodes, Meloidogyne spp were reported to parasitize Arabidopsis. In addition, *M. hapla* and *M. incognita* genomes data are publicly available. This has further paved way for plant-

root knot nematode interaction studies which entails a deeper understanding of plant responses to nematodes at cellular and molecular level (Sijmons *et al*., 1991).

1.2 Plant defense

In nature, plants often suffer from diseases caused by biotic stress agents like bacteria, fungi, viruses, nematodes and oomycetes as well as abiotic stress components which mainly encompass environmental factors like drought, salinity, Ozone, temperature among others. In addition, herbivores, both small and big animals, feed on or cause mechanical damage to plants, a scenario which is not considered as a disease even though it affects the plant in similar manner. Through evolution, plants have adapted to thrive in spite of their enemies by having an elaborate defense system which can be either constitutive or inducible (Dieter, 2008). Constitutive defense is the pre-existing measures against possible threats in plants. It is the main first line of defense against herbivores and pathogens. It is characterized by physical barriers like barks and waxy cuticle which are also adapted as thorns (*Carissa bispinosa*), spikes (*Acacia erioloba*), prickles (*Solanum pyracanthum*), shrinkage (*Mimosa pudica*), Milky latex (*Euphorbia pulcherrima*) and Trichromes (*Capsicum pubescens*) among others. The plant cell wall, in addition, is like an exoskeleton surrounding the plant cell and consists of cellulose microfibrils, pectin, hemicelluloses, proteins and lignin which all can vary in composition. These plant modifications ward off or cause allergic reactions to herbivore and prevent pathogen entry into the plant. In addition, volatile organic compounds such as toxic alkaloids, terpenoids, phenolic compounds and saponins are also produced by plants as a chemical defense whose odour, bitterness and reaction deters attackers. Chemical defense is usually utilized by plants in the event where the physical barriers are non-existent or have been overcome by the herbivores and or pathogen (Osbourne, 1996; Tierens *et al*., 2001). For example phenolic compounds like protocatechuic acid and catechol in scales of red onion inhibit conidia germination of *Colletotrichum circinans* on its surface.

Contrary to animals, plants do not have mobile immune cells and a somatic adaptive immune system. However, they are reliant on immunity within each cell which ignites defense responses upon invasion and thus referred to as induced defense. This is a kind of plant immunity which before and after pathogen invasion can be divided into primary and secondary immunity respectively. Primary defense occur before the pathogen entry inside the plant. At cellular level it is referred to as innate immunity due to its conserved nature and is characterized by Plant Recognition Receptors (PRRs), which recognizes a conserved signature ligand from the pathogen or the herbivore (Ausubel, 2005). These molecules from pathogens are called Pathogen Associated Molecular Patterns (PAMPs) and as such pathogen induced plant innate immunity is also referred to as PAMP Triggered Immunity (PTI). PRRs can also recognize plant indigenous molecules produced when pathogens cause mechanical cellular damage during infection and these molecules are called Damage Associated Molecular Pattern (DAMPs). Recognition of DAMPs elicits similar responses as would PAMPs of which a signal is transduced to the cell nucleus where activation of defense responses is induced. When a pathogen overcomes PTI, mainly by ligand modifications to avoid recognition by immune receptors, it henceforth gains access to the cell cytosol. Here it introduces secretions that are targeted to modify plant cellular processes or supress PTI in favour of the pathogen, which further increases invasion by more pathogen and growth of more mutualistic symbionts. These secretions have been reported to include various compounds particularly effectors and as such, the phenomenon is called Effector Triggered Susceptibility (ETS).

Some plants have evolved ways of preventing colonization of the plant cell by the pathogen by co-evolutionary acquiring the R-genes. R-genes (virulence genes) encode the polymorphic Nucleotide Binding and Leucine Rich Repeat (NB-LRR) proteins that directly or indirectly bind to pathogen effectors (avirulence factors), inducing defense responses and thus Effector Triggered Immunity (ETI) (Jones and Dangl, 2006). This phenomenon was first described in the model of recognition of specific resistance genes “gene-for-gene”. The

paradigm that activated immune responses in ETI occur faster, robust and prolonged compared to those in PTI (Jones and Dangl, 2006). The R genes functions quite similar to resistant genes conferring immunity in animals as NB-LRR proteins are broadly related to animal CATERPILLER/NOD Like Receptors (NLR) proteins. ETI mediates plant resistance against obligate biotrophs or hemi-biotrops but not necrotrophs, through a hypersensitive response (HR) characterized by cell death at points of infection (Matzinger, 2002; Glazebrook, 2005). So far there are only two types of cell death that have been described; vacuolar and necrosis cell deaths. In vacuolar cell death, a combination of autophagy-like process and release of hydrolases from collapsed lytic vacuoles causes removal of cell contents primarily during organ formation. Necrosis on the other hand occurs typically under abiotic stress and involves early rupture of the plasma membrane and shrinking of the protoplast. HR causes the pathogen nucleus to disintegrate into a homogenous mass and its cytoplasm dense. As a direct consequence, pathogen growth beyond the dead cell is halted. Generally, HR is meant to isolate the infection at the invasion point and thus prevent extensive infection and pathogen colonization (van Doorn *et al*., 2011). HR has been well studied in various diseases caused by different microbial agents like *Synchytrium endobioticum* causing wart disease of potato, *Phytophthora infestans* causing late blight disease of potato and *Pyricularia oryzae* causing blast of rice among others. R gene resistance is also associated with activation of Salicylic Acid (SA) signalling pathway which mainly involves three well known genes; Protein Arginine Deiminase (PAD) 4, Non-expressor Pathogenesis Related Gene (NPR) 1 and Enhanced Disease Susceptibility (EDS) 1. The high concentration of SA generated during ETI has particularly promotes NPR1 degradation and as such NPR1 is considered a repressor of ETI, which is contrary to its role in PTI as a positive regulator of SA-mediated basal resistance. Interestingly, HR activates of SA signalling throughout the plant an indication of the cross talk among ETI defense responses. SA induces activation of pathogenesis-Related (PR) genes directly involved in disease resistance against pathogens sensitive to SA dependent responses. This phenomenon is known as Systemic Acquired Resistance (SAR) which localizes infection by

the primary pathogen which in turn results in the induction of a wide spread and long lasting resistance to secondary pathogens in plant systemic tissues (Falk, 1999; Wildermuth, 2001; Zhang *et al*., 2003; Durrant and Dong, 2004). In addition to SA, some defense responses are dependent binaurally or singularly on Jasmonate (JA) and Ethylene (ET) pathways. These pathways occur parallel to one another with very few cases where they tend to have a negative interaction. JA-dependent signalling occurs through increased JA synthesis and consequently increases the expression of defense related genes such as Plant Defensin (PDF) 1 and transcription factors Ethylene Response Factor (ERF) 1, Related to APetala (RAP) 2, Jasmonate-INsensitive (JIN) 1 and Ethylene-Insensitive (EIN) 2 involved in defense responses. Cellulose synthases in the plant cell wall is involved in regulation of JA levels while JA-amino synthetase is required for conjugation of JA and several amino acids like isoleucine. The conjugated version of isoleucine is reported to be the active form of JA and thus JAR1 gene which encodes JA-amino synthetase is required for JA biosynthesis and in turn regulating JA levels. There is a complex hormonal cross talk between SA and JA/ET pathways of which most are confirmed to be activated in a mutually repressive manner and as such, resistance based on either pathway heavily depends on the pathogen involved. SA pathway-dependent defense responses are involved in resistance against biotrophic and hemibiotrophic pathogens; organism that rely on living tissues for nutrition. JA and ET pathways-dependent on the other hand responses against insect wounding and necrotrophs; organisms that obtain nutrients from dead cells (McDowell and Dangl, 2000; Wildermuth *et al*., 2001; Glazebrook *et al.,* 2003; Van Wess *et al*., 2003; Dong, 2004; Trusov *et al*., 2006). This phenomenon has been observed in various cases like during infection with *Pseudomonas syringae* where NahG transgene induced SA levels reduction triggers overexpression of JA-induced genes. At the same time, treating Arabidopsis with SA and JA at the same time abolished JA-mediated induction of the PDF1.2 gene, while infection by the hemibiotrophic bacterial pathogen *Pseudomonas syringae.* PV. *tomato* (Pst) DC3000, which enhanced SA production, led to reduced resistance to the necrotrophic fungal pathogen *Alternaria brassicicola* in neighbouring cells (Spoel *et al.,* 2003, 2007; Van der Does *et al*.,

2013). The antagonistic behaviour of these hormonal pathways has been reported to be highly dependent on SA-signalling gene (NPR1), SA biosynthesis, metabolism regulating JA-inducible transcription factor NAC and downstream transcription factors WRKYs and TGAs genes (Spoel *et al*., 2003; Li *et al*., 2004; Mao *et al*., 2007; Zander *et al*., 2010). The main player NPR1 protein contains a BTB/POZ and an Ankyrin repeat domains involved in protein-protein interaction. It is suggested that NPR1 regulates PR gene expression through direct interaction and as a cofactor of the TGA transcription factors (Zhang *et al.,* 1999, 2003; Zhou *et al.,* 2000). Regardless of their antagonistic relationship, SA and JA pathways are not always antagonistic even though in very few cases. One such case is in rice where JA signalling positively regulates plant resistance to the biotrophic pathogen *Xanthomonas oryzae* PV. *oryzae* (Xoo). This was suggested to be caused by activation of a common defence system by both hormonal pathways. Other studies have also found that their antagonistic relationship is highly dependent on their concentrations (Mur *et al*., 2006; Yamada *et al*., 2012; Tamaoki *et al*., 2013). Plant defense hormones i.e. SA, JA, and ET precisely regulates plant immune responses both locally and systemically thus coordinate defense in different parts of the plant and against different types of pathogens (Erb *et al*., 2012; Pieterse *et al*., 2012; Wasternack, 2013).

Besides hormonal signalling, some studies have reported that a phytoalexin such as camalexin (3-thiazol-2′yl-indole) as a plant antimicrobial effector in ETI often considered as a defense marker protein. Its synthesis is induced by pathogens such as *Pseudomonas syringae*, *Alternaria brassicicola*, and *Botrytis cinerea*, and some abiotic stresses, such as amino acid starvation. It has also been shown to inhibit the growth of fungal pathogens. Camalexin induction in Arabidopsis infected with *P. syringae* is dependent on the transcription factor *WRKY33*, which directly binds to the camalexin biosynthesis promoter gene PAD3. Defense signalling cascade involving MPK3/MPK6 signalling leads directly to phosphorylation of *WRKY33*, and this drives camalexin production in Arabidopsis infected plants (Ren *et al*., 2008; Qiu *et al*., 2008; Mao *et al*., 2011). PAD3 encodes cytochrome P450

monoxygenase CYP71B15, which is a camalexin biosynthetic enzyme and is currently utilized as a defense marker gene as well as *WRKY33* gene among others (Glazebrook, 2005).

1.2.1 PAMP Trigerred Immunity (PTI)

The term PAMP was first described in 1989 by Janeway in her then visionary theory of recognition. She proposed that microbial components are recognized by innate immune receptors allowing detection of infection. The theory was experimentally validated later and has become a standard that constitute legitimate contribution to understanding plant-microbes interaction (Janeway, 1989; Medzhitov and Horng, 2009; Medzhitov 2013). PAMPs have to date come to be well characterized following a certain criteria; PAMPs have a distinct structure, are essential for survival and are produced via pathways restricted within a given class of microorganisms. Since molecular patterns are not present only in pathogens, for example, they are also found in beneficial and mutualistic bacteria, the term Microbial Associated Molecular Patterns (MAMPs) is sometimes used (Janeway, 1989; Beutler, 2003; Medzhitov 2007). PAMP and DAMP recognition is considered as detection of “non-self-signals” and “self-molecules” since the molecule recognized originates from the pathogen and host respectively (Boller and Felix, 2009). Within the molecular patterns (also called elicitors), the PRRs recognize only small and conserved part of it. PRRs are adapted to recognize molecules of diverse nature like proteins, carbohydrates, nucleic acids and lipids among others. The first molecule to ever be clearly characterized as a PAMP is a short 13-amino acid peptide of a conserved fragment within a calcium-dependent cell-wall transglutaminase, from the oomycete *Phytophthora sojae* called Pep13. This PAMP elicits defence responses in *Solanaceae spp* (Nürnberger, 2004). Currently, there is at least one PAMP that has been reported from bacteria, fungi, oomycetes, Virus, nematodes, insects and parasitic plants. PAMP perception is specific and most of PAMP are restricted to a specific type of species, however there are some exceptions for example Necrosis- and

ethylene-inducing-peptide-1-Like Proteins (NLPs) and β-glucans. NLPs and β-glucan structure are present in multivariant organisms such as bacteria, oomycetes and fungi and induce PTI responses similarly (Ranf, 2017). Generally, even though most of PAMP involved in PTI are characterized, not all of their recognizing PRR are known. Nonetheless, almost all plant PRRs involved in PTI and reported so far are surface localized and exists as either Receptor-Like Kinases (RLKs) or Receptor-Like Proteins (RLPs). RLKs are composed of an extracellular domain that binds to specific region of a PAMP, a transmembrane domain which maintains the receptor at the cell membrane and an intercellular cytoplasmic kinase domain responsible for signal transduction. RLPs are structurally similar to RLKs but lacks an intracellular kinase domain hence for their PAMP induced signal transduction to be completed, they always recruits other RLKs or Receptor-Like Cytoplasmic Kinases (RLCKs) existing freely in the cytoplasm. Regardless of their nature as RLKs, RLPs or RLCKs, PRR extracellular kinases or PRR-associated kinases contain alteration in a conserved positively charged arginine (R) residue, located within a charge cluster. R neutralizes the negatively charged catalytic aspartate (D) next to it. Therefore, R blocks the catalytic function of D residue. This region of the kinase is called RD motif and it mediates phosphor-transfer during intracellular signalling. In close proximity to the RD motif is the kinase activation loop which when activated, produces negatively charged phospho amino acids that in turn overcomes the positively charged R residue leading to activation of kinase. Some PRR kinases don't have R which is mainly substituted by non-charged residues such as cysteine, glycine, leucine and phenylalanine residues. This region is commonly known as non-RD motif. Contrary to RD kinases, non-RD kinases don't auto-phosphorylate their kinase activation loop hence non-RD receptor are proposed to have a different activation mechanism, whose difference in functionality to RD kinases is currently unknown. Majority of Plant RLKs have RD kinase (Fig 4) which phosphorylates serine/threonine residues (Dardick *et al*., 2012).

Once RLKs recognize the elicitor, at cellular level, intracellular signalling is triggered via activation of Mitogen Activated Protein Kinase (MAPK) cascades to the nucleus leading to genetic reprogramming that induces early defense responses (Asai *et al*., 2002; Mishra *et al*., 2006). Among these responses is the rapid and robust expression of defense marker genes such as Pathogenesis Related (PR), Flg22-induced Receptor-like Kinase (FRK) 1 and WRKY genes (Asai *et al*., 2002; van Loon *et al*., 2006; Gust *et al*., 2007; Boudsocq *et al*., 2010; Ahuja *et al*., 2012; Bednarik, 2012). Reactive Oxygen Species (ROS) production like superoxide anions (O_2^-) also occurs within seconds to minutes thus populary referred to as oxidative burst. PAMP/DAMP induced oxidative burst has been observed in both vertebrates and plants as a main PTI induction characteristic where toxicity nature ROS damages or kills the pathogen. In addition, ROS production induces crosslinking within plant cell wall making them less prone to degrading enzymes from pathogens (Apostol *et al*., 1989; Apel and Hirt, 2004; Kohchi *et al*., 2009; O'Brien *et al*., 2012). The plasma membrane also become depolarized allowing an influx of extracellular Ca^{2+} in the cytosol (Ca^{2+} burst), which begis to occur at 30th and 120th second and peaks between 4 to 6 min after invasion (Jeworutzki *et al*., 2010; Ranf *et al*., 2011; Nomura *et al*., 2012). Ca^{2+} burst induces the opening of other membrane transporters allowing influx of H^+ and efflux of K^+, $Cl^–$, and NO_3^- which in turn increases the pH of the extracellular region to pathogen´s demise. At the same time, Ca^{2+} ions entering the cell cytoplasm from the apoplast activates calcium-dependent proteins such as Ca^{2+} Dependent Protein Kinases (CDPKs) (Boller and Felix 2009; Boudsocq *et al*., 2010; Jeworutzki *et al*., 2010). Callose is an amorphous homopolymer composed of (1, 3)-β-Glucan callose and is normally deposited between the plasma membrane and the pre-formed cell wall at the point of pathogen attack, upon PAMP recognition. Callose acts as a physical barrier blocking or slowing down invading pathogens from entering the plant However, its regulation is not well known as its biosynthesis is not described hence, there exist no mechanism of understanding how its deposition is induced as a response to PAMP perception (Radford *et al*., 1998; Luna *et al*., 2011; Kemmerling, 2012). PAMP perception also activates biosynthesis of ethylene which is an immunity hormone. PTI also alters

metabolite composition and the production of secondary defense compounds for example glucosinolates in fungi infected Brassicaceae spp (Bednarek *et al*., 2009; Sana *et al*., 2010).

Generally, as the first line of plant cellular defense, PTI is a stringent, robust and occurs within seconds to minutes. It is also very temperate and efficient to almost all non-host pathogens. Altogether, PTI responses contributes to basal resistance by preventing establishment of infection by pathogens, controls stomatal closure to prevent bacteria entry and surprisingly, PTI also inhibits growth of commensal microbiota, an indication that PAMPs presensce cut across a whole class for every organism, regardless if the organism is pathogenic or not (Melotto *et al*., 2008; Sawinski *et al*., 2013; Gourion *et al*., 2014; Rovenich *et al.*, 2014). In addition, PTI prevents microbial colonization by cutting nutrient supply and releasing anti-microbial compounds which in turn starve pathogens and reduces release of effectors into the cell (Chen *et al*., 2010; Wang *et al*., 2012; Xin *et al*., 2016; Yamada *et al*., 2016). PTI has also been associated with seedling growth inhibition due to the redistribution of plant resources from growth related processes to those leading towards defense (Gomez-Gomez *et al*., 1999; Boller and Felix, 2009)

Most of the PRRs are RLKs. RLKs have a monophyletic origin within the whole superfamily of plant kinases. In the sequenced Arabidopsis genome, the RLK (also named pelle) family formed based on similarity to the basic structure of animal receptor tyrosine kinases (RTKs), contains 610 RLK homologs representing about 2.5% of the annotated protein-coding genes (Arabidopsis Genome Initiative, 2000; Shiu and Bleecker, 2001). Among these, 193 RLKs do not have an obvious receptor configuration as determined by the absence of putative signal sequences and or transmembrane regions. The remaining 417 genes with receptor configurations have similar transmembrane and intracellular domains. However, their extracellular domains differ in their structural features particularly in the type and arrangement of its respective amino acids. These differences greatly contribute to their classifications into 21 subfamilies. The sizes of each the subfamily varies greatly however, Leucine Rich repeat (LRR) subfamily is the largest one containing 216 genes with LRR

motifs. The currently known PRR containing LRR ectodomains binds to peptides or proteins type of elicitors. Similarly, in animals, the most well characterized PRRs are the Toll-Like Receptors (TLRs) containing LRR extracellular domain. TLRs are involved in the sensing stimuli from bacteria, fungi, protozoa, and viruses. A well-studied example of all TLRs is TLR5 that interacts directly with its single microbial ligand flagellin (Felix *et al*., 1999; Hayashi *et al*., 2001; Smith *et al*., 2003). In plants, the best characterized receptor is Flagellin Sensing 2 (FLS2), which has a 28 LRRs containing ectodomain that directly binds to a 22 amino acid peptide (flg22) at the N-terminus of flagellin. Flagellin is building protein block of the flagellum which is the motility and virulent structure in bacteria. It is highly abundant and freely released from the wall of flagellum (Gomez-Gomez and Boller, 2000; Chinchilla *et al*., 2006; Yamaguchi *et al*., 2006; Zipfel *et al*., 2006). Flg22 binds to LRR3-16 section of FLS2 super helical ectodomain which directly interacts and forms a heterodimer complex with the LRR-RLK Brassinosteroid-Associated Kinase (BAK) 1 RD ectodomain; one of the five genes belonging to the Somatic Embryogenesis Receptor Kinase (SERK) family. Perception of flg22 by FLS2 initiates PTI responses of which ROS burst is the most immediate one. NADPH oxidases belonging to the Respiratory Burst Oxidase Homolog (RBOH) family; which contains 10 members in Arabidopsis, plays a crucial role in ROS production.

The induction of ROS production begins with BAK1/FLS2 heterodimer complex associating with and phosphorylating the RLCK Botrytis Induced Kinase 1 (BIK1), In turn BIK1 binds directly to the N-terminal domain of RBOHD, phosphorylating its residues S39, S339, S343 and S347 in a ligand -Dependent manner. RBOHD possess a core C-terminal region containing a trans-membrane domains and the functional oxidase domain responsible for superoxide production. Specifically, NADPH oxidases transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, leading to the production of superoxide (O2-).

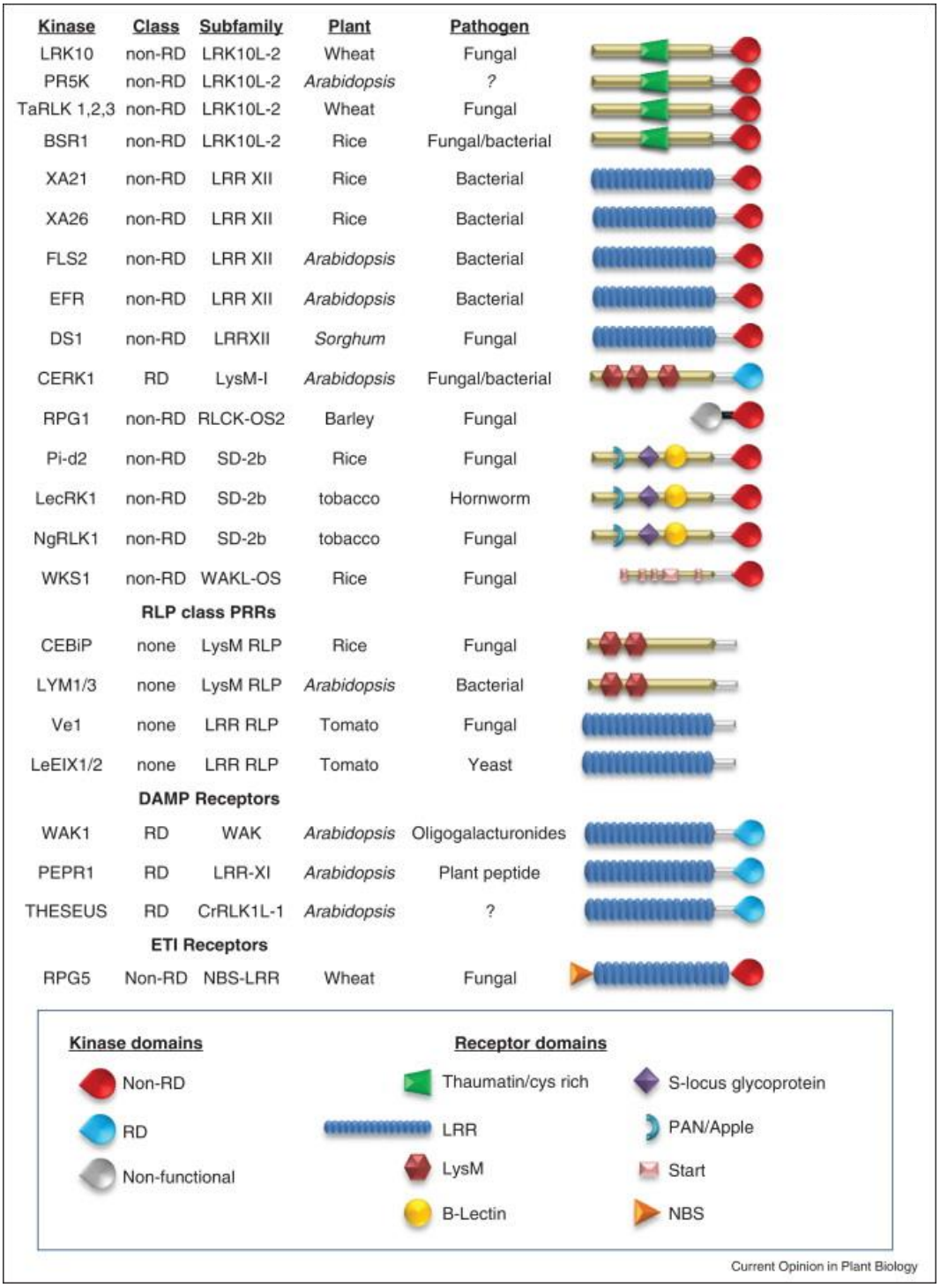

Figure 4. **Described and putative PRR receptors along with their domain organizations and kinase functionality**. Most plant and animal PRRs identified to date contain kinase domains. In addition, PRR kinases or PRR-associated kinases contain a positively charged and conserved arginine (R) residue located within a charge cluster, adjacent to the key catalytic aspartate (D) (the RD motif). Receptor kinases lacking the R and instead having an

uncharged residue such as Cys, Gly, Phe, or Leu, are referred to as non-RD. Kinase domain are connected to the extra-cellular domain by transmembrane domain. PRRs are grouped in different families based on the different components of their extra cellular receptor domain with majority belonging to LRR family. Atleast one PRR has been characterized from the whole microbial class of Oomycetes, Fungi and Bacteria within both monocots and dicots (Dardick *et al*., 2012).

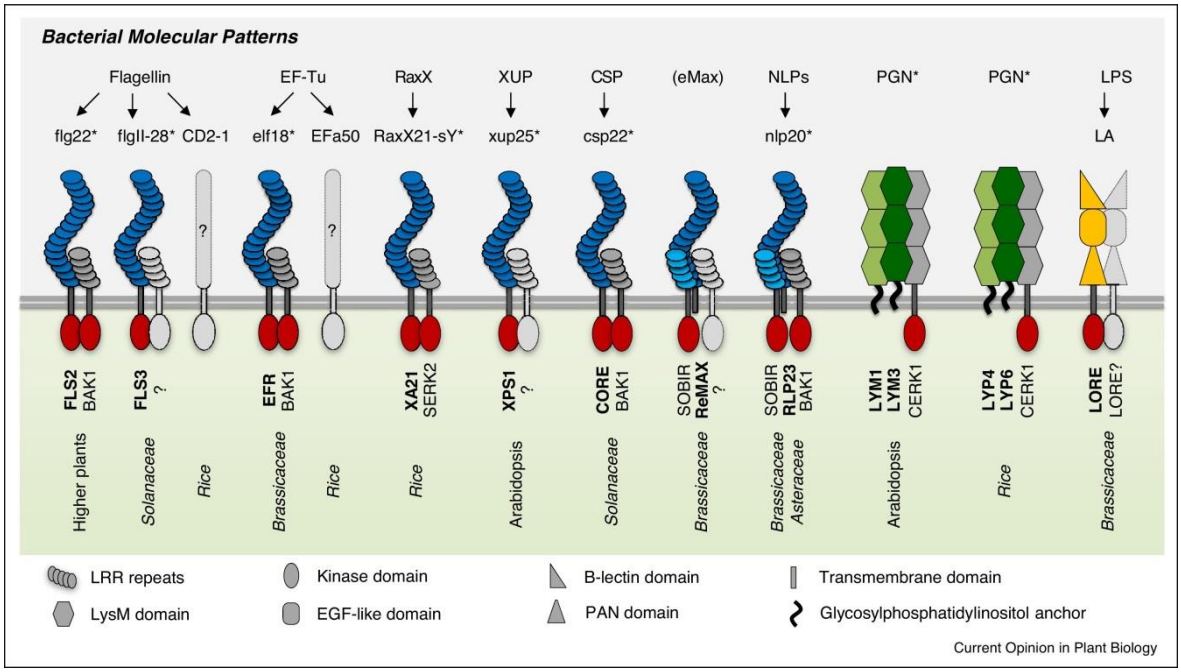

Figure 5. **Plants recognize different bacterial PAMPs through different types of plant cell surface receptors**. LRR receptors sense proteinaceous PAMPs. Flagellin which exist in three pecific epitopes; flg22, flgII-28, and CD2-1, are bound by FLS2 in Arabidopsis, FLS3 in tomato and an unknown receptor in rice, respectively. FLS2 Heterodimerizes with BAK1/SERK3 and other SERK-RLKs upon ligand binding. EF-Tu epitopes elf18 and EFa50 are perceived by EFR in family Brassicaceae and an undescribed receptor in rice, respectively. XA21, XPS1 and CORE recognize the bacterial RaxX21-sY epitope RaxX, XUP and CSP where XA21 constitutively interacts with rice co-receptor SERK2. RLPs ReMAX and RLP23 perceive the Xanthomonas protein eMax and the nlp20 epitope of NLPs, respectively. LysM family RLPs LYM1-LYM3 and LYP4-LYP6 recognize the N-

acetylglucosamin-containing glycan backbone of PGN in Arabidospis and rice, respectively. Lectin receptors facilitate recognition of the glycolipid LA moiety of LPS in Brassicaceae. LRR-RLPs ReMAX and RLP23 form constitutive dimers with the LRR-RLK SOBIR. RLP23-SOBIR, but not ReMAX-SOBIR, also associates with BAK1 in a ligand dependent manner.. FLS3, XPS1 and ReMAX-SOBIR presumably also interact with yet unidentified (SERK-like) LRR-RLKs. LysM-RLPs associate with CERK1 for signalling. Star (*) indicates existence of proof of direct PRR-ligand binding. (Ranf *et al*., 2017).

O2- is then converted to hydrogen peroxide (H2O2) by superoxide dismutase (Torres and Dangl, 2005; Marino *et al*., 2012; Suzuki *et al*., 2011, 2012). Apoplastic Ca2+ is reported to be required for PAMP-induced ROS production by binding to the EF-hand motif of RBOHD and phosphorylation by Ca2+ regulated protein kinases (Ogasawara *et al*., 2008; Kadota *et al*., 2004, 2014; Ranf *et al*., 2011; Segonzac *et al*., 2011; Marino *et al*., 2012). Other than BIK1, other RLCKs like PBS1-Like Kinases 1, 2 and 5 (PBL1, PBL2 and PBL5) and Brassinosteroid Signalling Kinase 1 (BSK1) also associates with the FLS2/BAK1 heterodimer complex and are then released to activate downstream immune responses. All in all, BIK1 is most well characterized positive regulator of PTI responses and induced resistance to *Pseudomonas syringae* (Lu *et al*., 2010; Laluk *et al*., 2011; Liu *et al*., 2013; Shi *et al*., 2013; Zhang *et al.*, 2010).

SERKs belong to subfamily II of the LRR-RLK family, containing only five members; SERK1, SERK2, BAK1/SERK3, BAK1-like (BKK1)/SERK4 and SERK5 in Arabidopsis. SERKs were originally described as embryogenic markers in *Daucus carota* (carrot) and later as potential co-receptors when their shape was identified to be complimentary to the spiral shape of LRR-RLKs which allowed ligand binding and receptor activation (Hecht *et al*., 2001; Brandt and Hothorn, 2016). BAK1/SERK3 was initially shown to be a positive regulator of the brassinosteroid growth signalling pathway via LRR-RLK Brassinosteroid Insensitive (BRI) 1. Similarly, BAK1 (SERK3) acts as a co-receptor upon flg22 perception essential for signalling activation in equally similar manner (Chinchilla *et al*., 2007; Sun *et al*., 2013). A lot of studies

are implicating SERKs in many signalling pathways especially as an increasing number of LRR-RLKs and LRR-RLPs type PRRs are recruiting BAK1 or other SERKs in their perception of ligands from different type of pathogens (Fig 5). This heavily suggests SERKs might be universal co-receptors for almost all LRR-RLKs. For example, in rice, BAK1 ortholog OsSERK2 forms a complex with the LRR-RLK XA21 receptor conferring resistance to the bacterium *Xanthomonas oryzae* (Chen *et al*., 2014). In tomato, BAK1 ortholog also interacts with the LRR-RLP Ethylene-Inducing Xylanase (EIX) 1 when bound to fungal xylanase initiating defense responses against *Cladosporium fulvum* (Liebrand *et al*., 2013; Santiago *et al*., 2013). In addition, BAK1 is recruited into a pre-formed Suppressor Of BAK1 (SOBIR1)-RLP23 complex in a Necrosis and ethylene-inducing peptide 1-Like protein (NLP) 20 dependent manner. NLPs are generally proteins present in many prokaryotes and eukaryotes (Fig 6). NLPs recognition induces the formation of a tripartite PRR complex that activates defense against the oomycete and fungal pathogens *Phytophthora infestans* and *Sclerotinia sclerotiorum* respectively (Albert *et al*., 2015). In DAMPs perception, LRR-RLKs PEP RECEPTOR1 (PEPR1) and its homolog PEPR2 recognize the wound-induced endogenous peptide AtPep1. However, only PEPR1 that has been reported to form a complex with BAK1 activates PTI like responses (Huffaker and Ryan, 2007; Yamaguchi *et al*., 2006, 2010; Krol *et al*., 2010; Tang *et al*., 2014*;* Ranf et al., 2017).

It is interesting that many different functions are dependent on five receptor proteins only hence begging the question why plant membrane signalling pathways depends on such few co-receptor kinases. Many scientist believe that to understand SERKs in plant signalling, the receptors supported needs to be identified or further studied, the perceived ligands characterized, specific signalling cascade in different LRR-RK pathways described and their regulation mechanisms by other cellular factors such as RLP, RLCK and phosphatases demonstrated (Schmidt *et al.,* 1997; Li, *et al*., 2012; Santiago *et al*., 2013; Sun *et al*., 2013; Wang *et al*., 2015; Brandt and Hothorn, 2016).

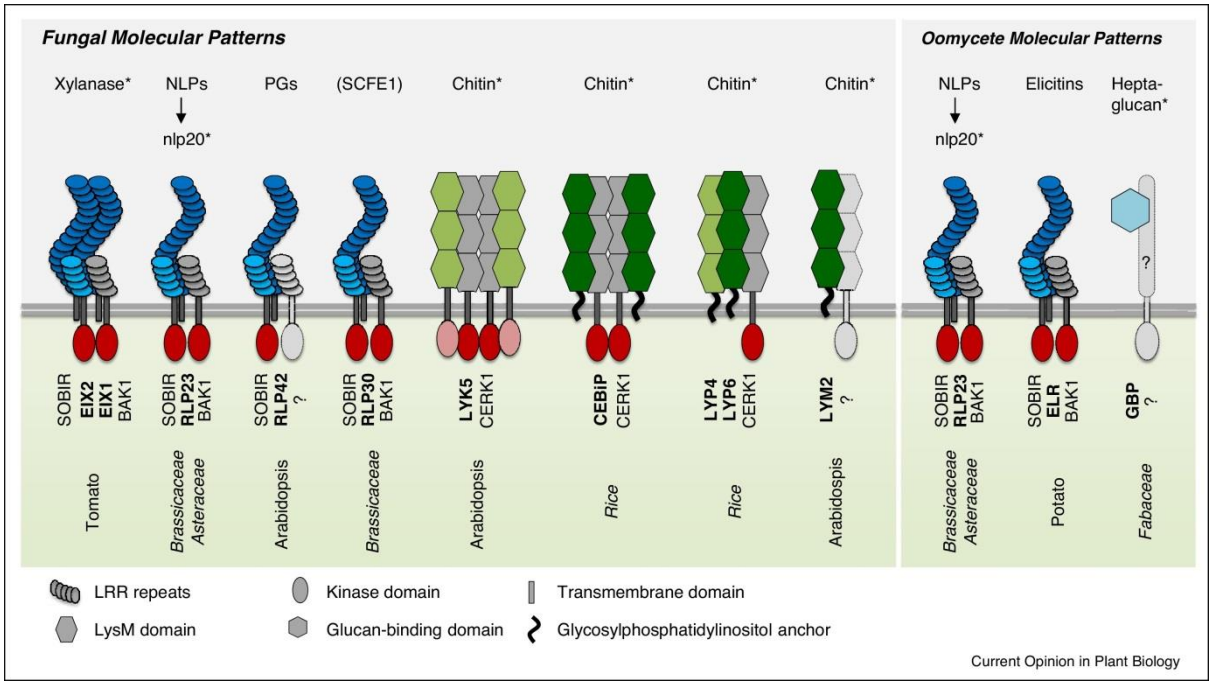

Figure 6. **Plant PRRs perceive different fungal and oomycete molecular patterns**. Tomato LRR-RLPs EIX1-EIX2 sense fungal xylanase and binds also to BAK1. RLP23, RLP30, and RLP42/RBPG1 recognizes NLP epitope nlp20, SCFE1, and endopolygalacturonases (PGs), respectively, and constitutively associate with RLK SOBIR and BAK1 as signalling co-receptors. RLP42-SOBIR presumably also interacts with a SERK-like RLK. Chitin oligomers are recognized by Arabidopsis LysM-RLP CEBiP, LYK5 and rice LYP4-LYP6, and recruites CERK1 for signalling in a ligand dependent manner. In Arabidopsis, chitin is sensed through LYK5, a pseudo-kinase, which recruits CERK1 in a chitin-dependent manner. Oomycete NLPs are also sensed through RLP23-SOBIR-BAK1. Elicitins are sensed through ELR in a BAK1-SOBIR-dependent manner. Heptaglucan fragments derived from oomycete cell walls are perceived through soluble GBP. GBP presumably associates with a yet unknown transmembrane protein for signalling. Star (*) indicates existence of proof of direct PRR-ligand binding. (Ranf *et al*., 2017).

Besides the LRRs, other PRRs` ectodomains contains Lysine Motifs (LysMs), lectin Motifs (LeMs) and Epidermal Growth factor (EGF). These ectodomains are involved in perception carbohydrates like bacteria Lipopolysaccharides (LPs) and fungal chitin as well as DAMPs

for example extracellular ATP and the plant-cell-wall-derived OligoGalacturonides (OGs). A typical example is the Arabidopsis LysM-RLK CERK1/RLK1/LYK1 which contains three LysM motifs and perceives a 7 t0 8 GlcNAc residues containing oligomers of fungal chitin. In rice, the chitin-binding protein is the LysM-RLP CEBiP containing a three extracellular LysM domains and a C-terminal tail. CEBiP homodimerizes to bind long chitin oligomers and activate defense in a similar mechanism as in Arabidopsis chitin receptor AtCERK1 (Kaku *et al*., 2006; Miya *et al*., 2007; Brutus *et al*., 2010; Willmann *et al*., 2011; Liu *et al*., 2012; Choi *et al*., 2014; Kouzai *et al*., 2014; Hayafune *et al*., 2014).

PTI has been widely studied in plants against fungi, bacteria and oomycetes. However, there are few case studies regarding PTI induced by insects, viruses and nematodes. Plants sense non-self-nucleic acids signals as viral double-stranded RNA during infection inducing not only PTI but also gene silencing. Viral dsRNA perception triggers a signalling cascade involving and dependent on SERK1 and a specific dsRNA receptor however, the mechanism behind this perception is still unknown and the signalling pathways involved are yet to be unveiled. All in all, PTI restricts virus infection and mediates antiviral resistance in plants (Niehl *et al*., 2016). Invasive parasitic plants have recently demonstrated to be recognized by host plants (Fig 7). The first report was done on Dodders (*Cuscuta spp*); a holoparasite that rap around almost all dicotyledonous plants except tomato and penetrate their stem with haustoria to their vascular bundles for nutrition. One of the dodder species *Cuscuta reflexa* was identified to encode a 2kDa peptide having an O-esterified modification; Cuscuta factor (CuF), perceived by the LRR-RLP Cuscuta receptor 1 (CuRe1) triggering PTI responses like ROS burst and production of ethylene in tomato (Fürst *et al*., 2016).

Nematode induced PTI is only sparsely studied. Nevertheless, Ascarosides abundantly found in whole nematode class was recently reported to induce PTI and Root knot nematodes have been shown to induce PTI in a BAK1 dependent manner which will be

discussed further below (Holbein *et al*., 2016; Ranf, 2017).

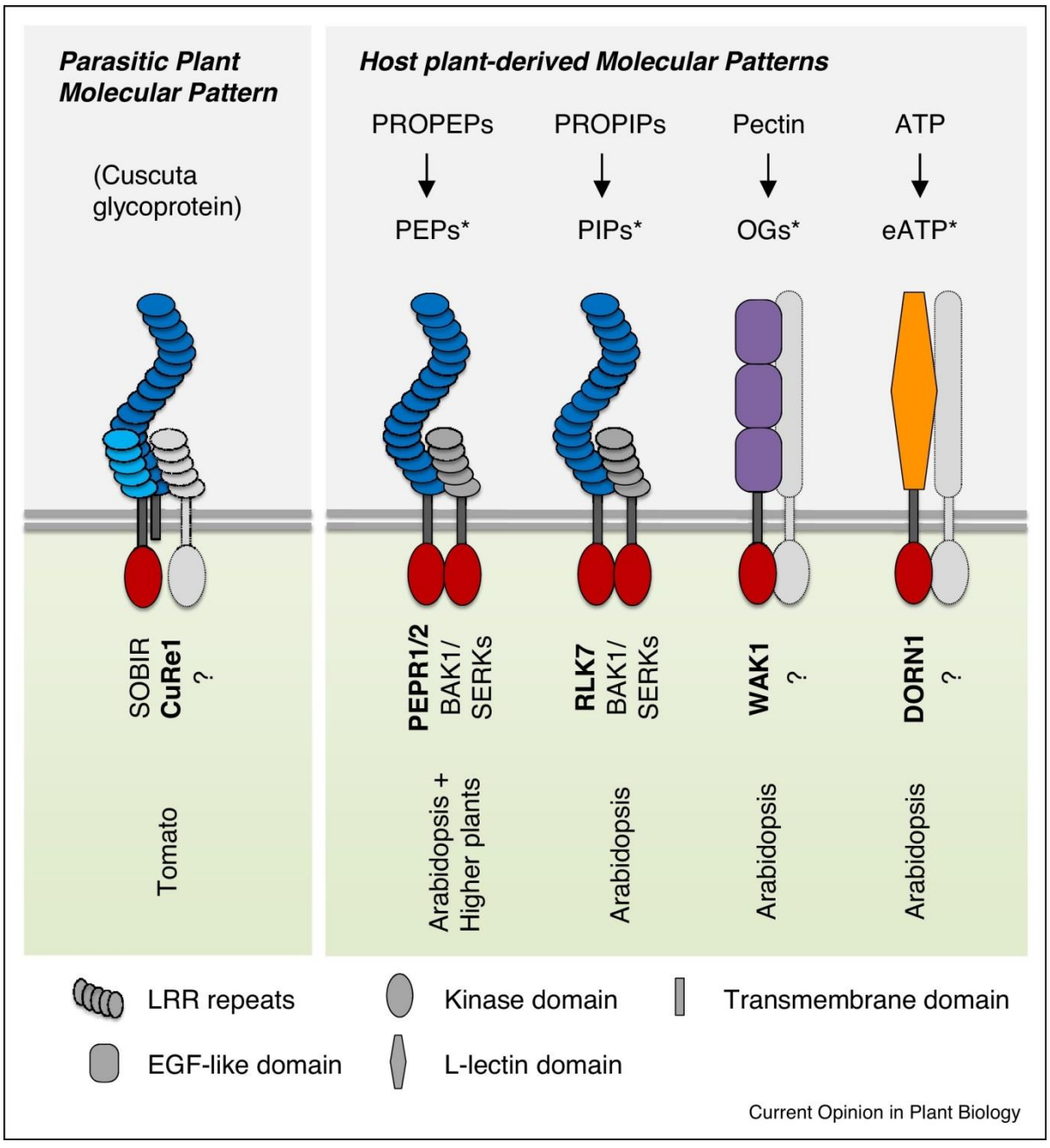

Figure 7. **Plant PRRs recognize non-self molecular patterns from parasitic plants as well as host-derived self molecular patterns**. The tomato LRR-RLP CuRe1 recognizes a yet unidentified glycoprotein from the parasitic *Cuscuta spp* and dimerizes with tomato SOBIR homologs and presumably SERK-like RLKs upon ligand recognition. LRR-RLKs PEPR1/PEPR2 and RLK7 PEPR1/PEPR2 and RLK7 associate with BAK1 and other SERKs to perceive endogenous PEP and PIP peptides derived from PROPEP and PROPIP

precursors, respectively. Pectin-derived OGs and extracellular ATP are sensed by RLK WAK1 and L-lectin-RLK DORN1, respectively. Star (*) indicates existence of proof of direct PRR-ligand binding. (Ranf *et al*., 2017).

Plants have to defend themselves to survive as well as reproduce to grow at the same time. Therefore understanding the regulatory mechanisms used by plants to balance growth and defense can improve plant breeding and engineering strategies for selection of the ideal genetic traits required to make the plants thrive. Growth-defense trade-offs mainly involves growth hormones such as auxin, Brassinosteroids (BRs), Gibberellins (GAs), and cytokinins. The best example depicting the molecular components involved in balancing growth is mainly observed in the relationship between FLS2 and the growth related BRASSINOSTEROID INSENSITIVE 1 (BRI1). BAK1 associates with both FLS2 and BRI1 receptors. It is thus believed that their competition for BAK1 incurs BR-mediated suppression of PTI defense (Belkhadir *et al*., 2012; Albrecht *et al*., 2012; Lozano-Durán *et al*., 2013). BRs are polyhydroxylated steroid phytohormones involved in various plant developmental processes like germination and senescence. BR stabilizes the growth receptor BRI1/BAK1 co-receptor complex, causing activation of their kinase domains (Li and Chory, 1997; Li *et al*., 2002; Nam and Li, 2002; Wang and Chory, 2006; Hothorn *et al*., 2011; She *et al*., 2011). The resulting phosphorylation events leads to inactivation of the glycogen-synthase-kinase-3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) kinase and thus activation of transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), to promote the expression of BR-regulated genes (Mora-Garcia *et al*., 2004; Tang *et al*., 2011). Upon PAMP recognition, BAK1 de-associates from BRI1 and forms a complex with FLS2, the transphosphorylation events that follow allows BIN2 to phosphorylates BES1and BZR1 thus blocking activation of BR-responsive genes and consequently growth inhibition (He *et al*., 2002; Wang *et al*., 2002; Yin *et al*., 2002; He *et al*., 2005; Yin *et al*., 2005; Vert and Chory, 2006). This in turn induces PTI responses. BZR1 transcription factor has also been linked in WRKY40-regulated genes as well as the cell

elongation related transcription factor HBI1. However, the mechanism involved in PTI suppression in favour of growth is not yet clear (Bai *et al*., 2012; Lozano-Durán *et al*., 2013). BR and GA mediated growth-defense cross talk functions in synergy to promote growth in response to environmental and developmental signals (Jaillais and Vert, 2012; Lilley *et al*., 2013). In presence of GAs, there is formation of a heterodimer of BZR1 and Phytochrome Interacting Factor 4 (PIF4) which binds to and activates promoters of growth related genes. Without bioactive GAs, A family of transcriptional repressors known as DELLA proteins binds and inactivates PIFs mediating defense (De Lucas *et al*., 2008; Bai *et al*., 2012; Gallego-Bartolome *et al*., 2012; Oh *et al*., 2012). Pathogen infection also activates auxin pathway including promotion of auxin biosynthesis genes and repression of AUX/ indole-3-acetic acid (IAA) genes and as such infection is enhanced (O'Donnell *et al*., 2003; Thilmony *et al*., 2006). This is achieved by heterodimerization of the AUX/IAA proteins with the AUXIN RESPONSIVE FACTORS (ARF) transcription factors. To survive the effects of pathogen induced or pathogen produced auxin, plants suppress auxin signalling during defense by inhibiting auxin F-box receptors that stabilizes AUX/IAA proteins and represses of auxin synthesis genes (Navarro *et al*., 2006). This suppression is partly due to microRNA miR393 which when induced by for example flg22, directly cleaves Transport Inhibitor Response 1 (TIR1) and two functional paralogs Auxin signalling F-Box proteins 2 and 3 (AFB2 and AFB3) transcripts (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Navarro *et al*., 2006). SA induced growth inhibition has been associated with suppression of auxin reception, import and export and signalling. GH3 enzymes; responsible for regulating auxin homeostasis by conjugating IAA with different amino acids are also induced by SA (Wang *et al.,* 2006, 2007). JA at the same time suppresses the expression of the auxin efflux carrier PIN formed 2 (PIN2) as well as it endocytosis and membrane accumulation (Sun *et al*., 2011). Lastly, resource allocation involved in growth-defense balancing is reported to occur at all levels, in prioritizing of carbon and nitrogen pools towards production of defense compounds. Some of them involved in protein folding and secretions gearing towards defense for example PR proteins are proposed to be regulated by TL1 BINDING

TRANSCRIPTION FACTOR 1 (TBF1) during PTI and SAR (Pajerowska-Mukhtar *et al*., 2012, Huot *et al*., 2015).

Although the overall importance of PAMP-triggered immunity for plant defense is established, it has not been used commercially to produce transgenic disease free lines. Nevertheless, there are reports of successfully transferring PRRs between two plant families and retaining their activity. A gene encoding a PAMP receptor does not introduce a novel defense mechanism into the plant. The transferred PAMP receptor merely allows the receiving plant to recognize infection, so it can respond with its own, natural immune system. For example, expression of EFR, a PRR from *Arabidopsis thaliana*, confers responsiveness to bacterial EF Tu in the solanaceous plants *Nicotiana benthamiana* and *Solanum lycopersicum*, making them more resistant to a range of phytopathogenic bacteria of different genera. This strategy can be utilized to confer more resistance to nematodes taking advantage of the natural innate immunity in the plant.

1.2.2 Plant defense against nematodes

For a long time, PTI induced by nematodes had remained *terra incognita* to nematology community. However, recent studies have sort to shed some light in the ability of plant to recognize nematodes (Fig 8). For example, beneficial and entomopathogenic nematodes such as *Steinernema carpocapsae* has been reported to induce resistance in Arabidopsis and *Hosta spp* characterized by increased catalase and peroxidase as well as overexpression of PR genes (Jagdale *et al*., 2009). In addition, some reports suggest that cell wall degrading enzymes produced by nematodes could cause PTI like responses induced by DAMPs. In addition, other reports have indicated that Root-knot nematodes cause apoplastic and cell membrane localized ROS production during early stages of invasion i.e. penetration and migration in tomato roots. This ROS burst was shown to be derived from the cell membrane localized RBOHD and RBOHF that are directly phosphorylated by BIK1 during PTI (Wojtaszek *et al*., 1997; Torres *et al*., 2002; Torres 2009;

Kadota *et al*., 2015). BIK1 on the other hand has been reported to be a positive regulator of root knot nematode induced PTI responses. In general and regardless of the components involved, reports have it that PTI due to root knot nematode could be dependent or independent of co receptor BAK1 and is linked to Camalexin and glucosinolates (Teixeira *et al*., 2016). All in all, a nematode derived compound which induces clearly recognizable and immediate effects as PTI responses had not been identified till recently when a group of defense signalling molecules present in a wide array of nematodes were characterized as PAMPs (Manosalva *et al.*, 2016). Ascarosides as they are referred are a family of small endogenous molecules whose key functions are upstream of conserved signalling pathways in developmental timing and behaviour in nematodes. For example acarosides acts as pheromones. The term “ascarosides was first coined when a specific type of lipid was detected for the first time in roundworms from family Ascaridia (Ludewig and Schroeder, 2013). They have been further described as glycosides derived from a dideoxysugar ascarylose linked to a fatty acid side chain. Specifically, they are also described as integrating building blocks from carbohydrate metabolism, peroxisomal β-oxidation of fatty acids and amino acid catabolism (von Reuss *et al*., 2012). Prior to the establishment of a reliable ascarosides naming system, compounds were named based simply by the length of their side chain for example a 10 carbon ascaroside was named C10 which of course led to confusion once more compounds having the same number of carbon molecules were identified. Fortunately, a new, easy to search, gene identifier naming system has been developed; Small Molecule IDentifiers (SMIDs), which consist of lower case letter depicting the general structure of the compound class, followed by the compound sign and ends with a number. For example icas#7 or hbas#10. The SMID database is publicly available for all small molecules identified from nematodes especially *C. elegans* (Srinivasan *et al*., 2012; von Reuss *et al*., 2012). In the study, among the 200 ascaroside molecules identified to date, ascr#18 was found to be the most abundant among cysts, root-knot and lesion nematodes. Ascr#18 induced PTI responses at very low concentrations (10 nM) in Arabidopsis just like responses caused by other PAMPs such as triggering SA and JA signalling pathways,

expression of PR related defense genes and increased resistance to other pathogens. These responses were also observed when other dicotyledonous and monocotyledonous plants such as tomato, barley and potato were treated with ascarosides at varying concentrations. In addition, Ascr#18 applied to the root not only induced defense genes in the roots but also resistance in the leaves, a phenomeno suggested to be caused by its translocation to the leaves, or the induction of a mobile signal that was translocated to the leaves. This remain to be proven since there is no reports of ascr#18 detected in the leaves so far. Nevertheless, the ability of Ascr#18 to induce PTI responses lead to the idea of a receptor capable of perceiving ascarosides both in roots and in shoot, and which is yet to be identified (Manoslava *et al*., 2016).

PTI is very important for plant survival. Therefore, plant parasitic nematodes, whether sedentary endo parasitic which have a prolonged interaction with the plants, or migratory ectoparasitic who have a very short interaction; require a PTI suppression mechanism for them to thrive in presence of defense responses and maintain feeding structures. Most successful biotrophs deliver effectors that inhibit PTI or PTI response. Comparative genomics approaches have allowed identification of these effectors. Root-knot nematode genomes are now available; for *M. incognita* and *M. hapla*, as well as cyst nematode *H. glycines* and *G. pallida*. Through prediction of effectors using these available data, and the confirmation of their expression in esophageal glands via In situ hybridization, effectors are believed to be secreted into the host through the stylet. Identification of effectors is important since deciphering their functions gives us an insight into their role in host manipulation (Abad *et al*., 2008; Opperman *et al*., 2008; Haegeman *et al*., 2012; Hewezi and Baum, 2013). Most characterized effectors specifically bind to or mimic plant proteins affecting hormonal balance, signalling and cell morphogenesis. Calreticulins (CRTs) are the only nematode PTI suppressors known that react directly due to a PAMP like elf18. CRTs are highly conserved calcium binding proteins present in both plants and animals and acts as Ca^{2+} binding chaperones, regulating Ca^{2+} storage and signalling. CRTs are located near nuclear

envelope, in the cytoplasm, or at the cell surface. They regulate numerous cell functions such as gene expression, cell adhesion and immunity regulation indirectly via calcium binding and or directly interacting with signalling proteins (Gold *et al*. 2010; Michalak *et al*., 2009). The calreticulin (CRT) Mi-CRT secreted from *M. incognita* is produced from the subventral glands of preparasitic J2 and in the dorsal gland of parasitic stages. It is secreted into plant via the stylet and accumulates at the cell wall of giant cells. It has been linked to suppressing normal elf18-induced callose deposition in Arabidopsis and reduced activation of defence-related genes (Jaubert *et al*., 2005; Jaouannet *et al*., 2012, 2013). That notwithstanding, the type III effector protein from *Pseudomonas syringae* AvrPtoB has also been shown to suppress PTI by the ubiquitination of FLS2 and the co-receptor Chitin elicitor receptor kinase 1 (CERK1) (Goehre *et al*., 2008; Gimenez-Ibanez *et al*., 2009). In addition, *Phytophthora infestans* effector AVR3a targets the host E3 ligase CMPG1 important for the downstream signal transduction pathway induced by INF1. This reveals the possibility that plant-parasitic nematodes could equally target the ubiquitination pathway as some of their effectors are similar to E3 (Gao *et al*., 2003; Bos *et al*., 2010). *H. schachtii* effector HS19C07 interacts with Arabidopsis UXIN INFLUX Transporter LAX3 resulting in reduced auxin transport in the syncytium and thus interfere with its development (Lee *et al*., 2011; Wang *et al*., 2011). Furthermore, sedentary endoparasitic nematodes secrete plant chorismate mutase homologs that are similarly suggested to affect auxins pool and root growth as well as the affecting the shikimate pathway, resulting in decreased SA and phytoalexin biosynthesis (Doyle and Lambert, 2003; Jones *et al*., 2003; Huang *et al*., 2005; Grunewald *et al*., 2009). *M. incognita* effector Mi8D05 interacts with a plant aquaporin Tonoplast Intrinsic Protein (TIP2) affecting nutrients and solutes transport that in turn interfering GC enlargement and nematode feeding (Xue *et al*., 2013). On the other hand, *H. schachtii* effector Hs10A06 targets spermidine synthase 2 to alters SA signalling and protects the nematodes from ROS and PR proteins in Arabidopsis (Hewezi *et al*., 2010, 2015). Suppression of PTI by diverse pathogens in a single host suggests that different pathogens

may target a small number of host components generally involved in, or that regulates plant defence responses.

Nevertheless, Effectors equally triggers ETI that counters the effects of PTI suppression and unlike nematode induced PTI; ETI triggered by nematodes is better understood. R genes proteins especially NB-LRR proteins that allow resistance to nematodes have been identified. However, very few nematode Avr proteins have been identified (Smant and Jones, 2011).

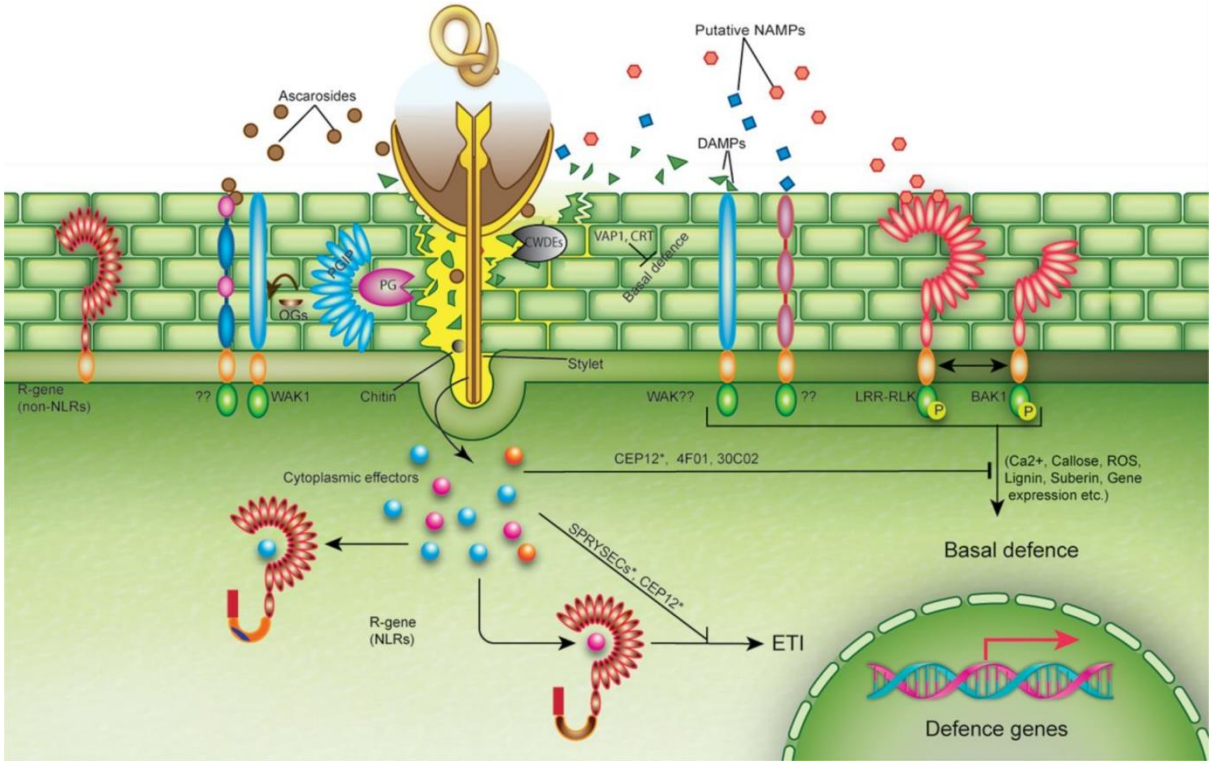

Figure 8. **Immune responses during plant-nematode interaction**. Nematode invasion causes cell wall damage, which consequently release damage-associated molecular patterns (DAMPs) and activates plant basal defence responses by Wall-Associated Kinases (WAKs). Nematodes secrete cell wall degrading polygalacturonases (PG) which interacts with plant PG-inhibiting proteins (PGIPs), to form small cell wall oligogalacturonides (OGs) that induces DAMP-associated immunity. Nematode Associated Molecular Patterns (NAMPs) such as ascarisides are perceived by unknown plasma-membrane localized pattern recognition receptors (PRRs) to induce Pattern Triggered Immunity (PTI)

characterized by Reactive Oxygen Species (ROS), callose, and lignin production. BRI1 Associated receptor-like Kinase 1 (BAK1) act as a co-receptor to unidentified LRR-RLK to initiate PTI. Nematodes, consequently, secrete apoplastic VAP1, CRT, CEP12, 4F01, 30C02 and SPRYSECs effectors to counter the immunity. Plants R-genes encoding Nucleotide binding Leucine rich Repeat, (NLRs) recognizes effectors to initiate Effector Triggered Immunity (ETI). In addition to NLRs, non-NLRs type R-genes also exists against nematodes. Star (*); indicates existence of proof of nuclear localization of effectors. (Holbein *et al*., 2016)

Similar to ETI caused by other biotrophic pathogens, Nematode effectors can induce HR which is targeted at the feeding structure or the cells around it for example in the case of responses induced by Mi-1 and Hero A in tomato to root knot and potato cyst nematodes respectively (Rossi *et al.,* 1998; Milligan *et al*., 1998; Sobczak *et al*., 2005). However, R genes incurring resistance for example, resistance by Rhg4 gene in soybean against H. glycines is due to a serine hydroxymethyl transferase (Liu *et al*., 2012). Several Cf genes (for resistance against *Cladosporium fulvum*; the leaf mold fungus), such as Cf-2, Cf-4, Cf-5 and Cf-9, have been suggested to encode extracellular receptors that perceive elicitor molecules secreted by the fungus (De Wit, 1992). Several Cf genes have been cloned and found to encode proteins with LRRs, which may function as extracellular, membrane-bound receptors (Thomas *et al*., 1998). Particularly, the extracellular plant immune receptor protein Cf-2 of the red currant tomato *Solanum pimpinellifolium*, was previously reported to confer resistance only to *C. fulvum*. Currently it is known that the root parasitic nematode *G. rostochiensis* also activate Cf-2-mediated disease resistance by perturbing the apoplastic papain-like cysteine protease Rcr3pim which is common component among the two pathogens. Apoplastic Rcr3pim is a molecular target of the Venom Allergen-like Protein (Gr-VAP1) of *G. rostochiensis* pathotype Ro1-Mierenbos juveniles, released during the early stages of nematode parasitism. However, how venom allergen-like protein Gr-VAP interacts with Rcr3pim of *S. pimpinellifolium*, in nematode virulence is not yet understood. VAPs constitute a monophyletic clade of cysteine-rich secretory proteins within the Sperm Coating

Protein/Tpx-1/Ag-5/Pr-1/Sc-7 (SCP/TAPS) superfamily. They are the most abundant released secretory proteins during infection by plant and animal parasitic nematodes (Lozano-Torres *et al*., 2012).

Just like in PTI, some proteins have been shown to supress nematode induced ETI. For example, at least one member of the SPRYSEC effectors family identified in potato cyst nematodes has been shown to suppress ETI in plants (Cotton *et al*., 2014). However, the mechanism behind SPRYSEC-19 mediated ETI suppression is still unknown. Other SPRYSECs from *G. pallida* also suppress ETI too. The RYSEC-19 effector from *G. rostochiensis* suppresses ETI induced by Gpa2 and the related Rx in the presence of the respective avirulence factor recognized by these R proteins (Postma *et al*., 2012; Mei *et al*., 2015).

Regardless of these known facts about plant interaction with nematodes, there is still more questions that remain to be answered. The significance of the economic importance of plant parasitic nematodes in agriculture is a driving force to try and better exploit genetics in crop improvement. That will require an understanding of plant defense and especially PTI which is still under-explored especially PTI activation, its induced signalling cascade and the components involved in its regualtion.

1.3 Arabidopsis thaliana

This plant is named after Johannes thalius; a physician from Nordhausen Thüringen in Germany. He discovered it in the Harz Mountains in 1577 and was the first to describe it, naming it *Pilosella siliquosa* (Thal's Gänsekresse). Carl Linnaeus (Carl von Linné) later in 1753 named the plant *Arabis thaliana* in honour of Thalius. In 1842, the German botanist Gustav Heynhold who worked in botanical gardens in Dresden and Frankfurt placed it in the newly erected genus Arabidopsis (Greek for “Like Arabis”). Thal cress or mouse-ear cress as is commonly known is a small flowering plant and a member of the mustard family (family

Brassicaceae) , with a wide natural distribution throughout Eurasia. Even though in 1873, Alexander Braun described a double flower phenotype when he reported the first mutant, it was Erna Reinholz who undertook her PhD work in Prof. Friedrich Laibach lab in Frankfurt, who isolated the first induced Arabidopsis mutants. All in all, it was Laibach; a German botanist and founder of the experimental Arabidopsis research, who drove attention on the plant since the 1930s. He particularly proposed the potential of *Arabidopsis thaliana* as a model organism for genetics. The growth of Arabidopsis research has over the last 40 years been remarkable, rewarding, and transformative. Even though Arabidopsis has been found to have no direct importance to agriculture, it has many characteristics which make it a usable model and a reference point while deducing genetics, cellular, and molecular biology of flowering plants. Important features include a short generation time where the whole life cycle starting from seed germination to maturation of the first seeds takes 6 weeks. It is small rosette plants that range from 2 to 10 cm in diameter and 20 to 25 cm tall. Flowers are 2 mm long and self-pollinates and as the bud opens, crossing can be achieved by releasing pollen on the surface of stigma. Mature seeds are 0.5 mm long and are produced in thin fruits called siliques; 5 to 20 mm long. A silique contains 20 to 30 seeds. This prolific seed production through self-pollination ensures easy reproduction and production of lots of seeds. About 750 different ecotypes have been obtained from natural populations for experimental purposes. However; the Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular studies (Greilhuber *et al*., 2006; Koornneef and Meinke, 2009). Arabidopsis has its whole genome sequenced which is relatively small (114.5 Mb/125 Mb total). Arabidopsis genome has an extensive genetic and physical map on its 5 chromosomes. The Arabidopsis Information Resource (TAIR) preserves and updates a database of genetic and molecular biology data for *A. thaliana* including the complete genome sequence along with gene structure, expression and protein information. In addition, it provides the gene DNA sequence, seed stocks, genome maps, physical markers, genetic markers and related publications. The function of the gene is updated as research articles are released. Efficient transformation methods utilizing Agrobacterium tumefaciens makes

Arabidopsis very easy to genetically manipulate and thus easy to study. A huge genomic resources and mutants lines are a readily available from Stock Centres. For example, the Arabidopsis Biological Resource Center (ABRC) at the Ohio State University, USA and Nottigham Stock Centre (NASC) at the University of Nottingham's Sutton Bonington Campus, in the English county of Nottinghamshire. Arabidopsis has been described as a host of various pathogens and as such it has been utilized for studies in plant pathology. Plants in an entire species could confer resistance to all isolates of a microbial species for example nematodes and they are as such referred to as non-host or species resistance. The breakthrough in establishing a plant nematodes interaction model was achieved when culture conditions for successful infection and development of nematodes was established for cyst nematodes *H. schachtii*, *H. trifolii*, and *H. cajani*, root-knot nematodes *M. incognita* and *M. arenaria* as well as migratory nematode *P. penetrans* on Arabidopsis (Sijmons *et al*., 1991). Currently, this model plant is used in plant nematode interaction studies, majoring in *H. schachtii* and *M. incognita* as the target pathogens.

Figure 9. **An illustration of different growth and development stages in *Arabidospsis thaliana***.

1.4 Objectives

Many reports have clearly demonstrated that nematode like other pathogens can trigger PTI responses. However, the PRRS involved in nematode PTI remain elusive. In our study, we focused on:

- Characterization of receptor genes upregulated due to nematodes.
- Identification of putative receptors that recognizes conserved molecular signature from nematodes.
- Deducing the signalling components involved in induction of PTI due to nematodes.

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PLOS | PATHOGENS

RESEARCH ARTICLE

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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

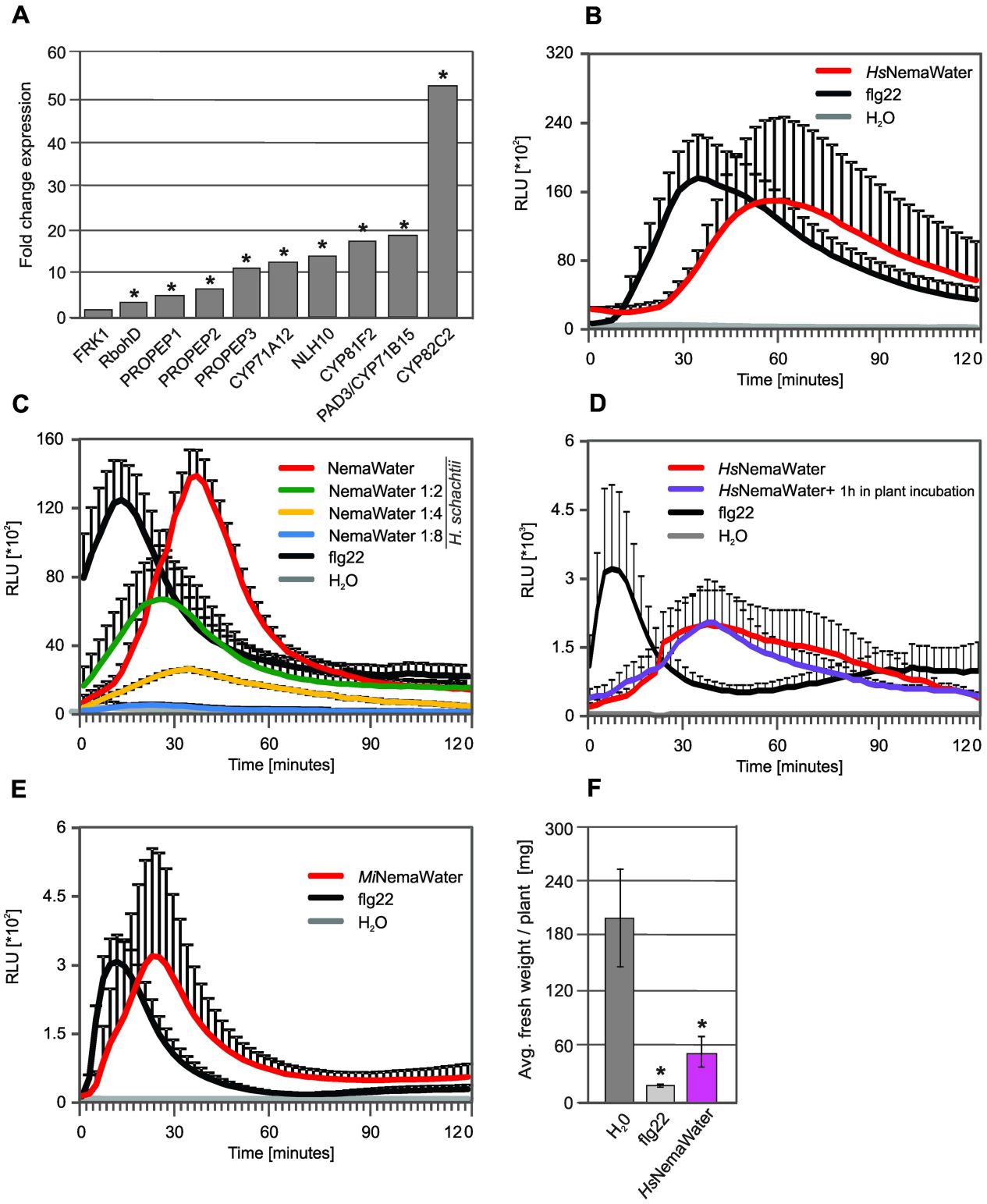

Fig 1. Nematode infection induced defense responses in plants that are characteristics of PTI. (A) Expression of PTI marker genes in microarray analysis upon nematode infection in migratory stage. Root segments from uninfected roots were used as control. Values indicate fold change compared with control. 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*.

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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).

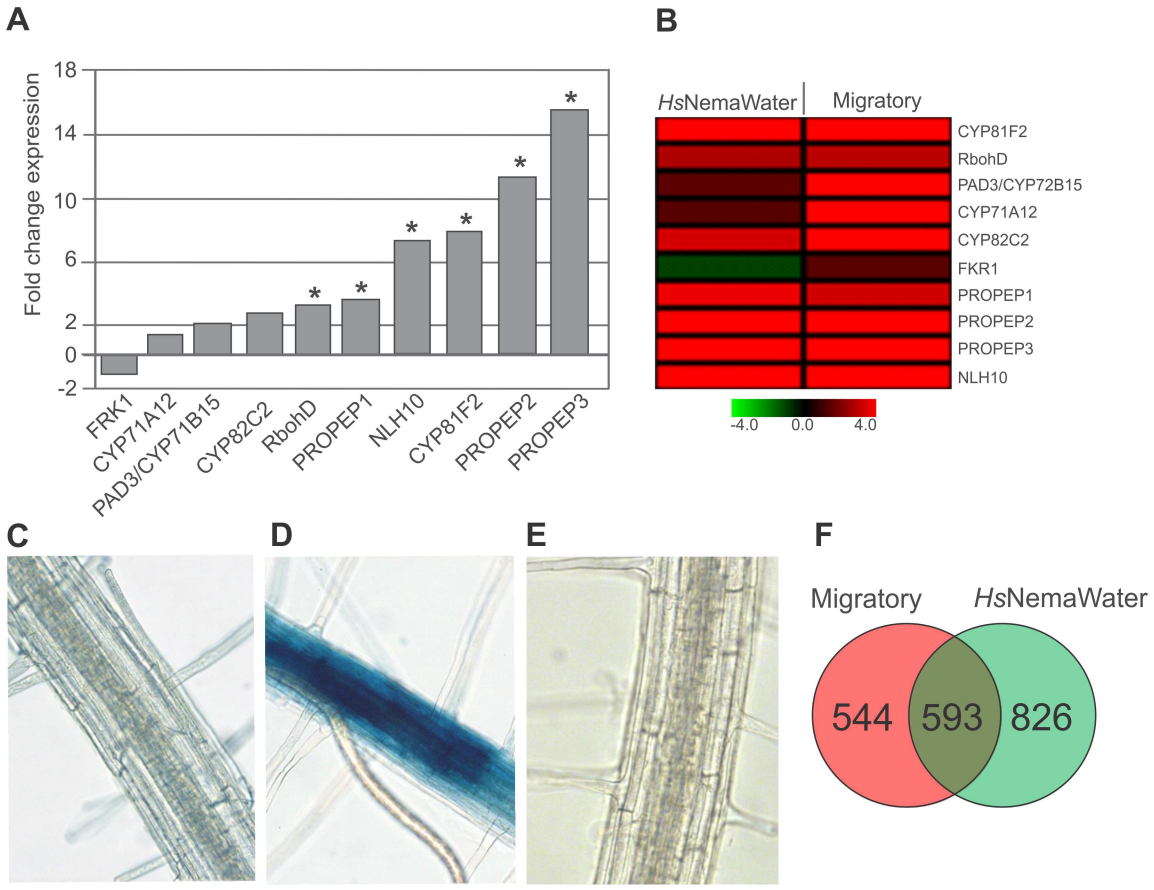

Fig 2. NemaWater treatment induced defense responses in plants that are characteristics of PTI. (A) Expression of PTI marker genes in microarray analysis upon *Hs*NemaWater treatment. Root segments from uninfected roots were used as control. Asterisk indicates significant difference to control *(FDR* <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.

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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

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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).

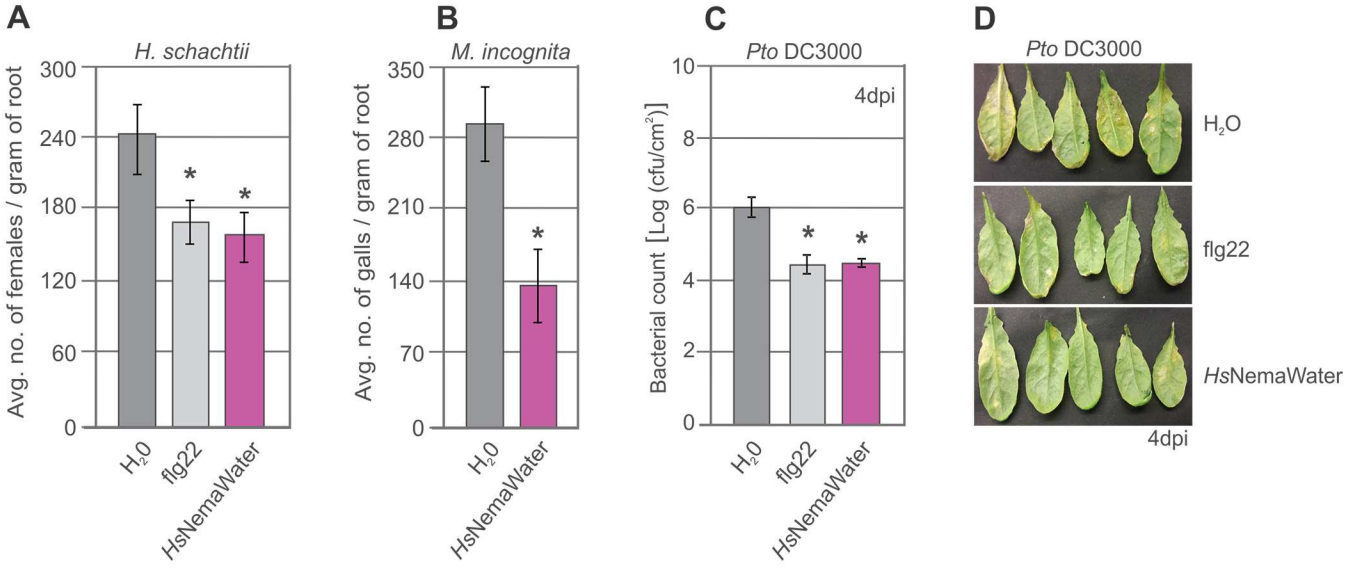

Fig 3. Pre-treatment with NemaWater induces resistance to pathogens. (A-B) Roots of Col-0 plants were treated with water or *Hs*NemaWater prior to infection and number of females were counted at 14 dai for cyst nematodes and number of galls were counted at 19 dai for root-knot nematodes. Bars represent 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).

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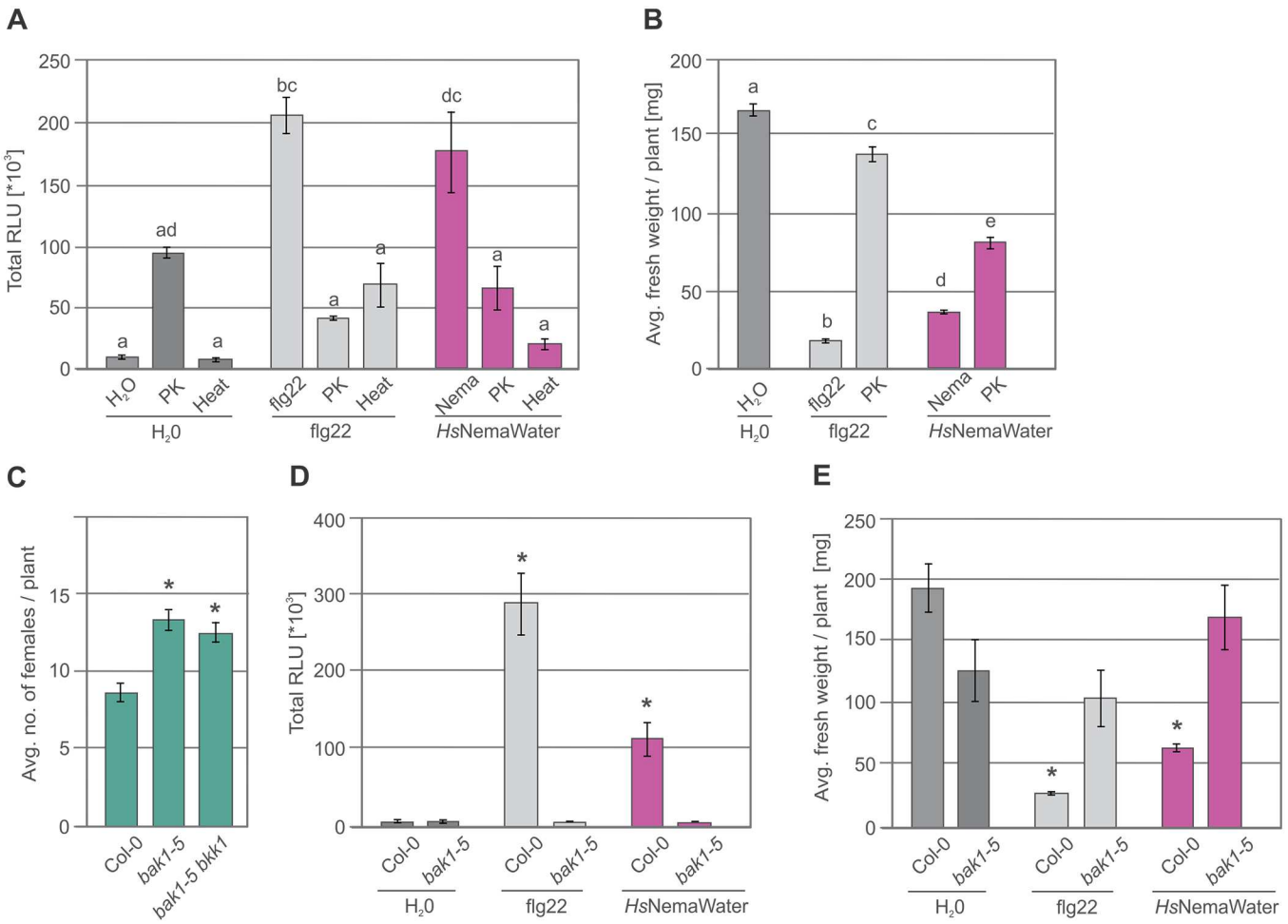

Fig 4. NemaWater treatment induced PTI responses were reduced strongly upon proteinase K, heat treatment, and in *bak1-5* plants. (**A**) Effect of Proteinase K and heat on production of ROS burst in root segments from Col-0 plants treated with water, *Hs*NemaWater or flg22. ROS burst was measured by 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).

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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

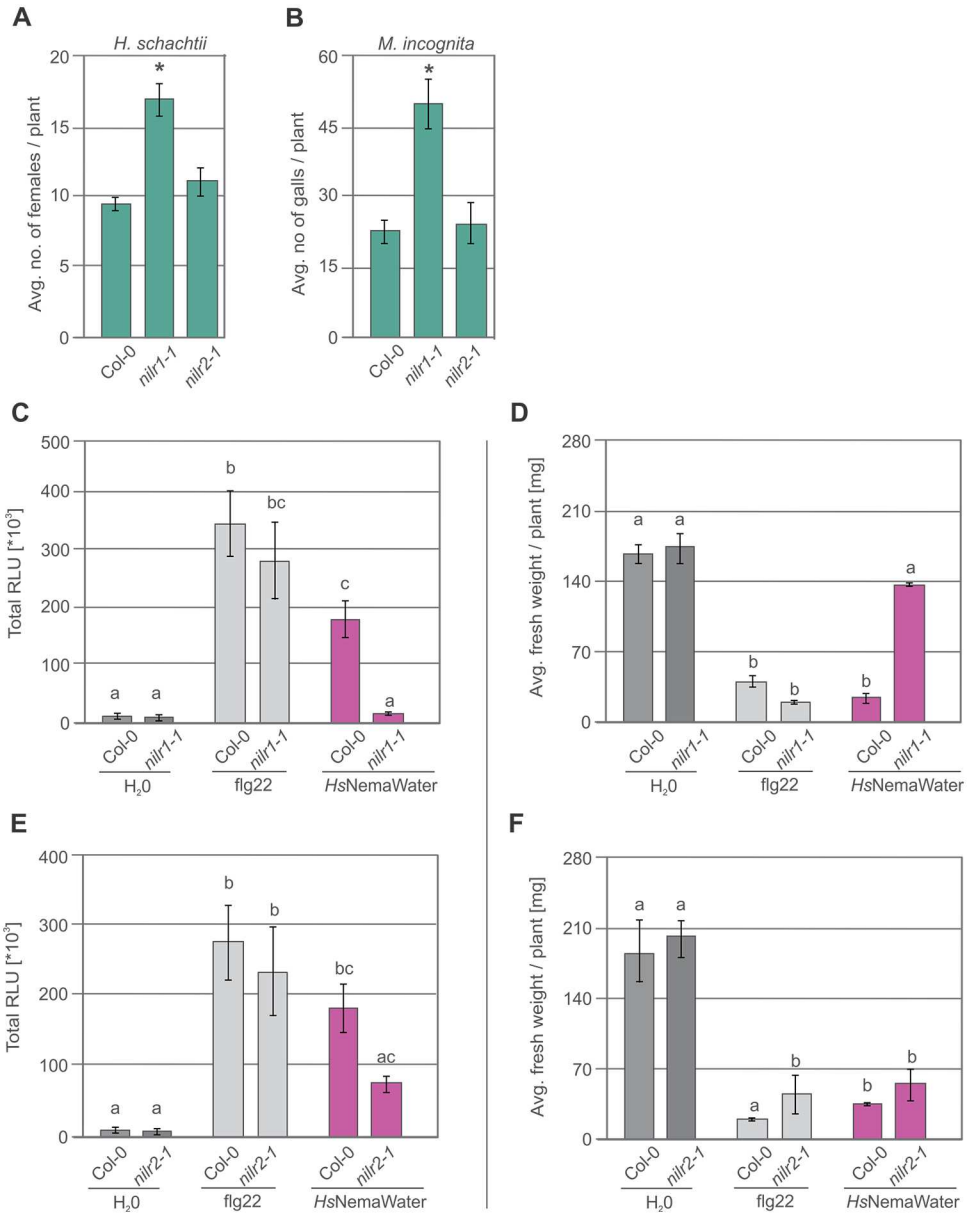

Fig 5. Knock-out *nilr1-1* enhances susceptibility to nematodes. (**A**) Average number of female nematodes induced by *H. schachtii* per plant in Col-0, *nilr1-1* and *nilr2-1*. Bars represent mean ± SE for three biological replicates. (**B**) Average number of galls induced *by M. incognita* per plants 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.

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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

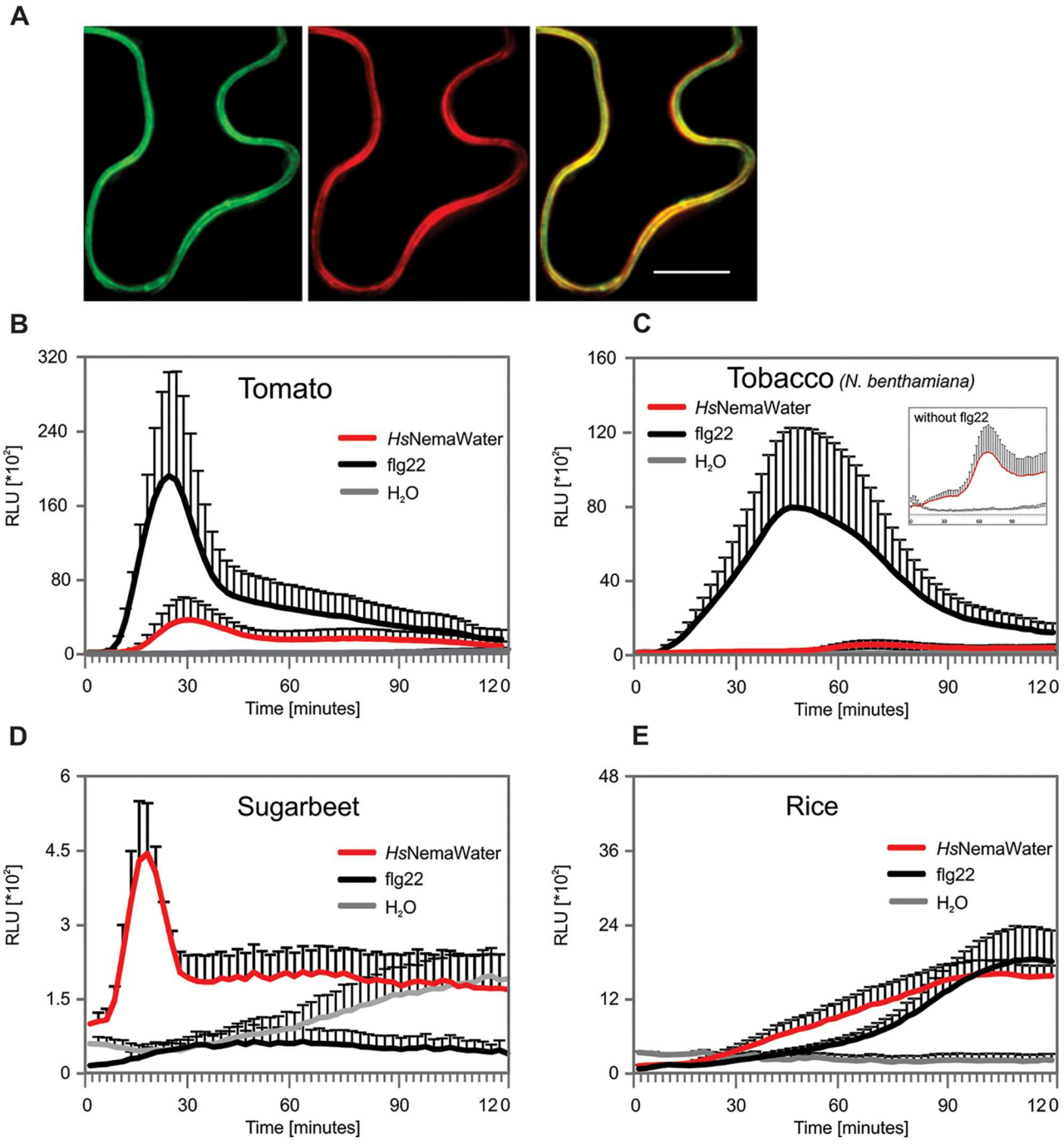

Fig 6. NILR1 is localised in plasma membrane. (A) Confocal microscopy of epidermis of *Nicotianna benthamiana* transiently expressing *35S:NILR1-GFP* and plasma membrane marker *35S:PIP2A-mCherry*. Scale, 50 μm. (B-E) Leaf discs from tomato (B), *N. benthamiana* (C), sugarbeet (D) and rice plants were 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.

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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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Identification of putative plant receptors involved in PTI against plant parasitic nematodes

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Abstract

Plant parasitic nematodes are of great economic importance for they cause massive losses in agriculture. A credible solution to their control begins by understanding how plants defend themselves against the pathogen. The first line of plant defense is induced by recognition of Pathogen Associated Molecular Patterns (PAMPs) by Plant Recognition Receptors (PRRs). We previously reported the first receptor that recognized nematodes; NILR1. However, considering the large number of receptor genes that were reported to be up regulated due to nematodes, we sort to explore further to identify more nematode receptors. Here we report four genes; CRK10 (At4g23180), CRK19 (At4g23270), NILR3 (At1g53440) and NEK5 (At3g20860), essential in PAMP triggered immunity against sedentary plant parasitic nematodes as their loss of function mutants were hyper susceptible.

Introduction

Plant parasitic nematodes are a major cause of food insecurity worldwide (Nicol *et al*., 2011). The most economically significant species belong to the cyst and root-knot nematode classes. They infect a majority of plants belonging to family brassicaceae and cause disease symptoms in the plant characteristic to those of malfunctioning root system like stunted growth, galling or formation of cysts in the roots and excessive formation of lateral roots. Above the ground, there is stunted development of leaves, foliage yellowing and wilting (Jones *et al*., 2013). Management of these nematodes has for a long time involved utilization of chemical based nematicides, which are not only expensive and harmful to the environment but also nematodes acquired resistance against them (Morris *et al*., 2016). The most effective and efficient control strategy is believed to be the production of resistant varieties. To achieve this, understanding plant nematode interaction and especially plant defense is inevitable. Plant defense exist as innate immunity and Effector Triggered Immunity (ETI) where the latter is induced by effectors secreted into the cell by the pathogen once it overcomes the innate immunity. While ETI is widely studied against nematodes, very little is known about nematode-induced innate immunity. As the first level of defense before the pathogen enters the plant cell, innate immunity is generally characterized by Plant Recognition Receptors (PRRs), which perceives conserved molecular signatures commonly known as Pathogen Associated Molecular Patterns (PAMPs). PAMP-triggered Immunity (PTI) has been well studied in all other patho-systems and it involves a series of cellular events including Reactive Oxygen Species (ROS) and calcium bursts, activation of Mitogen Associated protein Kinases (MAPKs), and callose deposition in the cell wall among others (Jones and Dangl, 2006). It is not until recently when the first nematode molecular pattern was identified as ascarosides (Manosalva *et al*., 2016). This family of small-molecule pheromones is characteristic of the whole Nematoda phylum, however, the receptor recognizing ascarosides is yet to be identified. In addition, we recently reported a receptor that is involved in recognition of nematodes; the membrane-localized NILR1 as described in

chapter 2 is a receptor like kinase belonging to the LRR super family. Lack of NILR in Arabidopsis rendered the plant susceptible to nematode infection. However, the PAMP whose recognition is mediated by NILR1 remains unknown. These discoveries, however, have shed new light on nematode-induced PTI and have paved the way for further studies. Nevertheless and regardless of these discoveries, previously reported transcriptomic data at nematode migratory stage and due to a nematode diffusate; NemaWater, revealed a huge number of upregulated genes involved in various biological processes such as response to stimulus, death and immune system response (Mendy *et al*., 2017). Among these genes, 51 belong to the Receptor-Like Kinase (RLK) family suggesting that there are multiple receptors involved in nematode induced PTI. Here we analysed the role of 25 of these genes in nematode infection that had not been previously studied. Expectedly, a number of genes belonged to Leucine-Rich Repeat (LRR) family similarly to NILR1. Another family heavily implicated was Cysteine-rich Receptor-like Kinases (CRKs) family formed by the Domain of Unknown Function 26 (DUF26) RLKs, which have been suggested to play important roles in the regulation of pathogen defence and programmed cell death. In addition, NIMA rElated Kinases (NEKs) implicated in cell cycle control showed potential involvement in nematode induced defense.

Results

CRKs and NILR3 play a role in immunity against nematodes.

Among the 51 RLKs genes upregulated in two microarray data previously reported (Mendy *et al*., 2017), we selected 25 genes (Table 1) whose role in nematode infection was unknown. Loss-of-function mutants were obtained and screened for infection against *H. schachtii*. Infection was demonstrated by deducing the average number of females per plant in percentage to Col-0 at 100%. Among the 25 mutants tested, there were 2 mutants of CRK genes; CRK10 (At4g23180) and CRK19 (At4g23270). *Crk19 (At4g23270)* was significantly susceptible to infection by *H.schachtii* compared to wild type Col0 (Fig. 1). On the contrary to

Crk19 (At4g23270), *crk10 (At4g23180)* showed no difference with Col-0 when infected with *H. schachtii*.

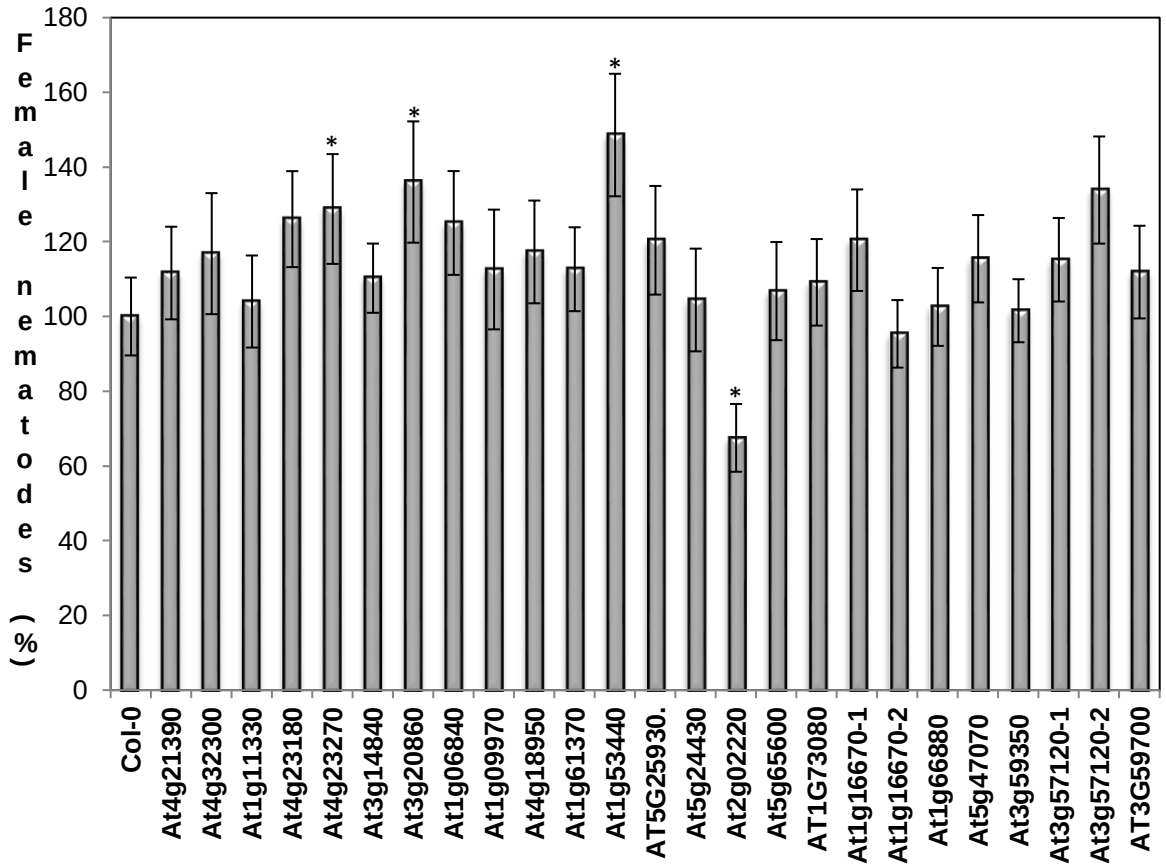

Figure 1. **The average number in percentage of female *H. schachtii* nematodes per plant in Col-0 and 25 RLKs TDNA-insertion lines.** Bars represent mean ± SE for three biological replicates.

To identify their involvement in nematode perception besides that of cyst nematode, we analysed *CRK10* (*At4g23180*) and *CRK19* (*At4g23270*) mutants for infection with root-knot nematode *M. incognita*. Interestingly, our data showed no difference in susceptibility of *crk19* (*At4g23270*) to infection in comparison to wild type Col-0 (Fig. 2). The opposite was true for *crk10 (At4g23180)* which was significantly hyper susceptible to infection with *M. incognita* compared to Col-0. Similary to *crk19 (At4g23270),* One mutant of an LRR gene; *nilr3* (Nematode Induced LRR-RLK 3) *At1g53440*)), was only significantly hyper susceptible to infection by *H. schachtii* and not to *M. incognita* compared to Col-0 (Fig 1 & 2).

NEK5 role in PTI is conserved within sedentary plant parasitic nematodes

Among the 25 mutants tested for susceptibility to both *H. schachtii* and *M. incognita*, one was a loss of function mutant of NIMA-related kinase 5 (NEK5). In contrast to CRK mutants, *nek5 (At3g20860)* was significantly hyper susceptible to infection by both *H. schachtii* and *M. incognita* compared to wild type Col-0 (Fig 1 & 2).

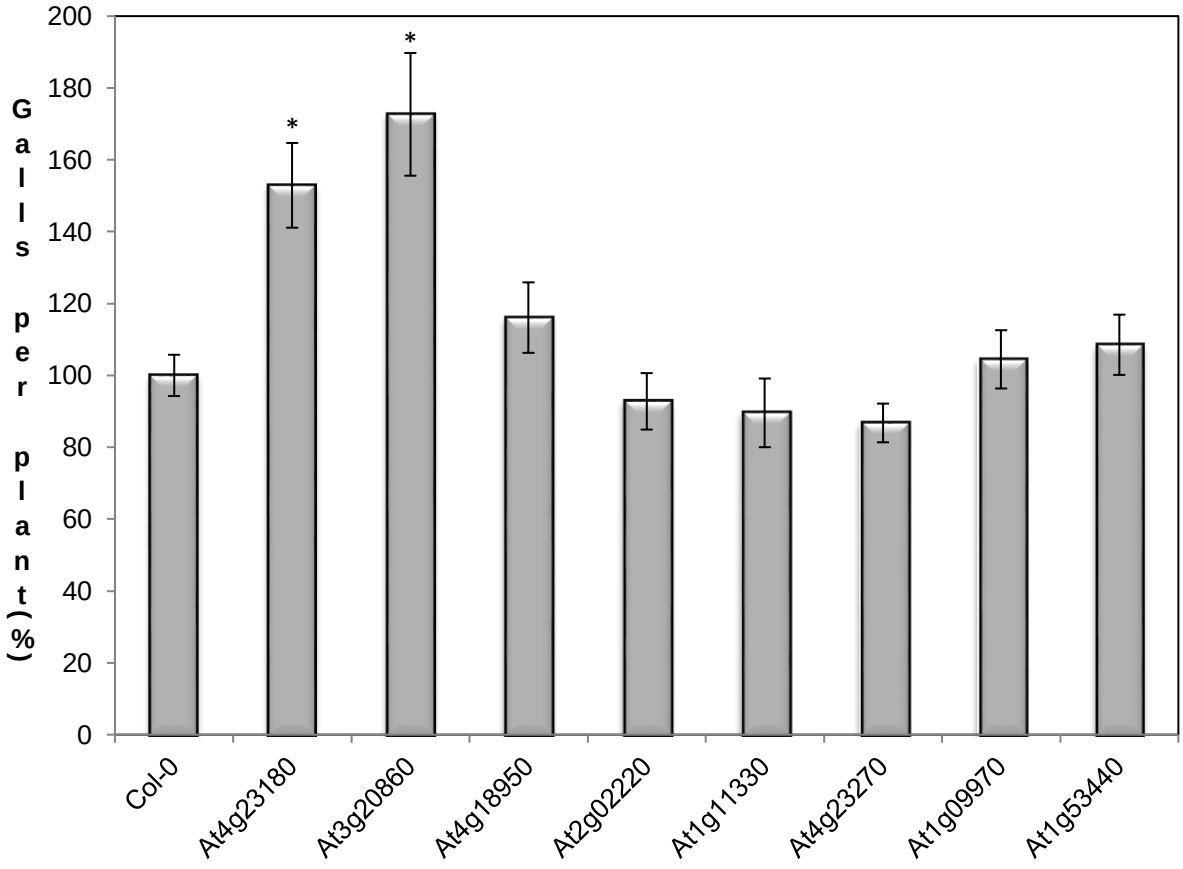

Figure 2. **The average number in percentage of galls induced by *M. incognita* per plant in Col-0 and 8 RLKs TDNA-insertion lines**. Bars represent mean ± SE for three biological replicates.

PSKR1 acts negatively to PTI during infection by cyst nematode *H. schachtii*.

Out of the 25 mutants tested, only 4 described above were significantly hypersusceptible to infection by either or both *H. schachtii* and *M. incognita*. Although transcriptome level of one of the LRR receptor gene was upregulated; Phytosulfokine receptor 1 (PSKR1) (At2g0220), its loss of function mutant (*pskr1* (At2g0220)) was resistant to infection by *H. schachtii* (Fig 1). Nevertheless, there was no significant difference in percentage galling due to M. incognita when pskr1 (At2g0220) was compared to wildtype Col0 (Fig 2).

Discussion

Recent studies on PTI in plants against nematodes have shed the light on a topic that was largely unknown. Demonstrating the ability of ascarosides to induce PTI like responses to great extent hinted the plants ability to recognize molecules embedded or released by nematodes. In addition, identification of NILR1 further demonstrated that plant receptors have ability to interact with nematode in activities that leds to induction of PTI responses. Here we analysed 25 other genes that had previously been reported to be upregulated by cyst nematode *H. schachtii*. CRK19 (At4g23270) and NILR3 (At1g53440) demonstrated roles in PTI induced specifically by cyst nematodes while CRK10 (At4g23180) only to root knot nematodes. Interestingly, NEK5 (At3g20860) was shown to play a role against both sedentary parasitic nematodes. Never In Mitosis A (NIMA) is a Ser/Thr protein kinase that was originally found in a mitotic mutant of *Aspergillus nidulans (*Oakley and Morris, 1983; Osmani *et al*., 1987). NIMA rElated Kinases (NEKs) are family mitotic kinases which exist conservatively in eukaryotes such as budding yeast (Saccharomyces cerevisiae), *Chlamydomonas*, *Tetrahymena*, mammals, plants among others (O'Regan *et al*., 2007; Parker *et al*., 2007). NEKs have a conserved N-terminal catalytic serine/threonine kinase domain, as well as a long basic C-terminal non-catalytic extension. They are mainly involved in various mitotic functions like mitotic initiation, spindle formation, centrosome separation

and cytokinesis in fungi and mammals. In plants NEKs are quite conserved having been successfully retrieved from poplar, Arabidopsis and rice whose genome contains nine, seven and six NEK members respectively (Vigneault *et al*., 2007). Many of the plant NEKs have been associated with hypocotyl development and flowering while some such as AtNEK6 has not only been involved in plant growth regulation and responses to ABA and high osmolarity during the seed germination stage, but also plays a role in salt tolerance and abiotic stresses (Sakai *et al*., 2008; Lee *et al*., 2010). However, except these few studies, functions of plant NEKs remain unknown, and their role in plant growth and stress response are still largely unclear. In our study, we demonstrated that *AtNEK5* plays a role in PTI against sedentary plant parasitic nematodes. It`s expression is the highest among all the AtNEKs and in roots, its mostly expressed in mature tissues. Fortunately, AtNEK5 is widely conserved in plants as it has homologues in rice *Oryza sativa* (Os) (OsNek6; Os02g37830) and poplar *Populus trichocarpa* (Pt) (PtNek6 grail3.0152000301 [b]) (Vigneault et al., 2007). While very little is known about NEKs in plants, NEK5 remain as the first receptor in the NEK family to be associated with pathogenic responses specifically against nematodes.

Unlike NEKs, CRKs are well known receptors. In the study, CRK19 and CRK10 indicated a role in defense against cyst nematodes and root-knot nematodes respectively. This phenomenon can be likened to various reports that have associated CRKs to biotic stress including but not limited to pathogen defence and programmed cell death (Acharya *et al*., 2007; Wrzaczek *et al*., 2010). CRKs are group of RLKs also referred to as DUF26 (Domain of Unknown Function 26; PFAM domain PF01657) RLKs and are characterized by a single or multiple repeats of DUF26 domain (also called antifungal domain since it has the antifungal protein ginkbilolobin-2 (Gnk2) from *Ginkgo biloba*) consisting of four conserved C-X8-C-X2-C cysteine motif in their extracellular domain (Fig 4). The conserved cysteine residues it's believed to maintain the CRKs` three-dimensional structure through disulphide bridges. It also forms zinc finger motifs to mediate protein-protein interaction and may be the target for thiol redox regulation (Idänheimo *et al*., 2014). This group is one of the largest in

RLK family in Arabidopsis containing 44 members arranged in several clusters of which 19 of them are simultaneously in chromosome IV starting with CRK5 to CRK24 excluding CRK9 (Fig 3) (Chen *et al*., 2001). The rest of the 23 CRKs from CRK1 to CRK46, are distributed within chromosomes I, III and V in Arabidopsis. Expression of several AtCRKs is not only induced during plant development but also by pathogens infection, Salicylic acid (SA), Ozone and Reactive Oxygen Species (ROS) (Czernic *et al*., 1999; Du and Chen, 2000; Chen, 2001; Chen *et al*., 2003; Acharya *et al*., 2007; Ohtake *et al*., 2000; Wrzaczek *et al*., 2010; Bourdais *et al*., 2014). For example CRK4, CRK5, CRK19 and CRK20 have been associated with defense against *Pseudomonas syringae* such as cell death and SA dependent responses to infection (Czernic *et al*., 1999; Chen *et al*., 2003, 2004; Acharya *et al*., 2007; Ederli *et al*., 2011; Yang *et al*., 2013). CRK-interacting protein; kinase-associated type 2C protein phosphatases is known to interact with these CRKs through its kinase-interacting FHA domain. Therefore, CRK19 could possibly have dual function as its ability to induce defense is confirmed not to be restricted to bacteria, but also due to nematode recognition. The close relation in CRK19 structure to CRK5, CRK4 and 20 and their joint interacting protein are high indications which are yet to be proven of their shared biological functions in nematode induced PTI as they are in bacteria defense responses. CRK10 on the other hand has been hypothesized to be cytokinin-regulated since it contains an adenine aptamer motif. Transcriptome analysis of genes differentially expressed upon cytokinin treatment has actually shown that CRK10 is downregulated by three folds. However, direct binding of cytokinin to the extracellular domain of CRK10 is yet to be proven (Grojean and Downes, 2010). Promoter analysis of CRK10 has been reported to contains a W-boxes recognized by the plant WRKY18 TF. In addition, WRKY proteins are important for inducible expression of CRK10 (Du and Chen, 2000). It is therefore highly possible that the role of CRK10 in nematode perception is related to WRKYs transcription factors involved in plant defense.

LRR-RLKs have been widely associated with perception of microbial ligands. In our study, NILR3 (At1g53440) indicated a role in plant defense against cyst nematodes. While the function of this gene remain mostly unknown, expression of NILR3 homology in cucumber Cucsa.057870.1 (referred to as probable LRR receptor-like serine/threonine-protein kinase) was altered in leaves of aphid-infested cucumber plants (Liang *et al*., 2015). Both Aphids and nematodes can cause physical disruption of tissues and as such releases Damage Associated Molecular Pattern (DAMP) that might be recognized by NILR3. Similarly, a common PAMP between nematodes and aphids could be involved.

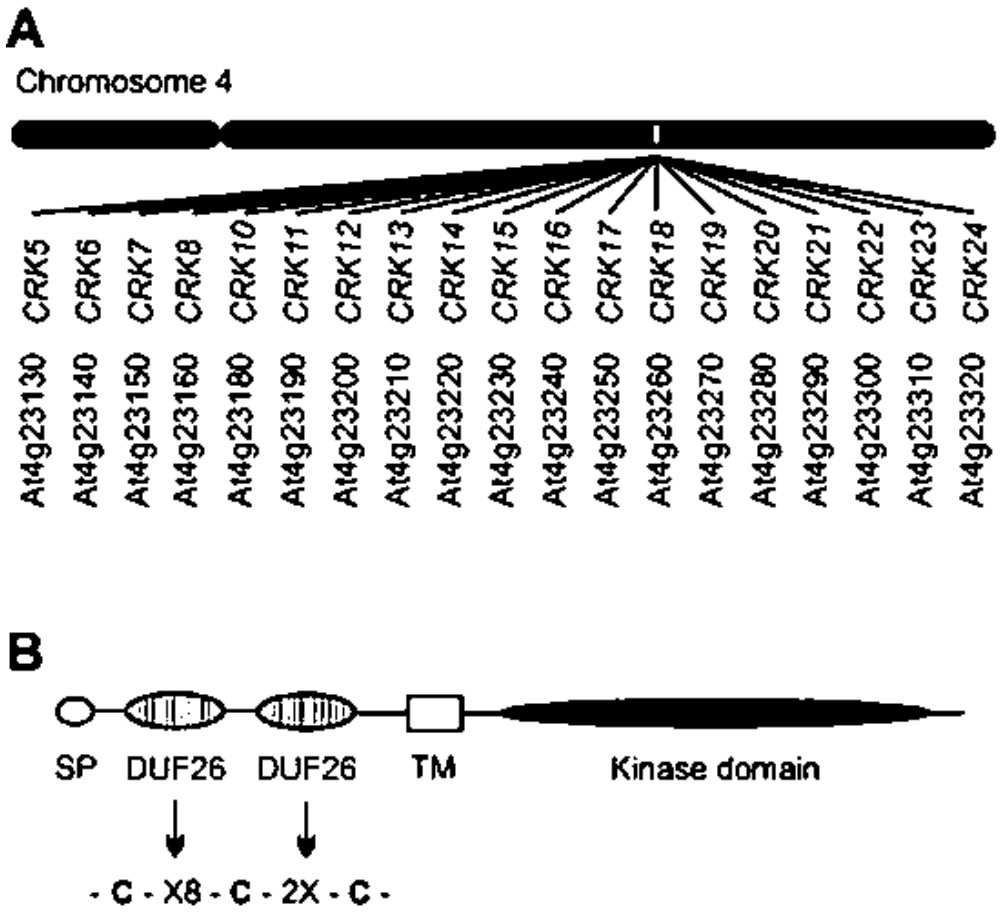

Figure 3. **Location of CRK10 and CRK19 genes in chromosome 4 and their domain structure**. (A) CRK10 and CRK19 are located in the largest tandem array on chromosome IV (CRK5–CRK24). (B) CRKs have conserved protein structure that includes a signal

peptide (SP), an extracellular domain containing one or more cysteine-rich DUF26 domains, transmembrane domain (TM), and intracellular kinase domain.

Our most intricately surprising finding in our study is the role of PSKR1 in nematode susceptibility regardless of its upregulated gene expression in our previously microarray data (Mendy *et al*., 2017). This receptor perceives Phytosulfokine (PSK); a Penta peptide of the sequence Tyr-Ile-Tyr-Thr-Gln containing a sulphate group on each of the two tyrosine residues (Sauter, 2015). PSK were first identified in cell cultures of Asparagus (Matsubayashi and Sakagami, 1996). PSK promotes somatic embryogenesis, pollen germination and adventitious root formation (Yamakawa et al., 1998; Chen *et al*., 2000; Hanai *et al*., 2000; Igasaki *et al*., 2003). PSKR1 has a putative GC catalytic domain within subdomain IX of its kinase a characteristic it shares with the DAMP receptor PepR1 (Qi *et al*., 2010; Kwezi *et al*., 2011). While PSK is also perceived to lesser extent by PSKR2, only PSKR1 is the main receptor and the most widely studied. Among the LRR receptor kinases, the PSK receptors PSKR1 and PSKR2 are closely related to the brassinosteroid receptor (Brassinolide Insensitive 1 BRI1) involved in growth. The leucine-rich repeats of PSKR1, PSKR2, and BRI1 contain an island domain which binds their ligands (Kinoshita *et al*., 2005; Shinohara *et al*., 2007; Clouse, 2011). Studies have revealed binding of PSKR1 to *Arabidopsis thaliana* H (+)-ATPase (AHA1) and AHA2, the two most highly expressed isoforms of AHA gene family of 11 members. In comparison, BRI1 similarly activates AHA1 and hyperpolarization of the plasma membrane in a brassinolide-dependent manner. Most interestingly, Just like BRI1, PSKR1 interacts with BRI1 Associated Kinase 1 (BAK1) to form a PSKR1/BAK1 complex. BAK1 binds to several LRR receptors recognizing ligand from numerous pathogens as it does to the growth receptor BRI1 and as such very vital in growth and defense especially in PTI simultaneously. Functionally so far, PSK signalling through PSKR1 regulates root and hypocotyl elongation of Arabidopsis seedlings (Matsubayashi *et al*., 2006; Kutschmar *et al*., 2009; Stührwohldt *et al*., 2011; Caesar *et al*., 2011). Besides this role, there are contrdadicting reports on PSKR1 role in plant defense. For example, loss of

function mutants of PSKR1 are more resistant to biotrohic bacteria *Pseudomonas syringae* pv. Tomato DC3000 and showed reduced formation of lesions (Igarashi *et al.,* 2012). Contrary, *pskr1* mutant was more susceptible to necrotrophic fungi *Alternaria brassicicola* by increased fungal growth unlike in wild type. In plant nematode interactions, *pskr1* mutants are observed to have impairment of root-knot nematode reproduction and the giant cells development is not fully differentiated or halted (Rodiuc *et al*., 2016). Likewise, here we report that loss of function mutant of PSKR1 was more resistant to biotrophic cyst nematode. The role of PSKR1 in Plant defense is thus pathogen-dependent and this antagonistic effect between biotrophic and necrotrophic pathogen resistance is linked to enhanced SA and reduced jasmonate pathways. PSKR1 therefore act similarly to BRI1 as they share regulatory elements and they both respond negatively to immunity responses during defense and growth cross talk. However, downstream signalling upon PSK perception is still unclear and the mechanism underlying these regulatory functions remain to be explored.

In conclusion, this study explores various genes that are involved in PTI and can possibly perceive nematode ligands. It is intriguing to find these four genes from families associated with pathogenic responses, playing a vital role in PTI. Further studies require elucidation of the components involve in their PTI roles, be it the PAMP recognized or the downstream signalling components.

Material and methods

Plant growth

Single T-DNA inserted knockout mutants for the listed RLKs genes were ordered from Nottingham Stock Centre (NaSC) (Table 1). Arabidopsis wildtype Col-0 and RLKs mutants' seeds were sterilized in 0.7 % NaOcl and the bleach extracted with 70% Ethanol. The seeds were rinsed 5 times with autoclaved distilled water and air-dried under the clean bench. Seeds were seeded in petri dishes (Fig 4A) of modified KNOP and or Murashige and Skoog (MS) media and then incubated in long day conditions (16hrs light/8hrs dark).

Table 1. **List of RLKs TDNA insertion mutants screen for susceptibility to nematodes.**

Gene count	Order Number	Mutant type	Locus	Gene count	Order Number	Mutant type	Locus
1	N872213	Sail-51-E04	AT4G30340	15	N663996	Salk-130548C	At1g53440
2	N862547	Sail-321-F05	AT3G59700	16	N678301	salk-028536C	At5g24430
3	N664269	Salk-147351C	At4g21390	17	N685170	salk-008585C	At2g02220
4	N686377	Salk-076637C	At4g32300	18	N686653	salk-111817C	At5g65600
5	N677232	Salk-143489C	At1g11330	19	N675275	salk-057158C	At3g09830
6	N663753	Salk-116653C	At4g23180	20	N800003	CS800003	AT1G73080
7	N661711	Salk-019639C	At4g23270	21	N2030753	GK-878D10-1	At1g16670
8	N671724	salk-094512C	At3g14840		N2030754	GK-878D10-10	At1g16670
9	N654909	salk-134409C	At1g06840	22	N414335	GK-150C07-8	AT5G47070
10	N663387	Salk-094492C	At1g09970	23	N446527	GK-485F03-4	AT3G59350
11	N660433	Salk-054652C	At3g20860	24	N423042	GK-241A02-7	AT1G66880
12	N663922	Salk-126675C	At1g61370	25	N412564	GK-131G12-7	AT3G57120
13	N860331	Salk-091274	AT5G25930		N412564	GK-131G12-10	AT3G57120
14	N684335	Salk-099335C	At4g18950				

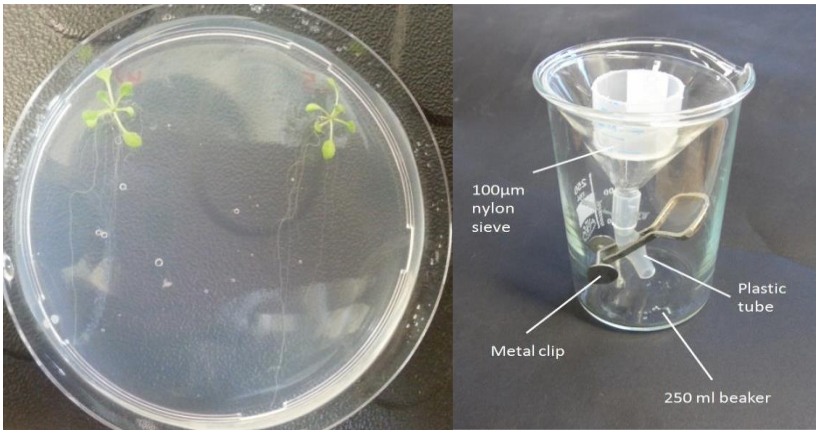

Figure 5. **12 days old Arabidopsis plants on KNOP medium (Left). Hatching of *H. schachtii* in its chamber made up of a 100µm sieve in a funnel.** The J2s hatch from the cyst in 0.05% zinc chloride (Right).

Nematode infection

H. schachtii assay: J2s hatched from cysts of a sterile *H. schachtii* culture (Fig 5B) and were sterilized using 0.03% mercury chloride (HgCl2) for 3 minutes after which, they were rinsed thoroughly with autoclaved distilled water.

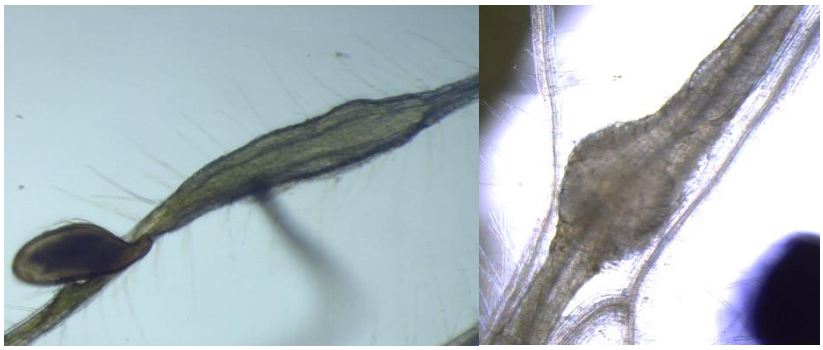

Figure 6. **An illustration of *H. schachtii* female at 14 days post infection and its syncytium (Left) and galling at 21 days post infection with *M. incognita* (right)** whose sizes were measured as a parameter for susceptibility to nematode.

Approximately 60 to 70 sterile J2 were inoculated to 12days old mutant and col0 wild type in plants knop medium under sterile conditions. 12 plants were used per genotype while the

experiments were done independently and in triplicates. The number of females per plant was counted at 14 days post inoculation (Fig 6A).

M. incognita assay: *M. incognita* eggs were extracted from heavily galled tomato (*Solanum lycopersicum* cv. Money-maker) plants from a greenhouse propagated culture. Tomato plants were blended in 1.5% NaOCl2 for about 3 minutes and rinsed with tap water. The paste was passed through a stack of sieves; 250µm 150µm 100µm 50µm, 20µm. The eggs were collected in 500 ml Erlenmeyer flask and surface sterilized in 10% NaOCl2 for 3 minutes. Eggs were rinsed with sterile tap water and the Sterile eggs suspension was incubated in a sterile 500ml glass chamber with 150 µL Nystatin (10,000 U/ mL) and 2mL gentamycin sulphate (22.5 mg/mL) in a total volume of 30 ml. The chamber was stored at room temperature in the dark for 4 days. The hatched J2s were incubated in 0.5% (w/v) streptomycin-penicillin for 20minutes and similarly with 0.1% (w/v) ampicillin-gentamycin solution. J2s were rinsed with autoclaved distilled water and subsequently incubated in 0.1% (v/v) chlorhexidine for 3 minutes after which they were thoroughly washed with autoclaved distilled water. Approximately 100J2s were inoculated on each plant in MS media. 12 plants were used per genotype and Col0. Experiments were done independently and in triplicates. In each experiment, the numbers of galls were tallied (Fig 6B).

Statistical analysis

Data were statistically analysed using SigmaPlot v 14.0. Statistical analysis included one-way Analysis Of Variance (ANOVA) (Dunnet t-test) of average number of females per plant for *H. schachtii* infection assay and number of galls per plant for *M. incognita* infection assay.

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Chapter 4

Utilizing BAK1 to mine components involved in nematode induced innate immunity

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Abstract

Plant innate immunity involves recognition receptors that perceives ligands derived from pathogen. Ligand perception initiates a signalling cascade that leads to changes in expression of various defense genes and defense responses in plants. Upon ligands recognition, Plant Recognition Receptors (PRRs) forms heteromeric complexes with other receptor kinases, receptor proteins and cytoplasmic kinases that aid in phosphorylation and signal transduction. Several studies have implicated the SERK LRR-RLKs as co-receptors not only to growth and development associated receptors but also many PRRs involved in plant immunity. One particularly is SERK3/BAK1 which has been described as a universal co-receptor to different pathogens induced basal immunity including bacteria, fungi and oomycetes. BAK1 has been reported to play a role in plant innate immunity against nematodes. We suggested that as a co-receptor, BAK1 interacts with other receptors and possibly other components while initiating defense signalling. This study, not only confirm the role of BAK1 in nematode induced basal defense, but also that it equally forms a complex which can be analysed to determine the specific proteins involved.

Introduction

Plants have a complex immune system that consists of two layers of receptors aimed at preventing penetration of pathogens in the plant and subsequent disease. The first layer involves Plant Recognition Receptors (PRRs), which recognizes conserved molecular signatures from pathogens. The second level occurs once the first line of defense is crippled by pathogen secreted effectors (Avr Proteins) and is characterized by effectors recognition by intracellular Nucleotide-Binding Receptors (NLRs/ R gene proteins) and as such referred to as Effector Triggered Immunity (ETI) (Win *et al*., 2012).

Various studies have clarified many details about functioning of PRRs and identity of Pathogen Associated Molecular Patterns (PAMPs). Majority of identified PRRs in plants are either Receptor Like Kinases (RLKs), which have an extracellular ectodomain, a transmembrane domain and a C-terminal cytoplasmic kinase domain or Receptor Like Proteins (RLPs) which are similar to RLKs but lack the cytoplasmic kinase domain (Shiu and Bleecker, 2001; Macho and Zipfel, 2014). In addition, most of the identified PRRs extracellular domains are Leucine Rich Repeats (LRR), which is the largest group of receptors in Arabidopsis. PAMP recognition triggers a series of events such as Reactive Oxygen Species (ROS) burst, Calcium burst, increased extracellular pH and cell wall reinforcement by Callose disposition. In addition, activation of Mitogen -Activated Protein kinases (MAPK) and Calcium-Dependent Protein Kinases (CDPKs) occur which in turn regulates the activity of relevant nuclear transcriptional factors associated with induction of defense gene expression (Macho and Zipfel, 2014).

PRRs have been shown to recruit other receptors regardless of their nature as RLKs, RLPs and/or other cytoplasmic kinases upon PAMP perception to form heteromeric complexes most of which are LRR-RLKs of the Somatic Embryogenesis Receptor Kinase (SERK) family. They are characterized by an LRR domain with five residue repeats, followed by a Ser-Pro-Pro (SPP) motif, the serine and proline rich domain, a single membrane-spanning domain, a cytoplasmic kinase domain and a small C-terminal tail. Among its five members (SERK1-5), SERK3 was identified to form a complex with the brassinosteroid; plant hormones with roles in growth and development, receptor Brassinosteroid Insensitive1 (BRI1) and thus renamed BRI1-Associated Kinase 1 (BAK1). Besides brassinosteroid signalling, BAK1 has been implicated in other developmental processes like in photo morphogenesis, root development and stomatal patterning (Whippo and Hangarter, 2005; Du *et al*., 2012; Meng *et al*., 2015; Jordá *et al*., 2016). In immunity, BAK1 has been confirmed to associate to an array of LRR-RLKs perceiving PAMPs derived from various pathogens for example Flagellin Sensitive 2 (FLS2) and EF-TU Receptor (EFR) that perceives a 22 epitope of the flagellin (flg22) and elongation factor Tu (EF-Tu) from bacteria respectively. In addition, BAK1 is associated with LRR-RLPs for example, RLP23, BAK1 and Suppressor of BRI1 interacting receptor kinase 1 (SOBIR1) forms a tripartite complex upon recognition of a conserved a 20-amino-acid fragment of Necrosis and ethylene-inducing peptide 1-Like Proteins (NLP20) present in several prokaryotic and eukaryotic species (Monaghan and Zipfel, 2012; Zhang *et al*., 2013; Chen *et al*., 2014; Albert *et al*., 2015). BAK1 has also been associated with resistance in tomato where the membrane bound immune receptor Ve1 recognizes secreted fungus *Verticillium dahliae* effector Avirulence on Ve1 tomato (Ave1) (Fradin *et al*., 2009; Zhang *et al*., 2013). Some reports have suggested that the specificity of these multiple functions of BAK1 in development, immunity and cell death is determined by some amino acid residues of its ectodomain and specific proteins interacting with BAK1 (Halter *et al*., 2014).

Like most of the plant pathogens, Plant Parasitic Nematodes (PPN) are important in agriculture as they cause great damage to plants that leads to incurring of massive losses in the tune of 100billion dollars. To effectively control PPN, understanding plant immunity especially nematode induced PTI is inevitable. Unfortunately, very little is known regarding PAMP recognition by PRRs and the signalling components involved including BAK1. Nevertheless, few reports have sort to shed to a topic that for a long time remained elusive. For example, BAK1 has been associated with induction of PTI defense responses against root knot nematode in Arabidopsis equally associated with camalaxin and glucosinolate pathway (Teixeira *et al*., 2016). Similarly, silencing the Arabidopsis homologue of BAK1 in tomatoes led to plants being hyper susceptible to root-knot nematodes linked to absence of PTI (Peng and Kaloshian, 2014). In our most recent publication, the Nematode Induced LRR-RLKs 1 (NILR1) activity was suggested to be BAK1 dependent due to their similar PTI responses to cyst nematode *Heterodera schachtii* (Mendy *et al*., 2017). These evidences highly indicate a central role of BAK1 in nematode induced PTI. However, direct binding or PRR to BAK1 and the nematode derived PAMP recognized remain to be studied.

In this study, we aimed at identifying the role played by BAK1 at molecular level upod induction by nematodes. We also sort to deduce the components that form a complex with BAK1 in nematode induced PTI. This is essential for identification of RLKs and or RLPs in plants involved as well as for understanding the mechanism behind signal transduction. We aimed at utilized ImmunoPrecipitation (IP) techique, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in finding BAK1 protein interactors.

Results

BAK1 forms a complex induced by nematodes

Knowing that BAK1 is required in nematode recognition (Teixeira *et al*., 2016; Mendy *et al*., 2017), we proposed that BAK1 do form a heteromeric complex upond ematode ligand perception similary to those formed in other pathosystem. We obtained *Arabidopsis thaliana*

transgenic plants expressing AtBAK1 from the strong 35S promoter, fused translationally to green fluorescent protein (GFP) (35::BAK1-GFP).

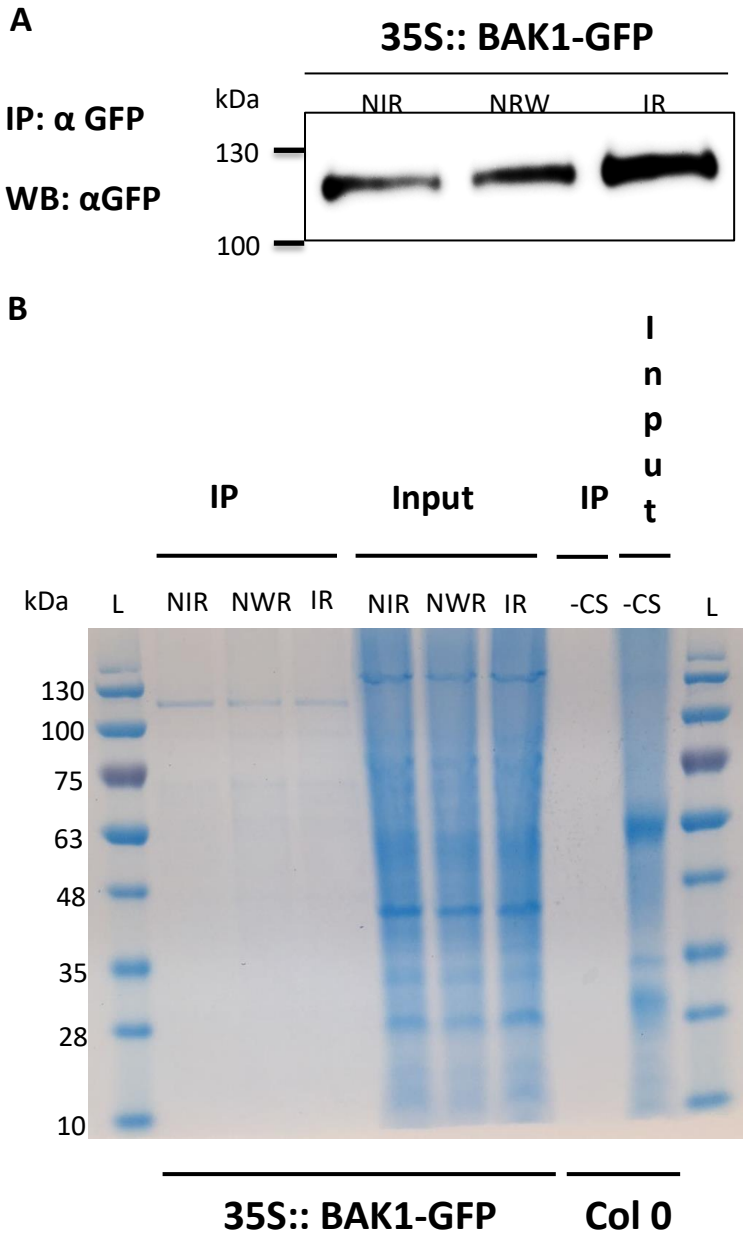

Figure 1**. BAK1 forms a protein complex induced by nematodes**. (A) Immunoblot from BAK1-GFP proteins indicating a signal at 120kDa position from both 35::*BAK1-GFP* NemaWater treated Roots (NWR) and 35::*BAK1-GFP* Infected Roots (IR). 35::*BAK1-GFP* Non Infected Roots (NIR) were used as negatively control to the treatments. The signal from the infected roots was much intense compared to the non infected roots.

(B) Coomasie blue stained gel showing pull down of 35::*BAK1-GFP* NemaWater treated Roots (NWR), 35::*BAK1-GFP* Infected Roots (IR) and 35::*BAK1-GFP* Non Infected Roots (NIR) forming a complex band at 120kDa position, different from the input sample. Col-0 whole plants were used as negative experimental control which showed no GFP pull down.

To identify proteins that form nematode derived PAMP (NAMPs) elicited complex with BAK1, we performed Co - Immunoprecipitation (coIP) experiments on Arabidopsis transgenic *35::BAK1-GFP* roots before; (35::BAK1-GFP Non Infected Roots (NIR)) and after infection with *H. schachtii* at 16hpi (*35::BAK1-GFP* Infected Roots (IR)) and treatment with NemaWater; a water solution obtained after incubation with sterile *H. schachtii* J2s for 24hrs (35::BAK1-GFP NemaWater treated Roots (NWR)). BAK1-GFP immunoprecipitated proteins as detected by Western blot with a α-HA antibody upon α-GFP antibody (Fig 1A) on the three protein samples tested; NIR, NWR and IR at 120kDa position. Similarly, SDS page was performed on the three samples and Coomassie blue staining which confirmed the immunoblot complex position that differed from the input sample and was absent in wildtype Col0 plants (Fig 1B)

Protein Identification

To identify the proteins contained in the BAK1-GFP pull down bands from the Nemawater treated, Infected root and Non infected gel bands, a tripsin digestion was performed to the extracted gel bands. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/MS/MS) is currently being performed to deduce the identity of the peptide sequence in the BAK1-GFP pull down bands for all samples.

Discussion

In recent years, coImmunoPrecipitation (coIP) has turned out to be the most direct technique to study protein-protein interactions in *vitro* in presence of antibodies against the target proteins. The technology utilizes the fundamental principle of the specific antigen-antibody

reaction where the entire intact protein complex is pulled out of solution as a first step in identification unknown members of the complex. Isolated proteins complex are digested into peptides before analysis by liquid chromatography-mass spectrometry (LC-MS/MS) for peptide characterization.

In this study, we report first time isolation of protein complex pulled by GFP attached to the the well known PRR co-receptor BAK1. Although the signal size is not an expression of difference in the constitution of the protein complex, in our study, nematode infected 35::BAK1-GFP roots pull down complex signal was observed to be much more intense compared to that of the non-infected (Fig 1A). The results went forth to demonstrate the ability of BAK1 to form a nematode induced protein complex. This can be likened to other similar studies whose approaches were similar. For example similar strategy was used to identify the components of downstream immune signalling by using a GFP tagged EFR (EF-Tu receptor) and subsequent identification of RBOH genes as part of its complex (Roux *et al*., 2011; Kadota *et al*., 2014).

Material and methods

Plant growth and nematode preparation

Seeds of Arabidopsis transgenic plants overexpressing BAK1 attached to GFP; 35s::BAK1-GFP, were kindly provided by Prof. Dr. Cyril Zipfel (The Sainsbury Laboratory, Norwich, UK). BAK1-GFP plants were grown in greenhouse conditions on sand soil mix of 80:20 ratios and after 8 weeks, seeds were harvested. Seed sterilization and seeding was done under sterile conditions in a clean bench.

1ml of 0.7% Sodium hypochlorite was used to sterilize the seeds for 5 minutes in a 2ml Eppendorf tube and the chemical extracted using 1ml 70% ethanol. The seeds were thoroughly rinsed with autoclaved double deionized water (ddH_20) and pipetted out on a clean filter paper in a petri dish. Seeds were air dried under the clean bench for 2hrs.

Knop medium was prepared according to table1, autoclaved and poured in 70mm diameter petri dishes. 3 seeds were planted per petri dish and incubated under long day conditions (16hrs light/8hrs dark).

Table 1**. A list of modified Knop medium ingredients and their quantities.**

Ingredients	Content	Mass L^{-1}	Volume L^{-1} Knop
Stock soluiton1	KNO3	121.32g	2ml
	MgSO4	19.71g	
Stock solution 2	Ca(NO3)2 x 7H2O	120g	2ml
Stock solution 3	KH2PO4	27.22g	2ml
Stock solution 4	FeNaEDTA	7.34g	0.4ml
Stock solution 5	H3BO3	2.86g	0.2ml
	MnCl2	1.81g	
	CuSo4 X 5H2O	0.073g	
	CaCl2 x 6H2O	0.03g	
	NaCl	2g	
Sucrose			20g
Daishin agar			8g

Simultaneously, Cysts from a sterile laboratory culture of *H. schachtii* were harvested and incubated in 3% zinc chloride for or 5days. Fresh J2s were sterilized using 0.03% mercury chloride and rinsed thoroughly with autoclaved ddH_20.

Nematode infection and sampling

Approximately 200J2s were inoculated per plant and incubated under long day conditions (16hrs light/8hrs dark).15hrs post infection (15hpi), long pieces of roots at nematode migratory stage were cut and frozen in liquid nitrogen. The sampling was repeated until a quantity of 9gs was achieved and labelled Infected Roots (IR). Equal amounts of uninfected roots were sampled in a similar manner and labelled Non-Infected Roots (NIR). The samples were crashed with mortar and pestle in presence of sand and 0.5 g polyvinylpolypyrrolidone (PVPP). The samples were preserved in -80^{0}C.

NemaWater treatments and sampling

Approximately 10,000 sterile J2s were incubated for 24hrs in 20ml of ddH20 while gently shaking. The nematodes were then discarded and the remaining solution labelled NemaWater. In 6 well plates, whole 10 days old plants were incubated with 3ml per well. The roots were cut and frozen in liquid Nitrogen. The process was repeated until 9gs of material was achieved and labelled NemaWater treated Roots (NWR). Non-treated wild type Col0 whole plants were collected in a separate 15ml falcon tube. The sample was crashed with mortar and pestle in presence of sand and 0.5 g polyvinylpolypyrrolidone (PVPP). The samples were preserved in -80^{0}C.

Protein isolation and ImmunoPrecipitation (IP)

A modified protocol by Kadota *et al*. 2014 was followed for protein isolation and immunoprecipitation.

50ml extraction buffer was prepared accordingly; 50 mM Tris–HCl pH 7.5, 100–150 mM NaCl, 10 % Glycerol, 5 mM DTT, 1 % (v/v) Protease inhibitor cocktail (P9599, Sigma), 0.5–2 % (v/v), IGEPAL CA-630, 1 mM $Na_2MoO_4 \cdot 2H_2O$, 1 mM NaF, 1.5 mM Activated sodium orthovanadate (Na_3VO_4) and 1 mM EDTA (Optional). 5gs of each of the BAK1-GFP NIR, NWR and Infected Roots IR samples was incubated in 10ml of extraction buffer and 1mM Phenylmethanesulfonyl fluoride (PMSF) for 2hrs at 4^0C while rotating at 10rpm. 2gs of wild type Col0 whole plant powder was used as an experimental negative control (-CS). The sample lysates were then centrifuged at 12000rpm 4 °C for 20 min using Beckman Coulter B409 - Optima L-90K Ultracentrifuge. The crude protein samples were each passed through a 20ml Bio-Rad empty fritted column into a fresh 15ml falcon tube. The protein samples were again centrifuged at 1500g for 1minute to completely remove PVPP and the supernatant transferred to a new tube.

200µl of each protein sample was used aliquoted in a low binding tube as an input sample. Input samples were spun at full speed for 10 minutes and the supernatant discarded. Elution was done by adding 40µl 2× NuPAGE LDS buffer with NuPAGE sample reducing agent then heating at 70^0C 15 min and centrifuging at full speed for 2minutes.

Preparation of agarose beads; approximately 120ul of chromatek GFP-Trap®_A beads were transferred into a low binding tube and washed 3 times with 600ml extraction buffer for each wash by spinning at 1000rpm for 1 minute and discarding the supernatant. The beads were then eluted with 1.2ml extraction buffer. 400µl of the beads was pipetted into each protein sample and incubated on a roller at 4^0C for 2hrs. Protein-beads mixtures were centrifuged at 500g for 1min and the supernatants transferred to another falcon tube. The beads were pipetted into low binding tubes using extraction buffer. The beads were washed with extraction buffer 3 times by centrifuging at 500g for 30sec at 4^0C. Beads were eluted by adding 80µl 2× NuPAGE LDS buffer with NuPAGE sample reducing agent and heating at 70^0C 15 min and after, centrifuging at full speed for 2minutes.

SDS page

20µl of each eluted sample was loaded into wells of NuPAGE Bis-Tris precast Gels from Thermo fisher using greiner bio one tips in NIR, NWR, and IR order in addition to 10µl Page Ruler™ Prestained Protein Ladder; 10 to 180 kDa. The gel was then run using ice cold 1X NuPAGE MOPs SDS running buffer in an XCell SureLock® mini gel running tank at Amp/A 20 for 150 minutes.

Western Blotting

The gel was removed from the cassette and stack together with 8 cm by 8 cm Immun-Blot® PVDF Membrane pre-soaked in methanol. Two 9cm by 9cm blotting papers and two same sized sponges were introduced on each side of the gel-membrane and the stack placed firmly XCell II™ Blot Module. The module was placed in an XCell SureLock® mini gel running tank and run in 1X SDS buffer with 20% methanol at 200 Amp for 70 minutes. The membrane was then blocked using 5% TBS Tween20-milk powder for 20 minutes. 3µl of primary antibody Anti-HA rat (Roche 11867423001)1:5000 was added and incubated at 4^0C overnight. The membrane was cleared off the blocking solution and washed three times with 15ml TBS-Tween at 4^0C room for 15minutes each time. 15ml of 5% TBS Tween20-milk powder with 3µl secondary antibody Anti-GFP mouse (Roche 11814460001)1:5000 was added into the membrane and incubated at 4^0C for 1.5hrs. The secondary antibody was washed off using 15ml TBS-Tween at 4^0C for 15minutes three times. The membrane was soaked evenly in 1ml of Enhanced ChemiLuminescence (ECL) solution prepared from Pierce™ ECL Western Blotting Substrate (Thermo fisher) according to manufacturer's instructions and spread on clear paper. Imaging was done by exposure to film using image quant LAS 4000 program connected to a BioSpectrum Imaging System with an increment of 30 seconds.

Coomassie blue staining and gel cutting for LC-MS/MS analysis

SDS page was repeated twice with the remaining 60µl of each sample in NIR, NWR, IR and –CS order as well as their respective eluted input samples. The gel was washed 4 times each with 100ml of autoclaved ddH_20 to remove excessive SDS. Each of the gels was treated with 15ml of SimplyBlue™ Safe Stain from Life technologies for 2 hrs while shaking gently at room temperature. The stain was washed off by incubating at room temperature with autoclaved distilled water overnight and then twice for 1hr each duration while shaking gently. The IP samples for NIR, NWR and IR at positions 130kDa, 120kDa and 100kDa were cut precisely under clean glass plate using a sharp scalpel and stored in -80^{0}C for LC-MS/MS analysis.

Protein Identification and characterisation using MS/MS data

A tripsin digestion was performed to the extracted gel bands. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/MS/MS) is currently being done to deduce the identity of the peptide sequence in the BAK1-GFP pull down bands for all samples

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Chapter 5

DISCUSSION AND CONCLUSION

The use of resistant cultivars is the most successful and preferable method to manage yield loss due to nematodes. To facilitate this, the importance to know and understand plant innate immunity can never be more emphasized. In the recent decades, various studies have sort to elaborate on the topic and the successful identification of acarosides as nematode derived PAMP (NAMP), the ability of nematode to induce PTI and association of BAK1 in nematode induced PTI has provided the evidence of PTI induction by nematodes.

In our studies, we have demonstrated the molecular events induced by nematodes and PTI-like responses that follow there after contributing to plant tolerance to nematodes. The induction of differentiated gene expression by NemaWater demonstrated presence of elicitors capable of inducing defense responses. Equally to NemaWater treatment, nematode attack at migratory stage caused the upregulation of several hormonal response gene involved primarily in JA/ET pathway which is active defense against necrotrophic pathogens and herbivorous insects (Kessler and Baldwin, 2002; Rojo *et al*., 2003; Glazebrook, 2005; Howe and Jander, 2008). This finding therefore confirmed the role of JA/ET pathway in promoting both nematode parasitism and damage associated responses even though nematodes are biotrophs. This was in contrast to SA pathway whose activation has been shown to inhibit nematode parasitism even though it has been shown to promote infection by biotrophic pathogens (Branch *et al*., 2004; Wubben *et al*., 2008). Similary, only slight increase in expression of SA biosynthesis genes was observed in our study

Even though similar trends were observed in both scenarios at nematode migratory stage and NemaWater treatment, there were some genes expressed specific to either of the treatment. Migration of nematode involves stylet movement forward and backward to facilitate nematode entry into the cell which in the process causes a lot of damage and would explain the upregulation of a share of genes at migratory stage specifically. Concurrently, a huge number of genes were upregulated due to treatment with NemaWater alone. Interestingly, nematode infection induces suppression of innate immunity and therefore upregulation of genes involved in these functions is thus expected mostly induced by secreted effectors by the nematodes into NemaWater. For example, in comparison to what is already known, the patterns of gene expression we observed in Soybean cyst nematode infected plants have been shown to suggest coordinated regulation of genes involved in parasitism. In addition, Calreticulin (CRT) *Mi*-CRT secreted from *M. incognita* triggers suppression of callose disposition and reduced activation of defence-related genes (Szakasits *et al*., 2009; Jaubert *et al*., 2005; Jaouannet *et al*., 2012, 2013).

We also demonstrated that nematode can induce PTI. One of the best characterized PTI signalling event is ROS burst which has so far proven to be a valuable tool to study plant immunity signalling components and regulatory mechanisms. ROS production is dependent on the membrane localized NADPH respiratory burst oxidase homolog D (RbohD) (Wojtaszek *et al*., 1997; Torres *et al*., 2002; Torres 2009; Kadota *et al*., 2015). ROS initiates a series of downstream signalling events crucial for triggering defense to reduce pathogen growth. The ability of NemaWater to induce ROS burst in wildtype Col-0 plants proved presence of PAMPs and activation of defense responses due to nematodes. That notwithstanding, NemaWater inhibited growth of wildtype Col-0 plants after treatment for 1hr. Growth inhibition is as a result of immunity and growth crosstalk where activation of immunity forces the plant to shift resources and nutrients towards defense ultimately reducing growth. Growth vs immunity has been associated to the functioning of brassinosteroid (BR) pathway. When BR is recognized by BRI1, there is formation of a

heteromeric complex with BAK1 that initiates a signalling cascade and phosphorylate BIK1, BSK1 and Brassinosteroid Suppressor 1 (BSU1). Phosphorylation of these components inactivates the negative growth regulators of BR pathway; Brassinosteroid-INsenstive 2 (BIN2), and activates some transcription factors BrassinaZole-Resistant 1 (BZR1) and Homolog of Brassinosteroid enhanced expression 2 interacting with increased leaf Inclination 1 (HBI1) (Fan *et al*., 2014). Brassinosteroid signalling and PTI have BAK1, BIK1 and BSK1 in common as part of their signalling (Lin *et al*., 2013). It is currently known that the roles of these components shift from growth to immunity or vice versa controlled by transcription factors BZR1 and HBI1 which acts in favour of growth in absence of a pathogen and vice versa (Gallego-Bartolome *et al*., 2012). Growth vs immunity has also been associated to hormonal crosstalk involving a shift in Gibberellins, Jasmonate and auxin. However, it remain unclear how the shift of hormones when PTI is activated results into changes in cellular processes that inhibits growth (Navarro *et al*., 2006; Eichmann and Schäfer, 2015).

Knowing that nematodes can thus cause PTI responses, we sort to find out if that translated into effects to overall infection due to nematode. In addition, we hypothesised those potential receptors genes involved in activation of PTI would be over expressed during nematode attack. Using reverse genetics especially use of knock-out mutations of genes is a commonly used strategy utilized in elucidating gene functions including but not limited to identification of novel PRRs (Shiu and Bleecker, 2001; Matsuda and Aiba, 2004; Bi *et al*., 2010). We demonstrated that among the 51 upregulated RLKs genes shared between NemaWater treatment and at nematode migratory stage, loss of function mutations of 5 of the genes rendered the plants hyper susceptible to a group of sedentary endoparasitic nematode attack. Susceptibility was illustrated by the female count per plant against wildtype as an increase in food supply and a constantly optimum environment favours nematodes differentiation into female cysts nematode (Wyss and Grundler, 1992; Lewis and Gaugler, 1994). For root knot nematodes, galling was the main characteristic used as a measure of

susceptibility. Mainly, these genes belonged to CRK-RLK, NEK and LRR-RLK families whose majority of gene members remain to be characterized. While NEK5 belonging to the NEK family was shown to play a role in PTI induced by both cyst and root-knot nematodes, there are no reports indicating or implicating this family to pathogenic responses. However, most studies have shown its plays a role in salt tolerance and abiotic stresses (Sakai *et al*., 2008; Lee *et al*., 2010). Members of this gene family involvement in tolerance to abiotic stress could be a hint as to their role in immunity against biotic stress too. In addition, NEK5 involvement is also a great indicator of that this family could contain potential PRRs which are yet to be characterized some probably involved in nematode triggered PTI. Unlike NEK family, CRKs have been linked to biotic stress for example programmed cell death, among others (Acharya *et al.,* 2007; Wrzaczek *et al*., 2010). Nevertheless, our study found out that genes belonging to this family can be species-specific in the role, since CRK19 and CRK10 expressed immunity against cyst nematodes and root-knot nematodes respectively. The CRKs` DUF26 domain possesses a conserved C-X8-C-X2-C motif whose Cys residue forms Cys bridges believed to be targeted for apoplastic redox modification (Ohtake *et al*., 2000; Chen, 2001; Bourdais *et al*., 2015). Members of this family such as the (PlasmoDesmata-Located Proteins (PDLPs), has also been associated with regulation of cell to cell communications and plant immunity (Amari *et al*., 2010; Lee *et al*., 2011; Caillaud *et al*., 2014). In addition, CRKs are transcriptionally induced in response to abiotic stresses such as salicylic SA, Ozone, salt, and drought treatments (Chen *et al*., 2003, 2004; Wrzaczek *et al*., 2010; Bourdais *et al*., 2015; *Yeh et al*., 2015). A group of CRKs are also strongly induced in response to pathogens and PAMP treatment such as CRK28 and CRK29 (Wrzaczek *et al.,* 2010; Bourdais *et al*., 2015). CRK28 for example is dependent on the co-receptor BAK1 and associated with FLS2 forming a complex to coordinate enhanced plant immune response against bacteria. Members of this family is thus a potential player in immunity (Yadeta *et al*., 2017). Further studies into the mechanism of binding of these receptors to PAMPs remain to be elucidated on.

Among all subfamilies in the RLKs family, the LRR-RLK gene family is most widely associated with pathogenic responses and disease resistance. Expectedly, in our study, 11LRR genes were upregulated due to nematode invasion. We studied four LRR genes; NILR1, NILR2, NILR3 and PSKR1 that were upregulated due to nematode invasion and NemaWater treatment. However, further studies into infection with cyst nematodes revealed NILR2 to play no role in nematode induced PTI. NILR3 homology in cucumber Cucsa.057870.1, referred to as probable LRR receptor-like serine/threonine-protein kinase was one of the genes with altered expression in leaves of aphid-infested cucumber plants (Liang *et al*., 2015). NILR3 not only was upregulated due to nematode attack and NemaWater treatment, but also potrayed a role in nematode infection. Since both aphids and nematodes induce damage, it remains to be demonstrated if the role of NILR3 in PTI is due to PAMP or DAMP perception. Intrigingly, PSKR1 was observed to act against immunity in nematodes. Several recent reports have shown that sulfated peptides are important signaling molecules utilized by plants to integrate growth and development programs with stress responses. Activation of stress responses comes at the cost of reduced growth. Improper regulation or prolonged activation of stress responses can lead to stunted growth and even cell death. PSKα perception by PSKR1 leads to the downregulation of SA-related responses after biotrophic pathogen infection, thereby preventing an over-induction of this particular signaling pathway that would otherwise reduce the fitness of the plants and leave them vulnerable to necrotrophic pathogens (Mosher and Kemmerling, 2013). It is thus not surprising that plants lacking PSKR1 were more resistant to nematode attack due to absence of PSKR1-mediated regulation of PAMP responses.

Loss of function mutation of NILR1 gene rendered the plant more susceptible to a class of sedentary nematode attack. Its insensitivity to NemaWater during ROS bursts and growth inhibition assay confirmed it's important in PTI activation due to Nematode only. Just as it's predicted structural characteristics based on BRI1 model as having a transmembrane domain, NILR1 has been confirmed to be localized in the plasma membrane when

transiently expressed in *Nicotiana bethamiana* epidermis. Similarly to all secretory proteins processed from the endoplasmic reticulum, a signal peptide is required for their secretion and as such, NILR1 has a signal peptide in its predicted structure. The presence of an extracellular domain is where potentially a nematode PAMP of protein nature binds and it consists of 22 tandem LRRs residues that have a 76 amino acid Island Domain (ID) cysteine cluster with the pattern of Cx2Cx16C between LRR17 and LRR18. Although the precise ligand-binding sites of LRR-RKs are not understood, the extracellular Island Domain (ID) usually plays a more stabilizing role during ligand binding (Torii, 2004). The Ser/Thr kinase domain can be hypothesized to function in signal transfer to the intracellular regions. Generally, the structure of NILR1 mimics most PRRs that have already characterized for example FLS2 which has all 3 domains. These predicted structures are based on Arabidopsis genome. A BLAST search of amino acid sequence of NILR1 extra cellular domain against non-redundant protein of land plants indicated presence of homologues in different *Brassicaceae* family species and in both monocots and dicots. In our study, NILR1 homologous from tomato *Solanum lycopersicum* (*Sl*NILR1) was transiently expressed in the epidermis of *Nicotiana bethamiana* found to be localized in the plasma membrane suggesting structural similarity to NILR1 (Fig E in S2). In addition, treatment of tomato plant induced a ROS burst similar to that in Arabidopsis suggesting the tomato homologous elicit responses similar to those of AtNILR1. Nevertheless, the functions of these homologues from land plants and their similarity or differences with AtNILR1 remain to be studied. In addition, the PAMPs perceived by NILR1 is yet to be identified.

The potential of Co-immuno precipitation technology has recently being in the fore front in PTI studies especially in demonstrating the ability of a PAMP to physically bind to the receptor and the signalling components involved. This method can be used to characterize the PAMP in NemaWater that binds to NILR1 as well as the signalling cascade that follows after perception. The successful transformation of NILR1 into an expression vector and further expression in Arabidopsis is the first step towards attaining this objective (Fig A to D

of S2). In addition, NILR1 over expression lines are important in studying the physiological and functional changes due to NILR1 gene.

In confirmation to already available reports, BAK1 is involved nematode induced PTI. While there are reports of PTI induction independent on BAK1, knocking out mutation of the BAK1 gene rendered the plant hyper susceptible in our study. Since previous studies had reported similar findings due to root knot nematodes, our results showing the involvement of BAK1 in PTI induced by cyst nematode confirmed that BAK1 role is conserved. In all reported cases, BAK1 acts as a co-receptor to a stable PRR forming a complex. Using co-immunoprecipitation and western blotting techniques, we managed to pull down BAK1 and its associated proteins which froms a complex upon nematode perception. Protein-protein interaction studies have so far used these methods successfully to decipher formation of a complex induced by pathogens such as bacteria and fungi (Chinchilla *et al*., 2007; Albert *et al*., 2015). In our case, deducing the identity of the protein components of the BAK1 complex pulldown is underway.

CONCLUSION

Here we report a comprehensive study into basal immunity by nematodes. Characterization of NILR1 as the first ever reported nematode PRR is a broad step towards understanding PTI in plants induced by nematode among other receptors. In addition, implication of CRKs, NEKs, and LRRs in nematode Induced PTI potrays presence of multiple receptors that plays a role in nematode perception and the gene families mainly involved. Through the receptors described, it is possible now to screen for their perceived NAMPs. Interestingly, just like other reports, PSKR1 was observed to be a nematode triggered PTI regulator other than inducer a similar scenario to ther patho system. This demonstrates the similarity of nematode responses to other pathogens and the conserved nature of innate immunity in plants. The successful pull down of BAK1 complex on the other hand, was the first step towards identifying its interactors during nematode perception. A mass spectrometry analysis of the complex peptides would be required to identify the protein components involved which could further drive the understanding of BAK1 dependents nematode molecular signalling. In addition, it might as well show how immunity is regulated and players involved.

These findings bring us closer to understanding plant immunity and PRRs involved in PTI against nematodes. Since PAMP receptor molecules differ among plant species, the heterologous expression of PAMP recognition systems has been used to engineer broad-spectrum disease resistance to important bacterial pathogens. Increased resistance has been obtained using this strategy against a range of bacterial diseases in both monocots and dicots. Similar strategy can be utilized to generate transgenic plants expressiong PRRs that perceive nematodes which would confer fitness against parasitic nematodes.

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ANNEXES

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

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S1 Text

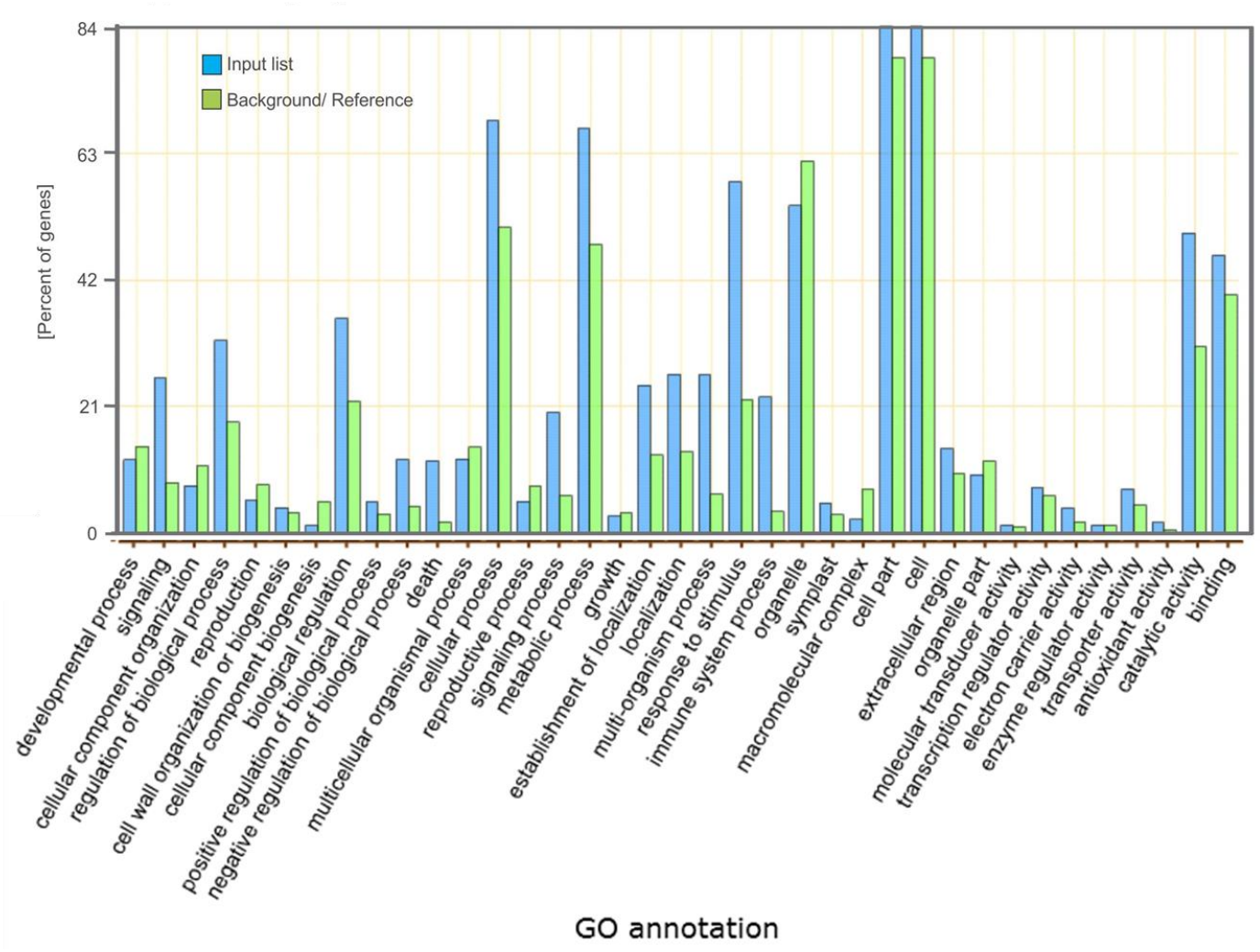


Fig A: GO categories preferentially upregulated during migratory stages of nematode infection.

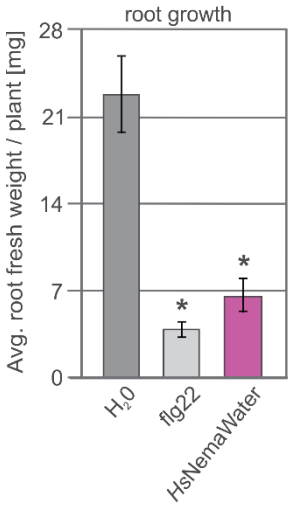


Fig B: Inhibition of root growth upon NemaWater treatment. 5-day-old Col-0 seedlings were incubated in water, flg22, or *Hs*NemaWater for seven days. Fresh weight of root was measured at 12 days after germination. Data were analyzed using *t-test*. Asterisk represent significant difference to water-treated control root segments (P<0.05). Hs, *Heterodera schachtii*.

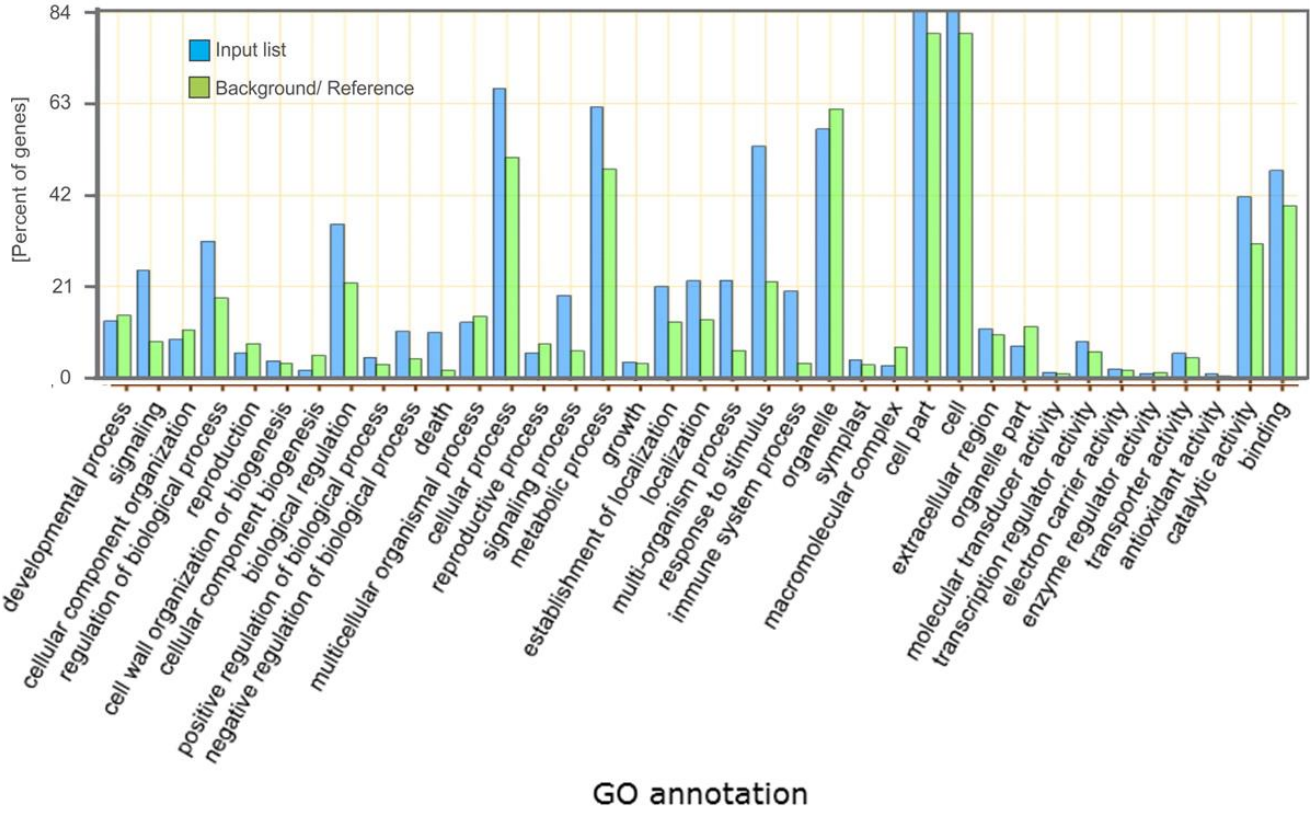


Fig C: GO categories preferentially upregulated upon NemaWater treatment.

Fig D: An illustration of our method for cyst nematode counting. Each Petri dish 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.

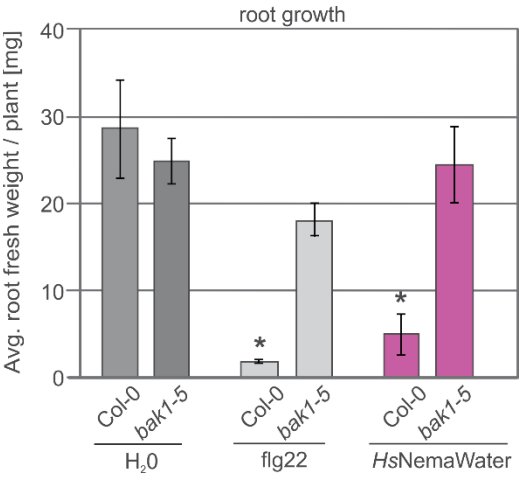


Fig E: Growth inhibition was impaired in *bak1-5* upon NemaWater treatment. 5-day-old Col-0 and *bak1-5* seedlings were incubated in water, flg22, or *Hs*NemaWater for seven days. Fresh weight of the root was measured at 12 days after germination. Data were analyzed using single-factor ANOVA and Dunnet's post hoc test ($P<0.05$).

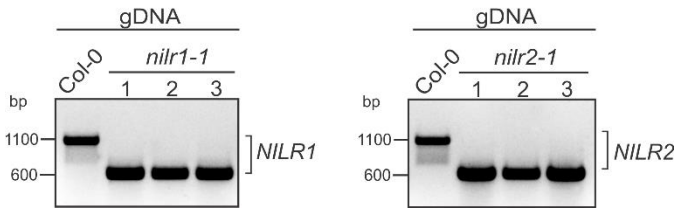


Fig F: Genotyping of NILR1 and NILR2 mutants. Genomic DNA of Col-0 or knockout lines (*nilr1-1*, *nilr2-1*) was PCR amplified using primers given in Dataset 6. The presence or absence of intact wild-type allele is shown.

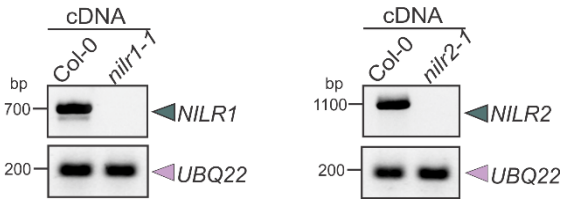


Fig G: RT-PCR for presence or absence of gene expression in Col-0 or knockout mutants. RNA from Col-0 or knockout lines (*nilr1-1*, *nilr2-1*) was extracted to synthesize single stranded cDNA. The presence or absence of expression is shown using primers given in Dataset 6. The upper and lower panel run separately.

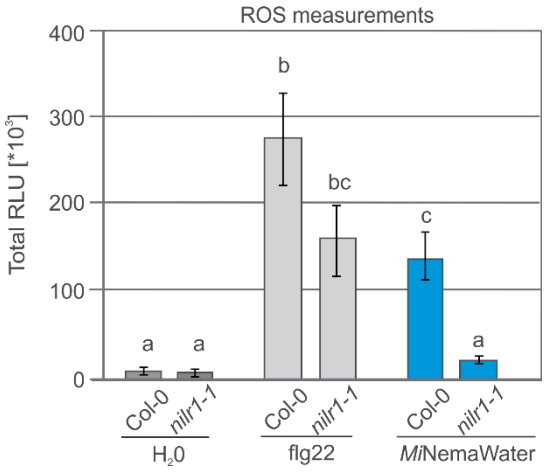


Fig H: Knocking of *NILR1* impair ROS burst to *Mi*NemaWater. Root segments from Col-0, and *nilr1-1* plants were treated with water, flg22 or NemaWater from *M. incognita* (*Mi*NemaWater) and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for twelve biological replicates. Columns sharing same letter are not statistically different.

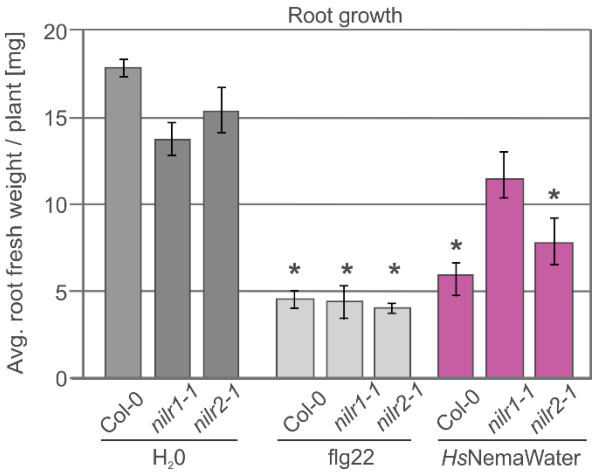


Fig I: NemaWater-induced growth inhibition was reduced strongly in *nilr1-1*. 5-day-old Col-0, *nilr1-1and nilr2-1* seedlings were incubated in water, flg22, or NemaWater for seven days. Fresh weight of the root was measured at 12 days after germination. Data were analyzed using single-factor ANOVA and Dunnet's post hoc test ($P<0.05$).

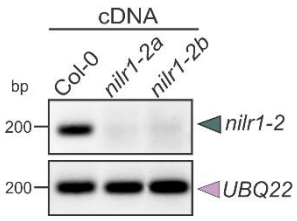


Fig J: Expression analysis of *nilr1-2* mutants. RT-PCR for presence or absence of gene expression in Col-0 or knockout mutants. RNA from Col-0 or knockout line (*nilr1*-2) was extracted to synthesize single stranded cDNA. a and b represent two independent plants. The presence or absence of expression is shown using primers given in Dataset 6.

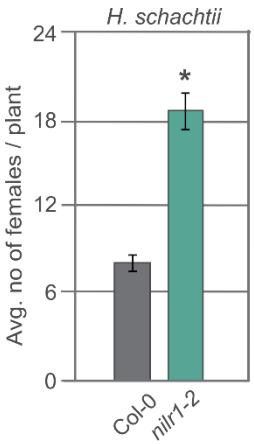


Fig K: Knock-out *nilr1-2* enhances susceptibility to nematodes. Average number of female nematodes per plant in Col-0 and *nilr1-2*. Bars represent mean ± SE for six biological replicates.

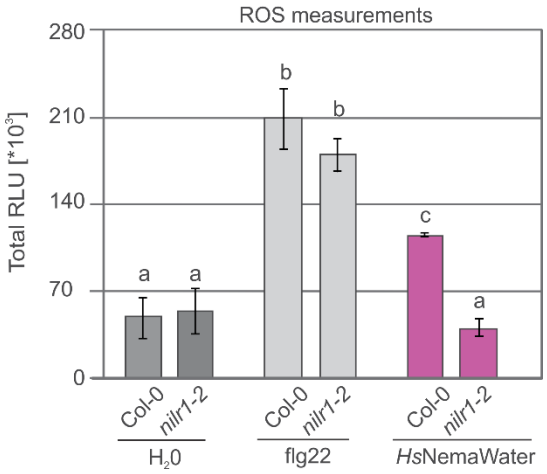


Fig L: Knock-out *nilr1-2* enhances susceptibility to nematodes. Root segments from Col-0, and *nilr1-2* plants were treated with water, flg22 or NemaWater from *H. schachtii* (*Hs*NemaWater) and ROS burst was measured using L-012 based assay from 0 to 120 min. Bars represent mean ± SE for three technical replicates. Experiment was repeated three times with similar results.

Signal peptide 1

TMVTRVIMTDDDSQSLCFLCFLFFFITAIAVAG

N-Terminus

35

DSLDSDREVLLSLKSYLESR

NPQNRGLYTEWKMENQDVVC

QWP GIICTPQRSR

LRR domain 1-17

88

VTGINLTDSTISGPLFKNFS

ALTE

LTYLDLSRNTIEGEIPDDLS

RCHN

LKHLNLSHNILEGELSLPGL

SN

LEVLDLSLNRITGDIQSSFP

LFCNS

LVVANLSTNNFTGRIDDIFN

GCRN

LKYVDFSSNRFSGEVWTGFG

R

LVEFSVADNHLSGNISASMF

RGNCT

LQMLDLSGNAFGGEFPGQVS

NCQN

LNVLNLWGNKFTGNIPAEIG

SISS

LKGLYLGNNTFSRDIPETLL

NLTN

LVFLDLSRNKFGGDIQEIFG

RFTQ

VKYLVLHANSYVGGINSSNI

LKLPN

LSRLDLGYNNFSGQLPTEIS

QIQS

LKFLILAYNNFSGDIPQEYG

NMPG

LQALDLSFNKLTGSIPASFG

KLTS

LLWLMLANNSLSGEIPREIG

NCTS

LLWFNVANNQLSGRFHPELT

RMG

Island domain I

493

SNPSPTFEVNRQNKDKIIAGSGECLAMKRWIPAEFPPFNFVYA
ILTKKSCRSLWDHVLKGYGLFPVCSAGSTVRTLKI

LRR domain (18-22)

SAYLQLSGNKFSGEIPASIS
QMDR
LSTLHLGFNEFEGKLPPEIG
QLP
LAFLNLTRNNFSGEIPQEIG
NLKC
LQNLDLSFNNFSGNFPTSLN
DLNE
LSKFNISYNPFISGAIPTTG
QVAT
LXXL[DN]
LSXNX[FIL][STE]
GX[FIL]
PX[SE][FIL][SG]RQNXX

690

FDKDSFLGNPLLRFPSFFNQSGNN
TRKISNQVLGNRPRT

Transmembrane domain

729

LLLIWISLALALAFIACLVVSGIVLM

Ser/Thr kinase

755

VVKASREAEIDLLDGSKTRHDMTSSSGGSSPWLSGKIKVIRL
DKSTFTYADILK
ATSNFSEERVVGRGGYGTVYRGVLPDGRE
VAVKKLQREGTEAEKEFRAEMEVLS
ANAFGDWAHPNLVRLYGWCLDGSEKILVH
EYMGGGSLEELITDKTKLQWKKRID
IATDVARGLVFLHHECYPSIVHRDVKASN
VLLDKHGNARVTDFGLARLLNVGDS
HVSTVIAGTIGYVAPEYGQTWQATTRGDV
YSYGVLTMELATGRRAVDGGEECLV
EWARRVMTGNMTAKGSPITLSGTKPGNGA
EQMTELLKIGVKCTADHPQARPNMK
EVLAMLVKISGKAELFNGLSSQGYIEM

Fig 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.

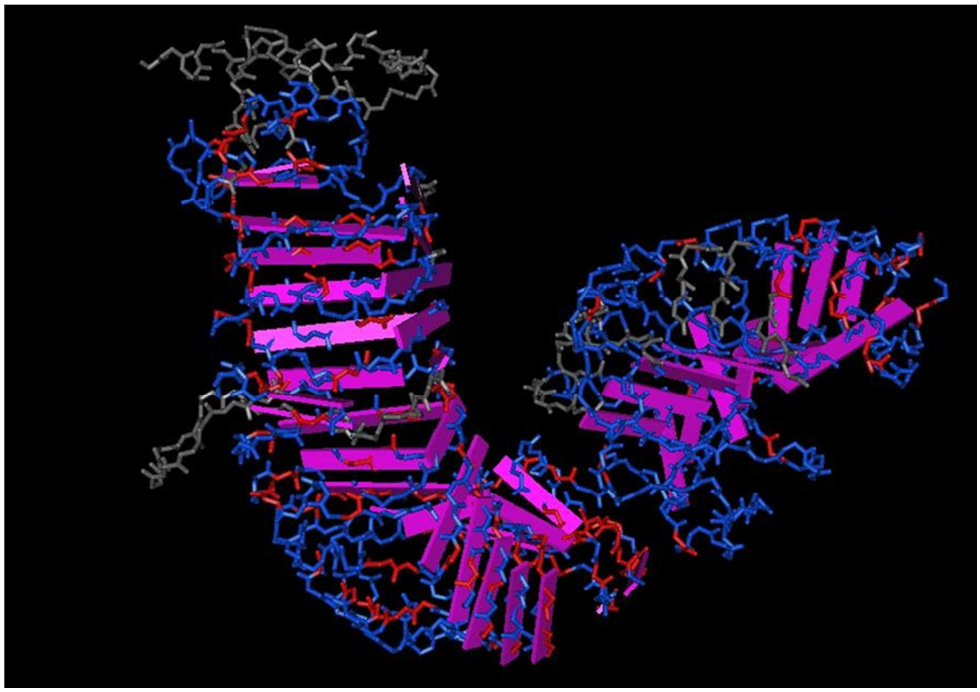


Fig N: A putative structural model for ECD of NILR1. The model was built using BRI1 as template. Conserved and similar residues between BRI1 and NILR1 are highlighted as red or blue respectively. Grey color represents additional residues. White dashed box represent Island domain

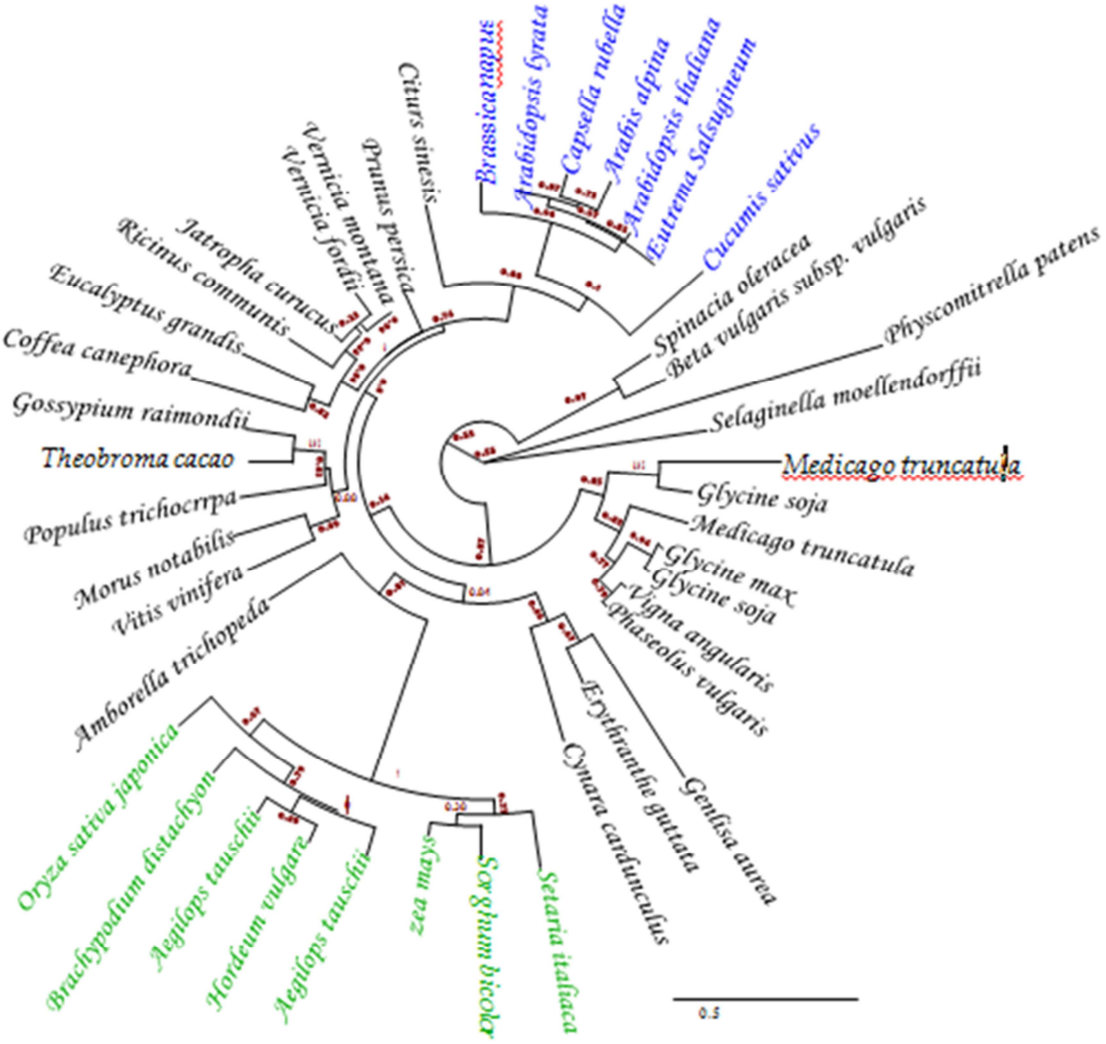


Fig O: Conservation of NILR1 in land plants. A phylogram tree generated from maximum-likelihood trees construction method based on alignment of sequence spanning NILR1's ECD. The number next to each branch (in brown) indicate a measure of support. The number varies between 0 and 1 where 1 represent maximum.

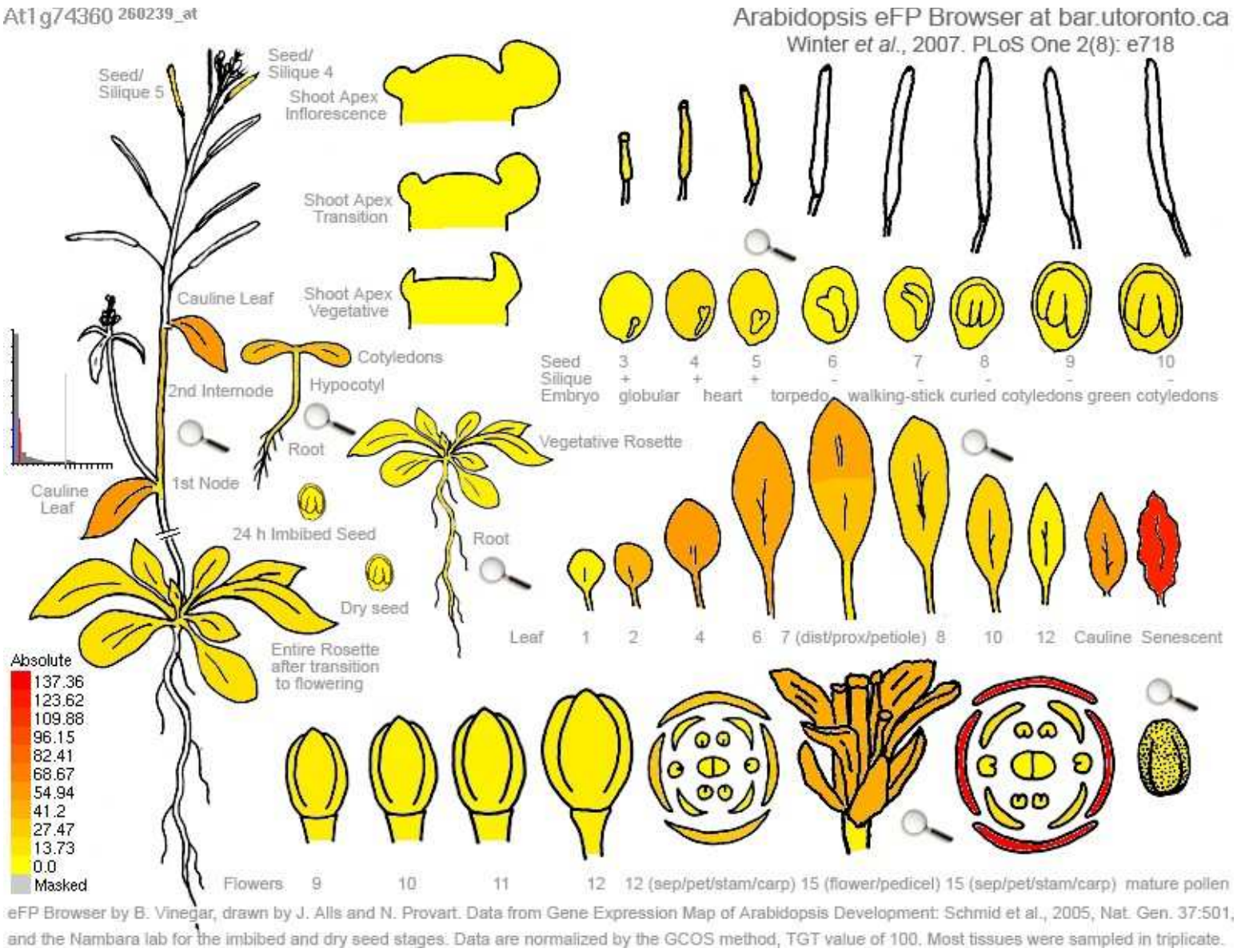


Fig P: Expression of NILR1 during development stages of plants.

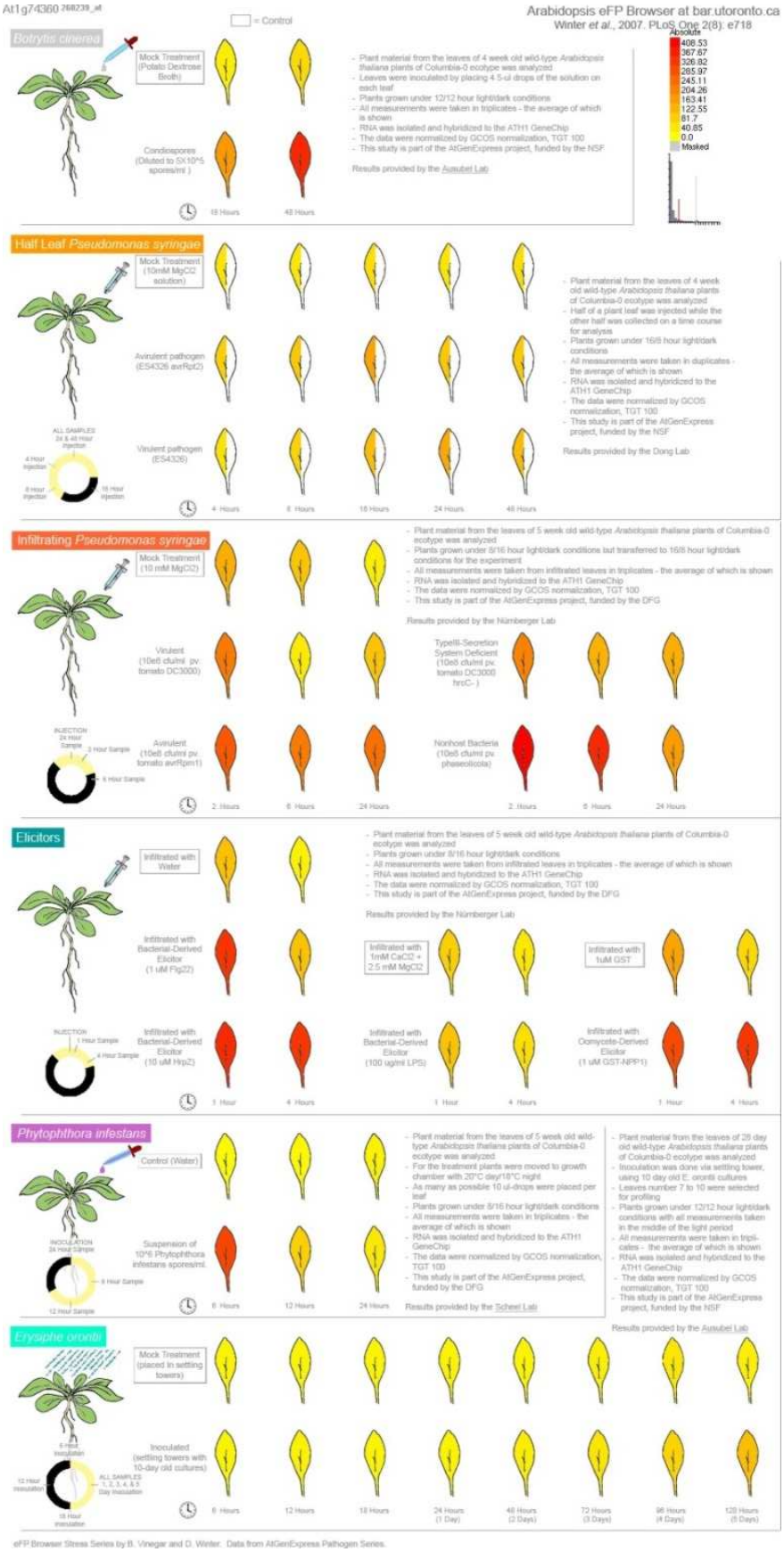


Fig Q: Expression of NILR1 under different biotic stress conditions.

S2

(i)

NILR1 Without stop codon

Forward primer

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGACTATGGTGACGCGTGTG

Reverse primer

GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTTCTATGTAACCTTGTGAAGATAAG

NILR1 With stop codon

Forward Primer

GGGGACA AGT TTG TAC AAA AAA GCA GGC TGCATGACTATGGTGACGCGTGTG

Reverse primer

GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACATTTCTATGTAACCTTGTGAAGATA
AG

(ii)

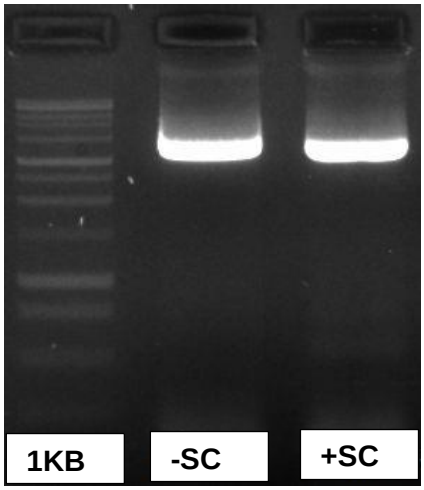


Figure A: **Amplification of NILR1 gene**. RT-PCR using 2 pairs of primers (i) with one amplifying the gene with stop codon (+SC) and another without (-SC) from wildtype Col0 genomic DNA (ii).

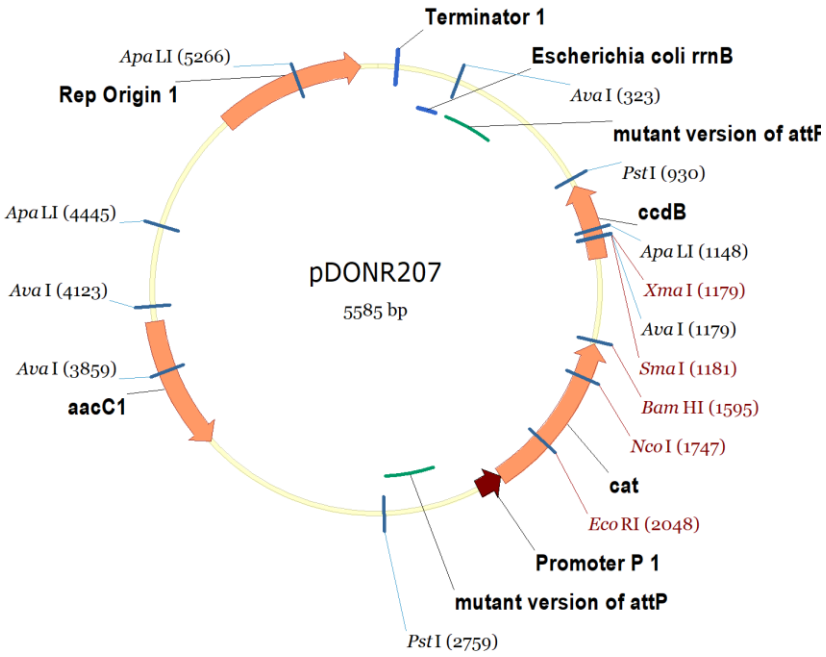
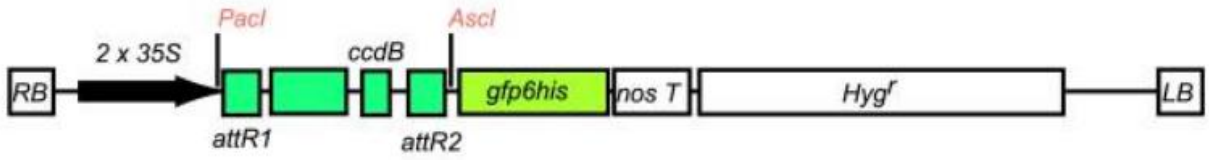


Figure B. **A genetic map of the donor vector used to deliver NILR1 gene into destination vector.** The amplified NILR1 fragments were cloned into pDONR207 using BP clonase (Invitrogen) according to manufacturer's instructions separately.

PMDC 83



PMDC32

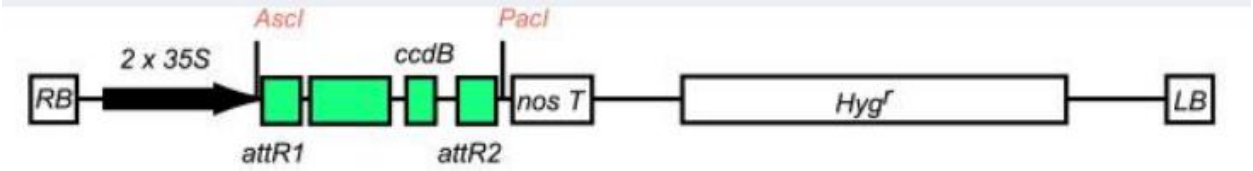


Figure C: **A map of destination vector used to over express NILR1**. BP cloning resultant pENTRY vectors (pENTRY/NILR1-sc) and (pENTRY/NILR1-sc) were used to clone NILR1 into the destination vector pMDC83 and PMDC32 respectively, via Gateway LR cloning using LR clonase (Invitrogen) according to manufacturer's instructions. In both cases, the gene replaced the ccdB gene and were regulated by the strong 35s promoter. Both vectors conferred Hygromycine resistance for selection of transformants

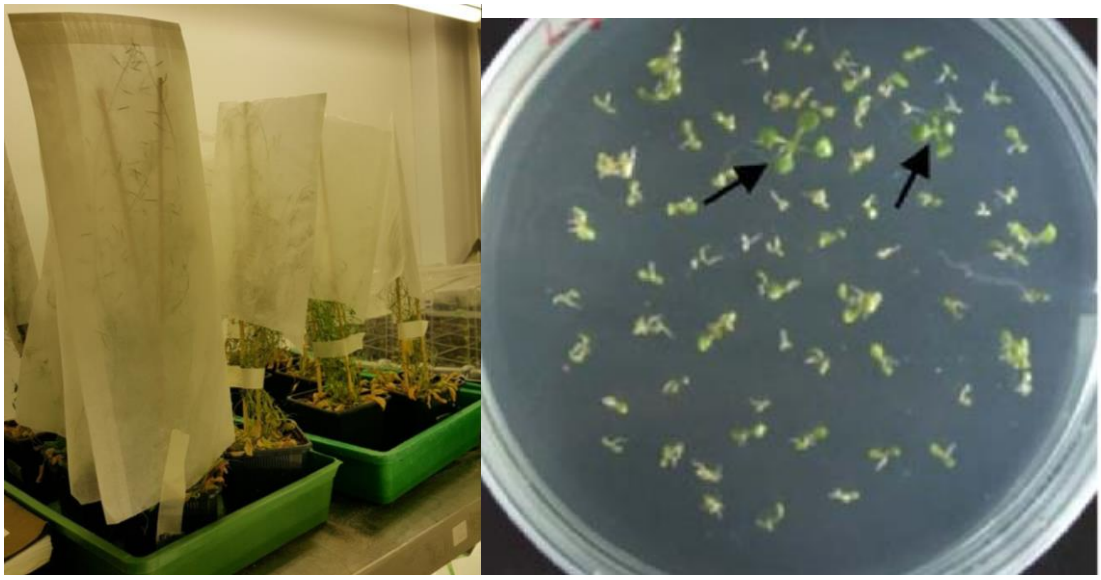


Figure D: **An illustration of transformation of Arabidopsis with NILR1**. The expression vector pMDC83 35S:NILR1-GFP and PMDC32 35S:NILR1 were transformed into Agrobacterium strain GV3101 to generate NILR1-GFP and NILR1 over expression lines respectively. *In vivo* transformation of Arabidopsis thaliana Col0 wildtype plants with each vector was performed via floral dipping. These plants were labelled as T0 and upon maturity, they produced T1 seeds. T1 seeds were selected in 25mg/ml hygromycine and the germinated plantlets transferred to soil. T1plants produced T2 seeds which were selected on hygromycine. Each transformant was transplanted into soil and each plant´s T2 seeds harvested individually.

(i)

GWAY-SlNILR1-F

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCAGAAGAGGAATCTGATATTCTTC
TTCT

GWAY-SlNILR1-R

GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAAATGAAGGAGAAGTGCTACGACT

(ii)

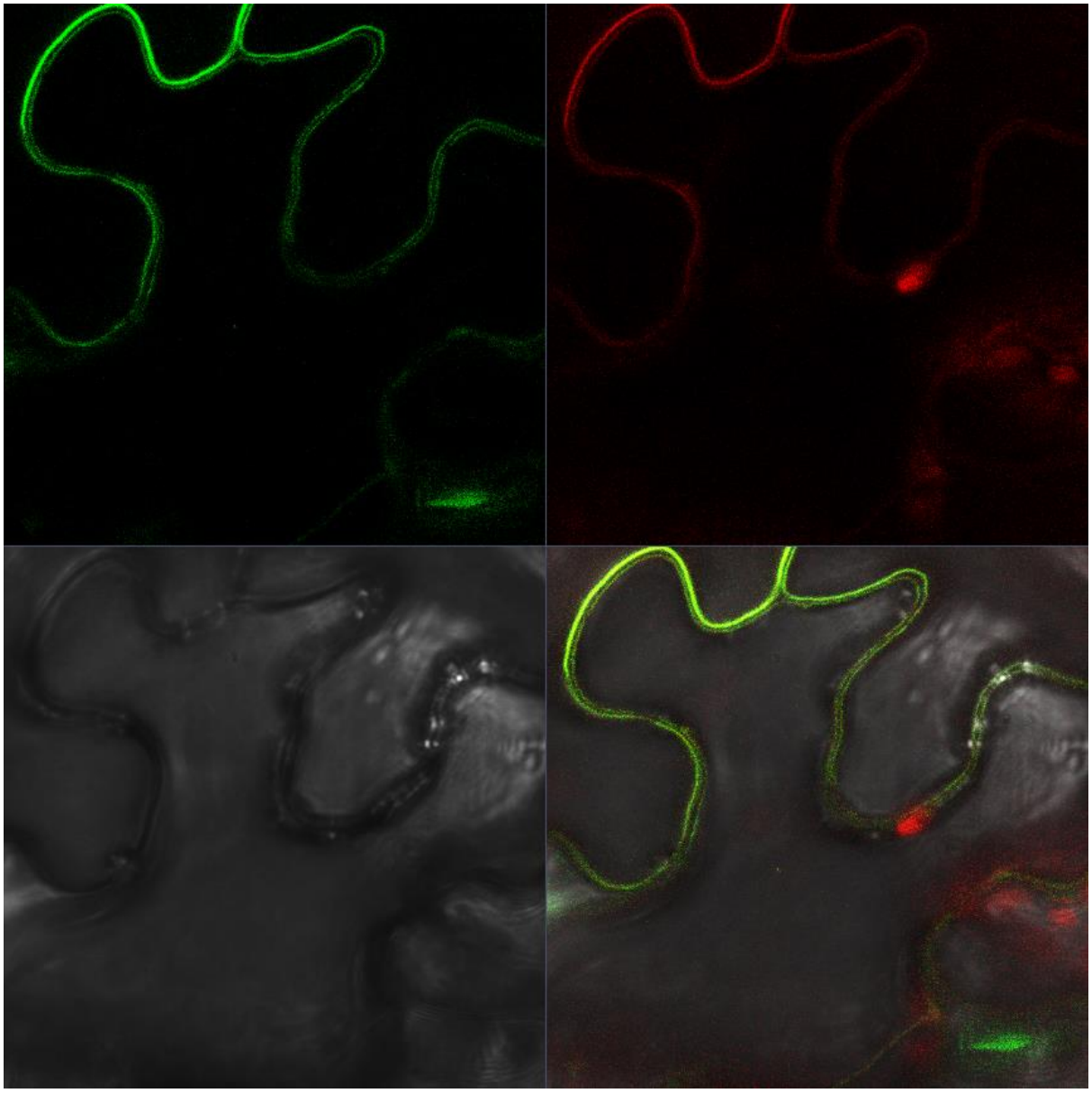


Figure E. **Localization SlNILR1 homologoue from tomato in epidermis of *Nicotina bethamiana***. *Sl*NILR1 gene was amplified using the primers (i). Amplified SlNILR1 gene was cloned into PMDC 83. The expression vector 35S:*Sl*NILR1-GFP was transformed into Agrobacterium strain GV3101 and co-infiltrated together with a plasma membrane mCherry marker 35S:PIP2A-mCherry into epidermis of 6-week old *Nicotianna benthamiana* leaves. The GFP and mCherry signal was detected using a confocal microscope (Zeiss CLSM 710). Similarly to mCherry plasma membrane marker (red), SlNILR1-GFP signal (Green) was detected on the plasma membrane where both signals overlapped (yellow).

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Publications

2017 Mendy B*, **Wang'ombe MW*,** Radakovic SZ, Holbein J, Ilyas M, Chopra D, Holton N, Zipfel C, Grundler FMW, Siddique S. Arabidopsis leucine-rich repeat receptor–like kinase NILR1 is required for induction of innate immunity to parasitic nematodes. PLOS Pathogens, 13: e1006284 (*First authors)