Identification and Characterization of PAMP/NAMP Receptors Recognizing Nematodes.

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of International Master of Science in Agro- and Environmental Nematology

Academic year: 2018 – 2020.

PREAMBLE OF MASTER'S THESIS IMPACTED BY CORONA MEASURES

Thesis Topic: Identification and Characterization of PAMP/NAMP Receptors Recognizing Nematodes.

This research aimed to identify and characterize more of the putative PRR genes to understand better what happens when a nematode invades a plant and how plant defends itself. The original thesis work plan included screening loss-of-function Arabidopsis mutants for selected putative PRR genes through an infection assay (at least three independent experiments) with sugar beet cyst nematode, *Heterodera schachtii*. However, prior to this step, the genotyping and expression check analysis is needed to confirm gene knockout in the mutants. The infection assay results would then be used to pick out candidate(s) to proceed to the next steps; the selection is made using the criteria of being significantly more susceptible to *H. schachtii* compared to wild-type genotype; Col-0. The selected candidate(s) is then to be tested further for other nematode-induced Pattern Triggered Immunity (PTI) responses in plants, including apoplastic ROS-burst assays using a well-known immunopeptide, flg22 and expression of PTI-marker genes using qRT-PCR.

However, given the Corona measures and considering the growing time and seed multiplication of the plants, I was only able to perform two replicates of the infection assays with nine selected putative receptor candidates. Additionally, genotyping and gene expression analysis were managed partly. I did not get the chance to do the ROS-burst assays and the qRT-PCR for the PTI-marker gene analysis. Nonetheless, the time away from the wet-lab gave me time to focus on dry-lab. I was able to do in-silico analyses on the putative PRR genes with a focus on establishing evolutionary relationships and characterization of the promising genotypes and also performed gene ontology on the putative PRR genes. I was also asked to write an extended literature review on my topic in place of the results I was not able to achieve; this explains why the thesis has a lengthy review of literature after the topic introduction as opposed to the Nematology Thesis Guidelines that do not require a literature review.

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

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1.SUMMARY

Plant-parasitic nematodes are one of the biggest threats to food security worldwide. A plausible approach to their control starts by understanding how plants perceive and protect themselves against nematodes. Effective plant defense against pathogens relies on the recognition of pathogen-associated molecular patterns (PAMPs) by surface-localized receptors (PRRs), leading to the activation of PAMP-triggered immunity (PTI). Extensive studies have been conducted to identify and characterize PRRs in various models of plant-pathogen interactions. However, not much is known about the PRRs in plant-nematode interactions. Until recently, only one nematode associated PRR has been discovered-NILR1. NILR1 is a membrane-bound Leucine-Rich Repeat protein that recruits BAK1 as a coreceptor for nematode perception. NILR1 was upregulated upon plant inoculation with infective second-stage juveniles of *Heterodera schachtii* and treatment with a one NemaWater, a cocktail of nematode elicitors. The loss-of-function of NILR1 led to increased susceptibility to *H. schachtii* in Arabidopsis. Besides NILR1, the study also showed that other plant genes were upregulated upon exposure to nematode elicitors, and this is the basis of this present study.

Based on the previous study, we picked out nine putative PRR genes that were upregulated at the migratory stage of infection and upon plant exposure to nematode elicitors, NemaWater in microarray analysis. Genotyping and expression analysis was performed to confirm homozygous GABI-KAT, SALK, and SAIL loss-of-function mutant lines. An infection assay was performed on loss-of-function mutants of these genes of interest to investigate their role in innate immunity and nematode resistance. In this work, we found two genes, SD2.5 (AT4G32300) and LRR-TPK (AT1G56120) are important for PTI against *H. schachtii* in Arabidopsis as their loss-of-function mutants showed significantly higher susceptibility. In-silico analysis of putative PRR genes revealed that all genes encode cell membrane-bound proteins, and gene ontology confirmed that at least eight of the nine genes have a biological function of response to biotic stimuli. With bootstrap values below 70%, there was no strong evolutionary relatedness between most of the putative PRR genes to NILR1 or BAK1. However, at 71% and 70%, SD2.5 showed evolutionary relatedness to NILR1and BAK1, respectively. BLAST analysis of SD2.5 and LRR-TPK confirmed the presence of homologs in the Brassicaceae family for both genes and on a more broad perspective in Eudicots only. Of note, however, was the presence of homologs in food crop species of *Brassica oleracea* for both genes and *Arachis hypogea* for SD2.5. Moreover, analysis of the protein motif of both genes on the KEGG Database revealed that SD2.5 has a B-lectin extracellular domain, a S-locus glycoprotein domain, a transmembrane domain and two intracellular domains; the Tyr-Ser-Thr amino acid domain and the kinase domains. LRR-TPK, on the other hand, is made up of nine extracellular leucine-rich domains, a Malectin and Malectin-like domain, a Podoplanin domain, a Tyr-Ser-Thr amino acid domain, and intracellular kinase domains

2.INTRODUCTION

Plant parasitic nematodes are one of the biggest threats to food security worldwide causing yield losses of up to 100B USD per annum (Kranse et al., 2020). The most notorious of these are the sedentary endoparasites comprising of the root knot nematodes and the cyst nematodes to whom belong the most agriculturally important genera of Meloidogyne, Heterodera and Globodera (Cooper & Eleftherianos, 2016). The obligate biotrophic nature of sedentary nematodes necessitates that they depend on living plant tissue for feeding and consequently reproduction towards their survival(Niu et al., 2016). However, the survival of the nematodes in a host plant comes with a hefty price which often results into plant disease and in cases of severe infestation wilting and death. The plant therefore mounts a counteracting defense against nematode infestation to ensure its own survival.

The first line of plant defense against pathogen invasion and establishment is the pattern triggered immunity (PTI). PTI involves the perception of pathogen molecules referred to as Microbial Associated Molecular Patterns (MAMPS)/ Nematode Associated Molecular Patterns (NAMPS) with the aid of surface-localized pattern recognition receptors (PRRs) (Monaghan & Zipfel, 2012) . In resistant plants, successful pattern recognition is followed by a ligand-dependent signaling cascade often marked by Ca^{2+} accumulation (Ranf et al., 2011) and coupled with MAPK signal transduction through the phosphorylation and dephosphorylation of cognate downstream proteins leading to transcriptional changes that effect immune response like ROS burst production, callose deposition, translation of defense genes associated with the Jasmonic acid (JA), Salicylic acid, and Ethylene pathways (Bigeard, Colcombet, & Hirt, 2015). On the other hand, virulent pathogens are capable of evading PTI through production of virulence proteins also known as effectors. Effectors are molecules released by the pathogen into the plant in a bid to frustrate the plant's immune responses and/or to ensure successful establishment of the pathogen (Vijayapalani et al., 2018). For the case of nematodes, effectors have been reported to interfere with PTI through degradation of molecules involved in signal transduction (Zhang et al., 2010) and the same have also been reported to manipulate the plant's genetic machinery to ensure successful establishment in their host through upregulation of plant genes that aid nematode feeding site formation; NFS (Grunewald et al., 2009).

Suppression of PTI by pathogen effectors can however initiate effector triggered immunity (ETI) in pathogen-adapted plants; this is in line with the Zig-Zag model as described by Jones & Dangl (2006). Here, the plant employs resistance genes (R-genes) that perceive avirulence proteins/effectors that may have earlier interfered with PTI (Cui, Tsuda, & Parker, 2015). Mechanism of effector recognition by R-genes in ETI is either through direct recognition where the defense protein attaches itself directly to the effector (Jia et al., 2009) or through indirect surveillance of perturbations in guardee proteins and/or decoys caused by the effectors (Chisholm, Coaker, Day, & Staskawicz, 2006). It is generally agreed that ETI is a more robust immune response than PTI because its hallmark is the hypersensitive reaction (HR) that is associated with cell death around points of infection thereby halting further growth and establishment of the pathogen. With regard to PTI and pathogen perception, a number of microbial associated pattern recognition receptors (PRRs) have been identified and characterized. The Arabidopsis flagellin-sensitive 2 (FLS2) is a well-studied point in case which perceives the widely conserved bacterial MAMP, flagellin (Chinchilla et al., 2007). The AtLYM1-AtLYM3-CERK1 complex and EF-Tu receptor (EFR) which perceives the Elongation Factor Tu in *Escherichia coli* are other examples of bacterial associated PRRs that have been identified and well characterized (Zipfel et al., 2006). The fungal MAMP chitin is reported to be perceived by the chitin elicitor binding protein (CEBiP) (Andrea et al., 2014) and the LysM-domain containing Nod factor receptor proteins; NFR1 and NFR5 (Eckardt, 2008).

To date however, only one nematode associated PRR has been identified and characterized. Mendy et al., (2017) reported that a leucine-rich repeat receptor-like kinase; NILR1 triggers a PTI response evidenced by ROS bursts and upregulation of PTI-defense gene markers upon perception of two nematode species; *Meloidogyne incognita* and *Heterodera schactii* (Mendy et al., 2017). From the above listed examples, it is apparent that little research has been done with regard to PTI and nematode perception in comparison to microbial pathogen perception. In this study, we therefore build on the previous work done in Mendy et al., (2017) and aim to characterize additional putative nematode associated PRRs that were that were reported to be upregulated at migratory stage of infection and upon plant exposure to nematode elicitors, NemaWater in microarray analysis.

3.LITERATURE REVIEW

3.1 Nematodes as organisms

Taxonomically assigned to the phylum Nematoda, also known as Nemata (Liu & Park, 2018), nematodes form the largest metazoan group. With over 20,000 identified species, nematodes can be found in almost any environment (Santos, Cardoso, & Maria, 2019). Nematode abundance in soil, fresh water and marine habitats is therefore proof of their ubiquitous nature. In fact, research reveals that nematodes are also present in the most unlikely areas to be occupied by life forms especially by any eukaryotes like the Antarctic (Tomasel et al., 2013) and in the deep and hot parts of the earth (Borgonie et al., 2013). Morphologically, nematodes are round worm-like animals that are unsegmented and from an ecological perspective, they belong to different trophic levels ranging from bacterivores, fungivores, plant-parasitic and predator (Emery, Reid, & Hacker, 2020), which could explain why nematodes have successfully adapted to living in diverse ecosystems.

The model nematode *Caenorhabditis elegans* is probably one of the best-studied animals. Its short life cycle, small size, easy cultivation and sensitivity to environmental changes are some of the traits that make the nematode a good model for studying a number of biological phenomena (Kruempel et al., 2020). Effects of toxic substances, aging and immune responses in animals are among the many areas of research that have been elucidated using this model organism (Ayech et al., 2020; Mchugh et al., 2020). In line with agriculture and nematodes, interest is directed to three groups of nematodes namely, the free-living nematodes that are used as bio-indicators of soil and environmental health, entomopathogenic nematodes used as biological control agents and the economically important plant-parasitic nematodes. Moreover, the entomopathogenic nematodes (EPNs) are soil-dwelling obligate parasites that are capable of infecting and killing some insect pests. They depend on the insect host to complete their lifecycle and have a specific association with certain bacteria (Eduarda et al., 2019). To date, three different genera of the EPNs have been identified; Steinernema, Heterorhabditis and the Oscheius (Baker, 2019). On the other hand, plant-parasitic nematodes are of particular economic importance because of the plant and crop yield losses associated with them. 4100 species of the plant-parasitic nematodes have been named to date with the biotrophic sedentary nematode species being the most damaging (Quentin et al., 2013).

3.2 Plant-parasitic nematodes (PPNs)

Plant-parasitic nematodes are majorly grouped on the basis of their feeding habits. Depending on whether they feed inside the root or outside of the same, they are categorized as endoparasitic or ectoparasitic, respectively (Liu & Park, 2018; Vieira & Gleason, 2019). Of the different nematode groups, the most economically important groups are the root-knot nematodes and the cyst nematodes which are also referred to as the sedentary nematodes (Cooper & Eleftherianos, 2016); this is because upon successful invasion and establishment of the infective second juvenile stage-J2s, they form feeding sites and the mature females became immobile feeders (Han et al., 2018)- thus the term sedentary. Second in line are the lesion and burrowing nematodes which fall under the migratory endoparasites; these migrate through roots and feed destructively on root cells (Owland & Chreiner, 2014), often killing the cells. Other economically plant-parasitic groups include the semi-endoparasitic (Wubben et al., 2010), the bulb and stem nematodes (Indarti et al., 2018), the seed gall nematodes and the foliar nematodes which also cause a fair share of yield losses although most of these are localized and not spread worldwide. Of note too, however, are the plant-parasitic nematode families that transmit viruses. These belong to the class Enoplea and are polyphagous ectoparasitic feeders in nature. They include; Longidorus, Paralongidorus, Xiphinema, Trichodorus and Paratrichodorus. The Nepoviruses responsible for a broad spectrum of disease in fruits and vegetable is transmitted by the Longidoridae while the Tobravirus causing the Tobacco Rattle disease is transmitted by the Trichodorids (Decraemer and Robbins, 2007).

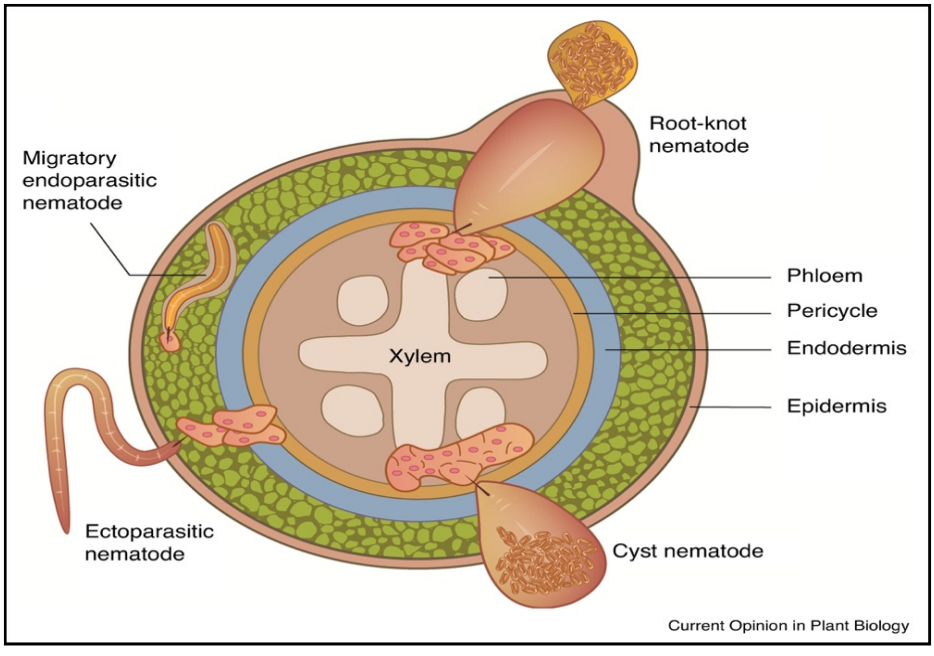


Figure 1: Classification of PPNs According to Feeding Habits. (Vieira & Gleason, 2019).

3.3 Cyst Nematodes (CN)

Cyst nematodes form one of the most economically important groups of plant parasitic nematodes. One report indicated that the CN genus Heterodera alongside the *Meloidogyne* spp are responsible for an estimated 10% decrease in total food production worldwide (Liu & Park, 2018). This particular group of plant parasitic nematodes acquires the name 'cyst nematodes' from the final mature stage of the females whose bodies enlarge to accommodate thousands of eggs. Upon death, the female body hardens to form a protective sheath around the eggs which can then remain viable for over a decade. This egg-containing body mass is referred to as the cyst (Siddique & Grundler, 2018).

As obligate biotrophs, cyst nematodes complete their lifecycle within a living plant. Once environmental conditions are favorable, eggs in the cysts hatch and emerge as J2s that invade the root. Their intracellular migration through the roots is characterized by breaking and puncturing of the plant cell wall (Shah et al., 2017) to reach the vascular tissue. Here, they select a single root cell that is transformed into a specialized feeding cell; the syncytium. After a suitable feeding site has been established, juvenile feeding and moulting occur in parallel to nematode maturation. Approximately two weeks later, they undergo three moults; the males then stop feeding and hatch out of the moults while females continue to feed and enlarge the female-induced syncytia. At full maturity, the males regain vermiform morphology and mate with the females while the females take up a lemon-shaped-body form that protectively houses the eggs. The eggs in the cysts can remain viable for up to over a decade; this is due to the protective covering offered by the cyst. The ability of the cyst eggs to remain dormant but viable for a long period of time in part contributes to the challenges associated with the control and management of cyst nematodes. (Ariyar et al., 2018; Siddique & Grundler, 2018; Vieira & Gleason, 2019).

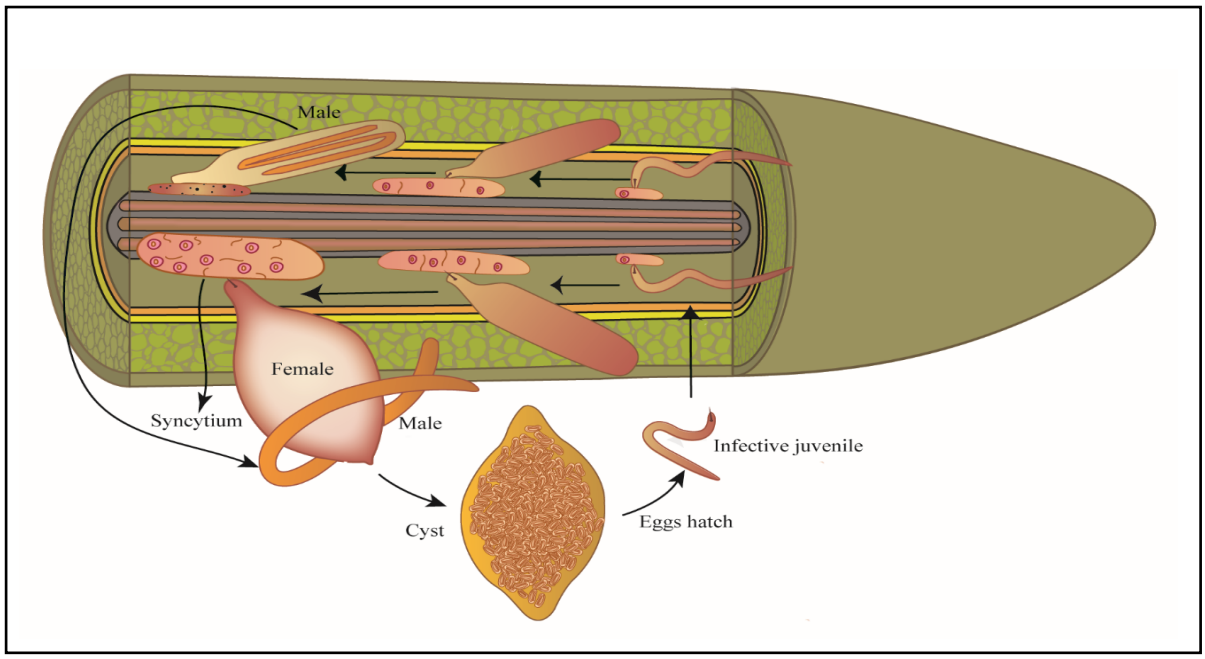


Figure 2: The life cycle of Heterodera schachtii (Siddique & Grundler, 2018).

As the sole food source of the sedentary CNs, the syncytium is referred to as 'the sink'; a term derived from the fact that nutrients from the leaves (the source) are drawn into the syncytium by the cyst nematode to facilitate feeding. Using radioactive carbon (^{14}C-labelled) and fluorescent probe Carboxyflourescent (CF), the Source-Sink Hypothesis was proven by Bockenhoff and colleagues; unidirectional translocation of these labelled molecules into the syncytium in the roots from the leaves where they were initially administered. To further investigate if the syncytium was the final destination for nutrients pooled from the leaves, the labelled molecules were introduced directly into the syncytium using microinjections and true to their hypothesis, the molecules were retained in the syncytium without moving out into neighboring cells (Bockenhoff et al., 1996). For syncytium enlargement, the selection of a single parenchyma or cortex cell is closely followed by the recruitment of neighboring cells through breakdown of adjacent cell walls and consequent fusion of the protoplast resulting in one large multinucleated cell; the syncytium. Structurally, syncytia have been reported to have dense cytoplasm, small vacuoles, multiple nuclei characterized with the generation of the smooth endoplasmic reticulum, mitochondria, ribosomes and plasmids. A single mature syncytium can accommodate a staggering number of about 200 nuclei (Kyndt, Vieira, & Gheysen, 2013). Besides the astounding structure of these specialized feeding cells, the increased metabolic activity therein is noteworthy. A phloem specific sucrose transporter gene-AtSUC4 was reported to play a crucial role in the early establishment and metabolic activity of

the syncytium, specifically in line with cell differentiation and supply of sucrose. Knocking-out AtSUC4 led to a significant decrease in the size of the females, confirming the importance of this gene in nutrient withdrawal (Grundler et al., 2007). For syncytium maintenance, virulent nematodes continue to subdue the plant's immune responses to facilitate their obligate biotrophic nature. In cyst nematodes, this is achieved by suppression of the salicylic acid and ethylene defense pathways. For example, with the aid of an effector- 10A06, cyst nematode *H. schachtii* has been reported to interact with the Arabidopsis spermidine synthase protein and this association is believed to be geared towards repression of SA-responsive genes (Hewezi et al., 2010).

3.4 Nematode control strategies

Numerous nematode control and management options have been investigated and implemented. These include chemical, physical, cultural and biological options (Jang et al, 2019; Ji et al., 2019; Sikandar et al., 2019). However, more often than not, it is challenging to get excellent results through the employment of one single control/management method. This is why an Integrated Pest Management (IPM) strategy is advised (Dixit, 2019). Comparatively, the single-handedly most effective control and management option is the chemical control (Ji et al., 2019). Sadly, this control strategy has a fleet of downsides to it (Aktar et al., 2009), environmental safety being at the center stage (Sasanelli et al., 2014) of the issues that arise concerning the use of chemical control strategies. With the increasing awareness of the negative effects of synthetic chemical control, the end of an era became more oblivious with the banning of many nematicides on the European market (Donley, 2019) and across other regions in the world (Mahere et al., 2014).

Contrarywise, with intensified research , biological control has gained popularity over the years due to its scientifically proven efficacy against target pests/pathogens and being environmentally friendly (Siddiqui & Mahmood, 1997). Biocontrol agents work through parasitism, production of toxins and enzymes that are harmful to the nematodes, competition for nutrients, conferring of systemic resistance to plants and promotion of plant health (Tian et al., 2007). For example, the fungi *Pleurotus eryngii* was reported to be effective against *Meloidogyne javanica* eggs. Through proteolytic and chitinolytic enzyme activity, the mushrooms and the extracts of the same reduced the number of intact *Meloidogyne javanica* eggs by 53% (Leite et al., 2017). Egg parasitizing fungi *Pochonia clamydosporia* and

Peocilomyces lilacinus have also been proven to be effective against plant-parasitic nematodes (America, 2004). Unfortunately, notwithstanding all the pros attached to biological control, the exponential adaption of this control technique is cut short majorly by a labor-intensive production process, technical difficulties associated with identification, characterization and upscaling of antagonistic organisms and the hefty cost of production (Paper et al., 2009). These later translate into the high price of the end product, making biological control option impractical especially in low income communities (Angbenin, 2016).

Besides biocontrol, pest management through the use of nematode-resistant plant cultivars is also largely vouched for (Mendy et al., 2017). Resistance to pathogens in plants is often controlled by one gene; the R-gene (Williamson, 1999). Resistant tomato cultivars carrying the *Mi-1* gene are an example of successful breeding for resistance against an array of plant-parasitic nematodes. The *Mi-1* gene confers resistance to three different Meloidogyne species of ; *arenaria, javanica and hapla* (Jablonska et al., 2007; Ox et al., 2015). Other resistance genes that have been used for resistance against nematodes from various sources include, HSI^{pro-1} from sugar beet, *Gpa2* and *Gro1* from potato, *Hero* from tomato and *Cre3* from wheat (Poch et al., 2006).

3.5 Plant Immune System and Pathogen Recognition

Unlike animals, plants do not possess mobile defender cells that can be mobilized to provide defense against a pathogenic attack on a cell(s) (Chisholm et al., 2006; Jones et al., 2013). Plants depend on a two-tier immune system characterized by innate immunity for a single cell and/or systemic acquired resistance where a plant is primed prior to a potential pathogenic intrusion. This 2-branch innate immunity is made up of the Pattern Triggered Immunity (PTI) and the Effector Triggered Immunity where the former is predominately associated with pathogen perception by membrane-bound pattern recognition receptors and the latter by intracellular Nucleotide Binding Receptor proteins also known as R-Proteins (DeYoung and Innes, 2007).

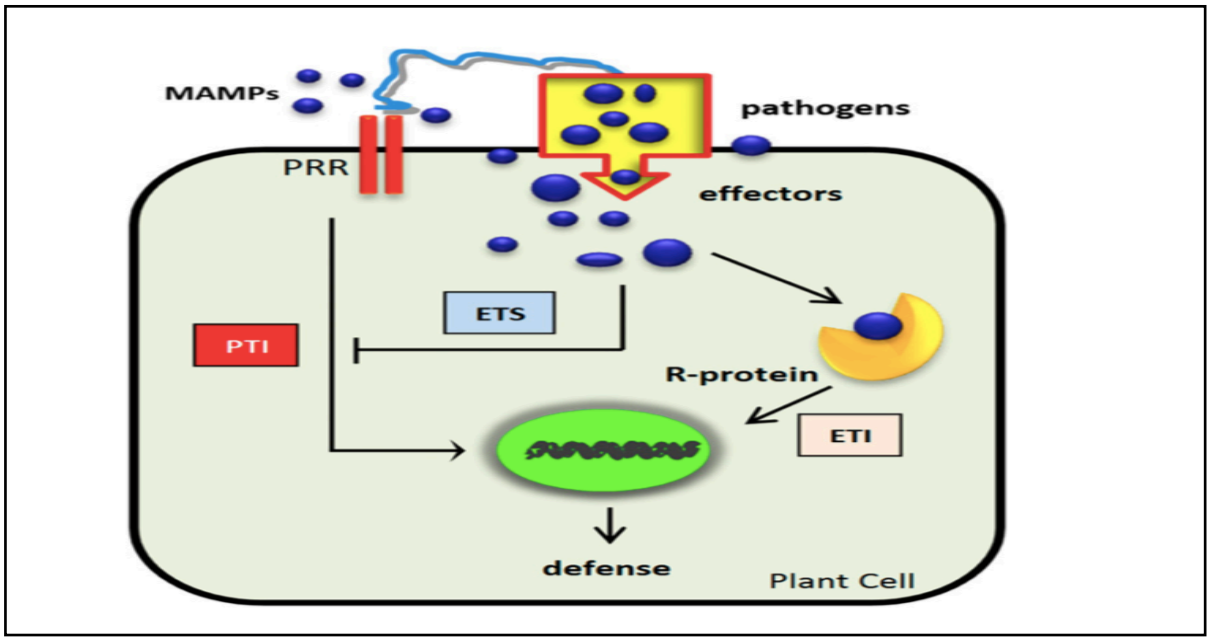


Figure 3:The Host-Pathogen Molecular Interaction. This face-off is referred to as the Zig- Zag model (Jones & Dangl, 2006).

PTI is initiated when a plant recognizes a non-self-invader (Bigeard et al., 2015) or a self-origin molecule in form of DAMPs (Tang et al., 2017). Pathogen associated molecular patterns dubbed PAMP or NAMP (Nematode Associated Molecular Pattern) for the case of nematodes (Choi & Klessig, 2016) are detected with the aid of plasma membrane-bound pattern recognition receptors; PRRs (Mott et al., 2017). PAMPs are molecules produced by pathogens and are vital for their own survival. For example, bacteria produce flagellin and fungal molecules lipopolysaccharides and peptidoglycans that make up fungal chitin are recognized by plants (Newman et al., 2013). These molecules are evolutionarily conserved over a broad grouping of the pathogens (Ye & Murata, 2016). For instance, nematodes have an analogous NAMP-ascarosides, that is conserved across free-living nematodes and the plant-parasitic genera alike (Gao et al., 2019). Ascarosides have been identified in nematodes as the molecule associated with kickstarting basal defense responses in plants (Klessig et al., 2019) upon recognition. However, recent research has brought to light a novel species-specific NAMP in the Pinewood nematode *Bursaphelenchus xylophilus*. Authors reported the BxCDP1 molecule as a NAMP that elicits PTI response in a number of plants like tobacco, Arabidopsis, and pine among others proving that the corresponding PRR must be a widely conserved protein in higher plants. Additionally, the reduced immune responses in the *bak1* mutants upon treatment with

BxCDP1 provided further evidence that this molecule is identified by a yet-to-be-identified LRR-RK or LRR-RLP that recruits BAK1 as a co-receptor (Hu et al., 2020).

As earlier mentioned, one of the key proteins in PTI response are the surface-localized pattern recognition receptors (PRRs) that kickstart the immune response upon pattern recognition. It should be noted however that these membrane-bound PRRs are capable of not only perceiving PAMPS/NAMPS but also detecting DAMPs (Damage Associated Molecular Patterns). DAMPs are plant molecules released as products of damage on plant cells; the molecules released could range from extracellular protein fragments to peptides, nucleotides and even amino acids (Hou et al., 2019). A point in case is the cyst nematodes that move intracellularly within the plant tissue breaking down the cell wall. The breakdown of cell walls releases a DAMP called oligogalacturonides that triggers a PTI reaction in plants upon its recognition by the PRR (Shirasu, 2019).

Contrastingly, ETI in plants emanates from the pathogen's ability to adapt over time and overcome the plant's PTI, ETI therefore kicks in upon PTI suppression by effectors. Jones and Dangl (2006), described this pattern as the Zig-Zag model which is roughly encapsulated into four phases. The first phase involves the recognition of PAMPs by the cell surface PRR leading to PTI. At phase two, the pathogen adapt/evolve and acquire effectors which counter the PTI making the previously resistant plant susceptible; this can also be referred to as Effector Triggered Susceptibility-ETS . Phase 3 is characterized by the bounce-back of the plant where it recognizes effectors using Nucleotide Binding-Leucine Rich Repeat proteins/receptors; NB-LRR (NLR) encoded by R-genes (DeYoung and Innes, 2007) . Successful detection of effectors ends in a NLR-triggered immunity aka ETI (Sato et al., 2019) birthing a hypersensitive reaction associated with programmed cell death. Natural selection plays a role in phase four by drawing the pathogen to escape ETI either through extinction/modification of already recognized effectors or by acquiring completely new effectors (Jones et al., 2006).

ETI-associated R proteins, are multi-domain receptors also known as Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR) (Lu et al., 2016). These proteins function as intracellular pathogen recognition receptors that are made up of three building blocks; the N-terminal domain, central NB-ARC domain and the C-terminal LRR domain (Jacob et al., 2013). The N-terminal domain can further be categorized into the TOLL/Interleukin 1 receptor (TIR) domain

and the Coiled-coil (CC) domain. These two categories are responsible for the two distinct types of plant NLRs notably; the TIR-type NLRs (TNLs) and the CC-type NLRs(CNLs) (Griebel et al., 2014)

Effector recognition by R-proteins is done either through direct or indirect recognition. With the direct recognition mechanism, the protein gets into direct physical contact with the effector which in turn leads to a cascade of downstream immune responses post effector perception. An example of this mode of recognition was reported in the molecular interaction between rice and the rice blast causal agent; *Magnoporthae oryzae* where the rice R-gene PI-TA encodes a protein that directly binds to fungal effector AvrPita (Jia et al., 2009) making rice resistant to this particular fungal strain.

The indirect recognition mechanism is characterized by the 'surveillance' action of the R-genes where they monitor perturbations and modifications in cognate guardee proteins or decoys that are targeted by the effectors . In *Arabidopsis thaliana*, the RPS-5 gene indirectly perceives the *Pseudomonas syringae* effector AvrPphB when it cleaves to the *A. thaliana* guard protein PBS1 (Chisholm et al., 2006). Another example of the indirect recognition of effectors was reported in tomato where the fungal effector Avr2 is recognized by the R-gene Cf-2. Rcr3 is monitored by Cf-2; upon binding and inhibition of the former by Avr2, the former is activated (Benvenisty et al., 2005). While it was previously thought that R-gene and effector association was specific with one gene being capable of perceiving only one effector, famously hypothesized as the gene-for-gene phenomenon , research has proven that one R-gene is capable of perceiving more than one pathogen especially through indirect recognition. Lozano-torres et al. reported the dual resistance conferred to tomato by Cf-2 through perception of two separately evolved effectors of fungus and nematode. The leaf mold fungus, *Cladosporium fulvum* and the cyst nematode, *Globodera rostochiensis* share a similar virulence target in tomato; $Rcr3^{pim}$. Effector-induced perturbation of Cf-2-monitored $Rcr3^{pim}$ by either the Gr-VAP1 or the Avr2 effectors therefore leads their recognition and consequently ETI in tomato (Lozano-torres et al., 2012).

3.6 Plant Cell Surface Receptors (PRRs)

Pattern recognition receptors (PRRs) are surface-localized plant proteins that are either receptor kinases (RK) or receptor-like proteins (RLP) (Li et al., 2016). The structure of the receptor kinases is made up of an extracellular ligand-binding domain, a single-pass transmembrane

domain and an intracellular kinase domain. While the receptor-like proteins (RLPs) have a similar structure to that of the RKs, they lack any obviously known intracellular signaling domain (Boutrot & Zipfel, 2017). The extracellular domains of the PRR are often used to categorize and group PRRs and these often dictate coreceptor choice and ligand perception.

In spite of a big proportion of the so far identified and characterized PRRs having Leucine Rich Repeat (LRR) ectodomains, RKs and RLPs have diverse ligand binding ectodomains. Lysin-Motifs (LysMs), Lectin-like Motifs and Epidermal Growth Factor (EGF) like domains, B-Lectin domain, S-domain and the Cys-Rich domain make up the other extracellular domain types used in MAMP and DAMP perception (Zipfel, 2014). For incidence, in Arabidopsis, the bacterial MAMP Peptidoglycan (PGN); a constituent of the bacterial cell wall is perceived by LysM-RLPs, AtLYM1 and AtLYM3 which form complexes with another LysM-RLK, CERK1 to achieve resistance against bacteria (Willmann et al., 2011).

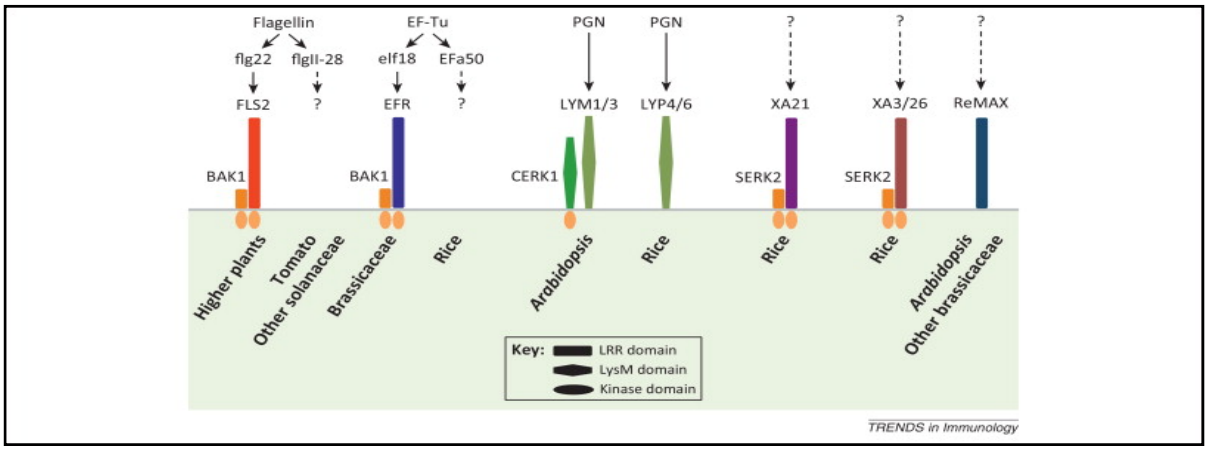


Figure 4:. Diversity of Plant PRR Extracellular domains (Zipfel, 2014).

In plants, a handful of PRRs has been identified and characterized to date. Among these include receptors specific for recognition of PAMPs and DAMPs. The Arabidopsis flagellin-sensitive 2 (FLS2) and the immunogenic 22-amino-acid epitome of the N-terminal of flagellin in bacteria is the oldest and best-studied PRR-PAMP pair (Chinchilla et al., 2007; Lu et al., 2009; Ranf et al., 2011). Another well studied PRR is the Arabidopsis EF-Tu receptor (EFR), which perceives the Elongation Factor Tu in *Escherichia coli* (Zipfel et al., 2006). EFR directly perceives the conserved N-acetylated epitome; elf18 that makes up the first 18 amino acid sequence of the bacterial protein Elongation Factor Tu (Niu et al., 2016; Stefanie Ranf et al., 2011; Zipfel et al., 2006). The RLK Protein PEPR1 receptor has also been reported to perceive DAMPs; specifically the AtPep1 DAMP (Bigeard et al., 2015; Choi & Klessig, 2016; Holbein,

Grundler, & Siddique, 2016; Hou et al., 2019). With regard to NAMP recognition, Mendy made a novel report on the role of a leucine-rich repeat receptor-like kinase; NILR1 that is necessary to trigger PTI reaction upon identification of two nematode species; *Meloidogyne incognita* and *Heterodera schachtii*. To prove this, he knocked out the NILR1 gene to create a *nilr1* loss-of-function mutant. Moreover, true to his hypothesis, the mutant was very much susceptible to both nematode species earlier mentioned upon infection (Mendy et al., 2017).

Unlike the above mentioned PRR-PAMP combinations that have been widely and intimately studied, the cognate PAMPS of a number of PRRs that have been identified is still elusive. The term 'Orphan PRRs' has been allocated to this group of PRRs. While it may be clear that these PRRs are upregulated during either bacterial or fungal invasion, the specific ligand associated with these PRRs remains unknown. For example, the B-lectin-type RK in rice confers resistance to *Magnaporthe grisae* but the associated fungal ligand is yet to be identified (Zipfel, 2014).

Numerous researches have proven that PRRs work in concert with co-receptors and receptor like cytoplasmic kinases (RLCKs) for a complete immune response beyond ligand perception. Ligand-induced heterometric complexes are formed between these molecules setting off subsequent intracellular signaling (Lu et al., 2009; Mendy et al., 2017; Monaghan & Zipfel, 2012). For example, the BIR1 associated receptor kinase (BAK1) associates with different LRR-receptors to aide PAMP recognition by plants. BAK1 is a member of the SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) containing only five LRRs. For instance, BAK1 was reported as a co-receptor recruited by a leucine-rich repeat RLK; NILR1 which perceives proteinaceous ligands of the NAMP ascarosides (Mendy et al., 2017). It was also reported to interact with LRR-receptors FLS2 to detect flg22 and EFR to detect elf18 in bacteria (Liu & Park, 2018; Manosalva et al., 2015; Roux et al., 2011). However, there is a consensus that prior to PRR-BAK1 association, PRR-RP forms a complex with SUPPRESSOR OF BIR1 (SOBIR1) protein and only recruit BAK1 upon ligand binding. A proof for this concept was provided by Albert et al. who reported that the RLP23 PRR forms a complex with SOBIR1 in the resting state of the plant and only mobilizes BAK1 to form a complex post nlp20 perception to induce immune signaling (Albert et al., 2015; Tang et al., 2017).

While the above stated examples may seem straightforward, it should be noted that these heteromeric complexes undergo a wide array of processes and they are not only used for ligand perception but also for the control and regulation of PRRs and their cognate co-receptors.

Beyond making associations, different components of these heteromeric complexes also dissociate to kickstart intracellular signaling and receptor activation. For example, the FLS2-BAK1-BIR1 complex elaborates the association-dissociation events where ligand-induced association of FLS2 and BAK1 leads to phosphorylation of BIR1 (BAK1-INTERACTING RECEPTOR- LIKE KINASE1) which in turn dissociates to mediate downstream PTI signaling (Tang et al., 2017; Tena et al, 2011).

In the absence of ligands, the plant employs a multi-layered regulation system that either keeps the receptors in an inactive state or completely degrades them. With the aid of LRR-RLK pseudo-kinases like BIR1 and BIR3, unnecessary PRR-Coreceptor associations are barred. Saijo et al. reported that the BIR1 restricts premature PRR-BAK1 association through forming a complex with BAK1 thereby keeping it inactive and unavailable for BAK1-SOBIR1 association, only dissociating with the co-receptor in response to flg22 when recruited by the ligand-bound FLS2. BIR3 has also been shown to form complexes with FLS2 and BIR1 in order to regulate PTI and intracellular signaling respectively (Saijo et al., 2018).
Additionally, in Arabidopsis, the BOTRYTIS-INDUCED KINASE1 (BIK1), a member of the RLCKs was also reported to have a regulatory effect on FLS2 and BAK1 by forming protein complexes with each in the absence of a ligand (Lu et al., 2009; Stefanie Ranf et al., 2014).

Aside the use of heteromeric complexes, regulation of PRR abundance is also achieved through PRR degradation. The association between U-box E3 ligases; PUB12 and PUB13 and BAK1 leads to the phosphorylation of the former which in turn leads to ubiquitination of FLS2 and its consequent degradation (Saijo et al., 2018). The negative regulatory effort of PUB12 and PUB13 on FLS2 was reported by Lu et al. indicating that FLS2-associated bacterial resistance was stronger in *pub12* and *pub13* mutants (Lu et al., 2011). PRR regulation through degradation is also realized through autophagy. For instance, ephemeral desensitization of FLS2 associated signaling is achieved by degrading the PRR through Clathrin mediated endocytosis (Mbengue et al., 2011). When a need arises however, PRRs are then replenished by denovo-synthesized receptors through the Exocyst Exo70B2 complex. However, Exo70B2 is also regulated by suppression by E3 ligases namely PUB22, PUB23 and PUB24 (Trujillo, 2018).

3.7 Immune Responses During Plant-Pathogen Interaction

The complete picture of PTI in plants comprises of ligand perception, intracellular signaling and defense responses against the pathogen identified. Ion fluxes across the plasma membrane, production of apoplastic ROS, activation of MAPK with consequent production of defense phytohormones like salicylic acid, jasmonic acid and ethylene are some of the immune responses post pathogen perception (Bigeard et al., 2015; Brantl, 1998; Stefanie Ranf et al., 2011; Shirasu, 2019; Zipfel, 2014). Intracellular signaling plays a pivotal role in achieving these immune responses; acting as a bridge between the start-point and end-point of any innate immune response. Intracellular signaling is primarily performed by the kinase domains of the receptors and co-receptors and the RLCK like BIR AND BIK1; rooted in phosphorylation and transphosphorylation of these membrane proteins and their cognate proteins downstream (Ranf et al., 2014; Rao et al., 2018; Tena et al., 2011).

One of the earliest signaling events post ligand recognition is the influx of calcium ions into the cytosol. Calcium is a regulatory molecule that activates Respiratory Burst Oxidative Homologue D (RBOHD); an NADPH oxidase which in turn produces hydrogen peroxide as an antimicrobial agent within the first 1-2 minutes of ligand perception, Mitogen Associated Protein Kinases (MAPK) and Ca^{2+} Dependent Protein Kinases 5 (CDPK5) towards intracellular signaling (Cristina et al., 2010; Hettenhausen et al., 2013). With regard to Calcium and early ROS production, Ca^{2+} bind directly to the conserved EF-hand motif of the RBOHD enzyme for initial activation (Ranf et al., 2011) . However, worth noting is the synergistic effort of BIK1 and CDPK5 which phosphorylate RBOHD at different sites to further promote and maintain RBOHD activation and consequent ROS production (Stefanie Ranf et al., 2014; Saijo et al., 2018). Aslam et al. provided proof for the importance of Ca^{2+} in innate immunity by reporting that pathogenic bacteria, in fact, produce Extracellular polysaccharides (EPSs) that confiscate apoplastic calcium and are therefore able to diminish MAMP signaling since the influx of Ca^{2+} into the cytosol from apoplast will be cut off or greatly reduced (Aslam et al., 2008).

BOTRYTIS-INDUCED KINASE1 (BIK1) is instrumental right from the onset of PTI after ligand perception and continues to play a role in later stages like activation of transcriptional factors. Sone et al. reported the role of BIK1 in modulation of downstream PTI responses. In this experiment they investigated the specific binding and phosphorylation sites of BIK1 with

the elf18 ligand, the EFR PRR and the BAK1 adaptor. They then proceed to make stable transgenic lines with mutations in these binding sites and study the role of BIK1 phosphorylation from ROS burst production to defense phytohormone production. Results indicated a drastic increase in the amount of Jasmonic acid (JA) and Salicylic acid (SA) in the mutant plants post treatment with a bacterial MAMP; Pst DC3000. An expression analysis also indicated a correlating upsurge of PDF1.2 and PR1; responsive genes which are associated with the Jasmonic acid and Salicylic acid respectively upon bacterial inoculation. This evidences BIK1 as the link between the bacterial resistance and downstream phytohormone regulation.

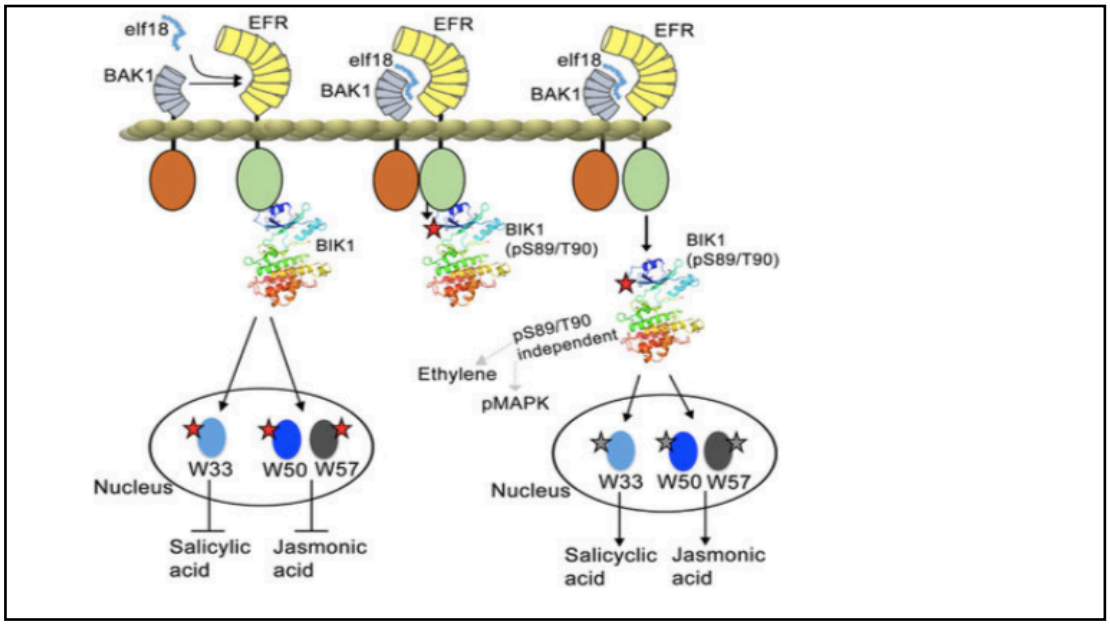


Figure 5: A Model of How BIK1 Modulates Downstream Signaling. (Sone et al., 2018).

Another interesting finding was that BIK1 is beyond a membrane bound protein as it also localizes into the nucleus where it binds directly to JA and SA associated transcriptional factors to regulate JA/SA responses. Co-expression and co-immunoprecipitation assays showed that BIK1 directly interacts with transcriptional factors WRKY30, WRKY50 and WRKY57 in the nucleus to modulate JA biosynthesis.

This experiment also further confirmed preexisting knowledge that BIK1 is not involved in MAPK signaling as the mutants were still able to activate MAPK phosphorylation at levels similar to those in the Col-0 wild type (Sone et al., 2018). The MITOGEN ASSOCIATED PROTEIN KINASE (MAPK) cascade machinery is employed by the plant as a signal transduction pathway in response to both abiotic and biotic stresses. These proteins relay information from sensors and or receptors that the cell membrane to intracellular. molecules associated with stress response. In plants, the cascade is made up of four sub-families; MAP4K,

MAP3K, MAP2K and MAPK, this is also the sequential order through which signals are passed from the receptors to the nucleus.

The signaling events in the MAPK cascade are characterized by phosphorylation and dephosphorylation events. Particularly, signaling from MAPKKK through to MAPK is achieved through a series of Threonine/Tyrosine and Serine/Threonine phosphorylation. As the last component of the cascade, MAPK is set into motion where it then localizes into the nucleus and through phosphorylation of transcriptional factors, it is able to activate them towards producing a response that is needed for the stress identified (Cristina et al., 2010).

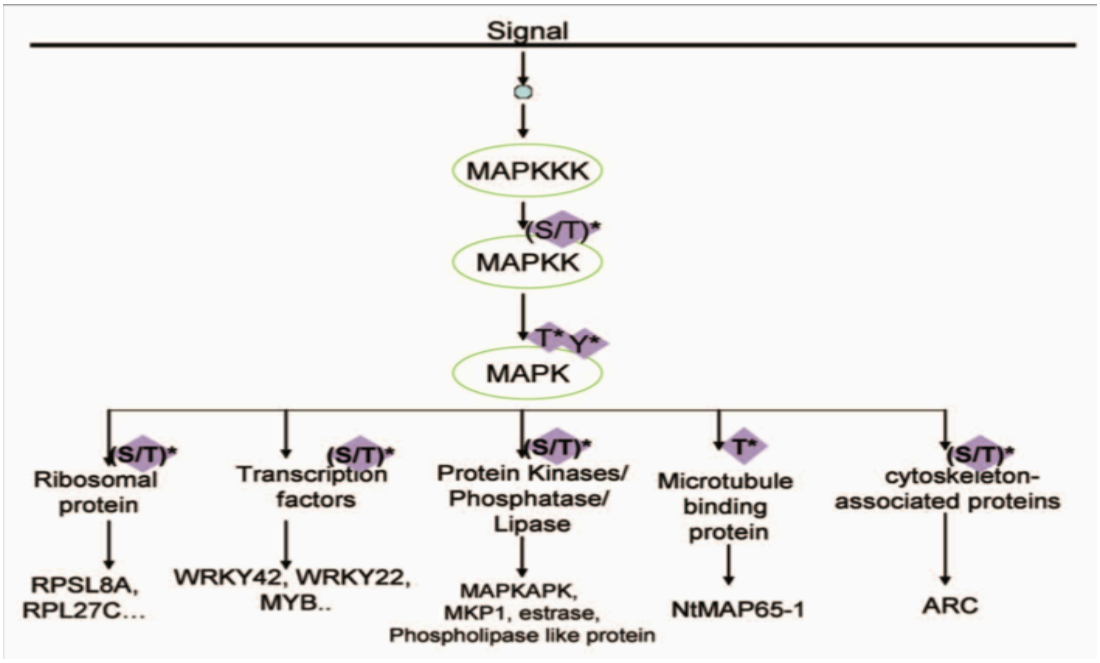


Figure 6: The MAPK Signaling Cascade in Plants (Asai et al., 2002).

FLS2-MAP3K1-MAP2K4/MAP2K5-MAPK3/MAPK4-WRKY22/WRKY29 is an example of a complete signaling pathway that is initiated upon flg22 perception by FLS2. FLS2 activates MAP3K1through phosphorylation of its Ser/Thr residues. Additionally, results of this study also provided evidence for the conservation of the downstream signaling mechanism as a fungal pathogen, *Botrytis cinerea* was able to induce MAPK mediated immune responses in the same fashion as *Pseudomonas syringae* (Asai et al., 2002). A proof of concept that MAPK activates downstream processes that birth defense responses was provided by Qiu et al. who reported that the Arabidopsis MAPK4 is able to modulate gene expression through releasing the transcription factor WRKY33 into the nucleus. In this study, it was established that upon FLS2 activation, MPK4 is activated which in turn leads to phosphorylation of the MKS1. WRKY33-

MKS1 complexes are then released into the nucleus where WRKY33 targets the promoter of PHYTOALEXIN DEFICIENT3 (PAD3). The PAD3 enzyme that is necessary for biosynthesis of an antimicrobial agent, camalexin is then encoded (Qiu et al., 2008).

A decent amount of research has proven that innate immunity is capable of conferring complete resistance to plants against biotic stresses. While many biological processes and details therein remain elusive, we can conclude that understanding the molecular interactions between pathogens and plants towards an applied approach remains a promising venture towards development of resistant crop cultivars and consequently the reduced use of environmentally unfriendly control measures like synthetic pesticides. Unfortunately, as the scoop of the research herein indicates, a big amount of innate immunity research has been focused on other pathogens besides nematodes. And yet plant parasitic nematodes remain a big threat to food security worldwide.

3.8Plant Immune Responses To Plant Parasitic Nematodes

Notwithstanding the fact that nematode associated PRR and PAMP are still largely orphan, a decent amount of research has been done towards elucidating plant immune responses to nematode invasion. These defense responses range from production of chemical compounds that either kill or repel nematodes, release of anti-nematode enzymes and physical measures like reinforcement of the cell wall. With regard to cyst nematodes, the destructive intracellular movement of infective J2s is one of the first triggers of the plant's immune response upon nematode entry in the root. Damage of the cell wall through mechanical puncture and employment of cell wall degrading enzymes leads to the release of the plant DAMP-oligogalacturonides (OG). OG sets off a PTI response often characterized with production of camalexin; a nematocidal chemical (Shah et al., 2017). Post OG perception, the plant counteracts cell wall degradation by the nematodes through releasing Polygalacturonase Inhibitor Proteins (PGIPs) that bar the catalytic action of these cell wall degrading enzymes employed by the nematode. For incidence, in Arabidopsis, inhibitor proteins from the PGIP family dubbed as PGIP1 and PGIP2 have been identified and characterized. PGIP1 was reported to be upregulated specifically during the migratory stage of *H. schachtii* at approximately 10hpi. *PGIP1* loss-of-function mutants showed more susceptibility to *H. schachtii* with a significantly higher number of females and larger syncytium size than the wildtype, this points to the role of this inhibitory protein in plant defense (Shah et al., 2017).

The plant's efforts to defend itself are persistent even when the nematode successfully evades prior immune responses and is able to get to its preferred feeding region of the roots. For endoparasitic nematodes, formation of a feeding site comes next in line after successive root invasion. At this point, the plant has also evolved mechanism to frustrate feeding site formation towards resistance. Using mutants of the ethylene signaling pathway, Marhavý and colleges provided evidence for the role of the ethylene pathway in inhibiting or delaying feeding site formation. The time period between root invasion by *H. schachtii* J2s and selection of an initial syncytial cell (ISU) was recorded using Long-term 4D confocal imaging and it was established that the J2s were able to establish feeding sites faster and more successfully in the ethylene signaling pathway mutants as opposed to the wild-type (Marhavý et al., 2019).

Toxins like phenolic compounds are also produced as a form of chemical ammunition in plant defense (Bigeard et al., 2015). Dhakshinamoorthy et al., reported the role of phenylphenalenone anigorufone, a phenolic compounds in the resistance of a banana cultivar to burrowing nematode; *Radopholus similis* (Dhakshinamoorthy & Ariama, 2014). The mode of action of phenylphenalenone anigorufone is rooted in the accumulation of the chemical around the infection site and consequently the localization of lipid complexes in the nematode's body upon ingestion (Hölscher et al., 2014).

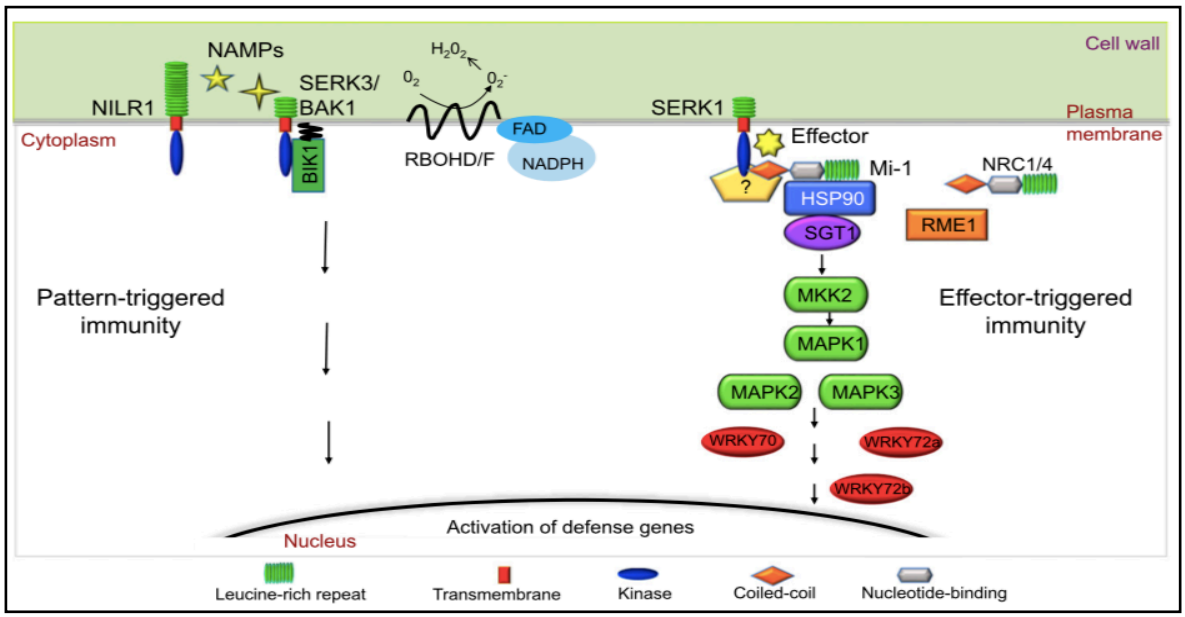


Figure 7: . Immune responses to Plant Parasitic nematodes.

Release of ROS can safely be regarded as a broad-spectrum primary immune response in plants against a wide array of pathogens and nematodes are no exception to this rule. In fact, ROS like hydrogen peroxide is produced almost instantaneously upon ligand recognition within an estimated 1-3 minutes (Cristina et al., 2010; Hettenhausen et al., 2013). From Mendy et al., hydrogen peroxide was reported to be a key component of ligand-dependent immune responses when *nilr1* mutants showed a significantly lower production in the ROS burst assays couples with increased susceptibility to *H. schachtii* (Mendy et al., 2017).

Besides the plasma membrane-bound proteins, NLRs have also been reported to play a role in orchestrating immune responses to nematodes. In fact, a consensus has been arrived to that ETI responses are generally more robust than the PTI responses. Effector recognition by R-proteins initiates a cascade of signaling events whose end product is a hypersensitive reaction (HR) often coupled with localized cell death at the point of infection (Cui et al., 2015; Jacob et al., 2013; Shirasu, 2019). A number of NLRs have been reported in association with nematode resistance to endoparasitic nematodes. Structurally, NLRs that have been identified with nematode resistance are either TIR-NLRs or CC-NLRs. For example, Mi-1.2, Mi-1.3, Hero-A, Gpa2 possess the CC domain while Gros 1-4 and Ma possess the TIR domain (Shirasu et al., 2019). In G. pallida, the Gp-RBP-1 effector initiates a HR with cell death post GPA2 perception, Mi-1 also confers resistance to RKN by inducing a HR, barring the formation of a feeding site and accumulation of H_2O_2 that has a direct effect on the nematode (Kaloshian & Teixeira, 2019).

To date, only one nematode associated PRR; NILR1 has been identified but its cognate NAMP ligand remains unknown too. While Mendy et al.(2017) focused on characterizing one PRR-NILR1, they identified a number of putative PRR genes through microarray assays whose role in nematode induced PTI is yet to evaluated and characterized. This research is, therefore, aiming to identify and characterize more of the putative PRR genes in a bid to better understand what happens when a nematode invades a plant and how the plant defends itself.

Successful identification of more PRR genes will take us one step closer to deciphering plant defense. This knowledge can then be transferred to breeding programs towards tailoring of sustainable and environmentally safe solutions to food insecurity.

4.METHODS

4.1Plant Material

Loss-of-function Arabidopsis mutants for putative PRRs were the plant material used. The selection of putative PRR genes was based on work done in (Mendy et al., 2017). The genes were upregulated in both microarray assay for the RNA extracted from infection assays performed using infective J2 of *H. schachtii* and Arabidopsis treatment with NemaWater.

Table 1: Microarray fold change of Putative PRR genes in infection assay with migratory J2s and upon treatment of Arabidopsis with NemaWater.

Sl. No.	Gene Locus	Gene Symbol	Gene Title	Migratory stage	NemaWater Treatment
GABI-Kat Lines					
1.	AT4G28350	LECRK-VII.2	L-TYPE LECTIN RECEPTOR KINASE VII.2, LECRK-VII.2	3.67	7.69
2.	AT1G14370	APK2A	APK2A, KIN1,KINASE 1, PBL2, PBS1-LIKE2, PROTEIN KINASE 2A	2.14	2.18
3.	AT1G16670	CRPK1	COLD-RESPONSIVE PROTEIN KINASE 1	2.34	2.66
4.	AT5G40170	RLP54	ATRLP54, RECEPTOR LIKE PROTEIN 54, RLP54	3.12	3.82
SAIL & SALK Lines					
5.	AT4G23180	CRK10	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 10, RLK4	2.62	5.03
6.	AT3G05360	ATRLP30	ATRLP30, RECEPTOR LIKE PROTEIN 30, RLP30	1.87	2.17
7.	AT1G56120	LRR-TPK	Leucine-rich repeat transmembrane protein kinase	2.16	2.76
8.	AT5G06740	LECRK-S.5	L-TYPE LECTIN RECEPTOR KINASE S.5, LECRK-S.5	3.08	2.35
9.	AT4G32300	SD2-5 (S-domain-25)	S-receptor kinase -like protein	2.19	2.79

4.2 Plant Growth

4.2.1 Growth Media

0.2 Knop solid media containing 20g L^{-1} sucrose, 8g L^{-1} Daichin agar, 1 ml L^{-1} Vitamin B5, 2 ml L^{-1} of stock solutions I-III , 0.4 ml L^{-1} of stock solution IV and 0.2 ml L^{-1} of stock solution V was used to prepare the growth media. Besides Vitamin B5, the other ingredients were dissolved in double distilled water and the pH of solution adjusted to 6.4 before solution was autoclaved; this was done to avoid degradation of Vitamin B5. Post autoclaving, the solution was cooled to approximately 50°c and Vitamin B5 was added prior to pouring the media into petri dishes. Addition of Vitamin B5, pouring and setting of media was done on a clean bench. The chemical composition of each stock solution used is provided in Table 2 below:

Table 2: Chemical composition of stock solutions used for preparing growth media

Stock Solution	Chemical	g/L
Stock solution I.	KNO_3	121.32g/L
	$MgSO_4 - 7H_2O$	19.71 g/L
Stock solution II.	$Ca(NO_3)_2 - 4 H_2O$	120 g/L
Stock solution III	KH_2PO_4	27.22 g/L
Stock Solution IV.	FeNaEDTA	7.34 g/L
Stock solution V.	H_3BO_3	2.86 g/L
	$MnCl_2$	1.81 g/L
	$CuSO_4 -5 H_2O$	0.073 g/L
	$ZnSO_4 - 7 H_2O$	0.36 g/L
	$CoCl_2 - 6 H_2O$	0.03 g/L
	H_2MoO_4	0.052 g/L
	NaCl	2 g/L

4.2.2 Seed Sterilization

Arabidopsis mutant seeds were surface sterilized with 0.6% sodium hypochlorite for 3 minutes, followed with 70% ethanol for 3 minutes with regular shaking then finally rinsed with sterile water 5 times before air-drying them in the hood.

Planting of sterile seeds was done using a sterilized set of forceps. Two seeds were planted on each petri dish and sealed with parafilm before taking them to the growth room.

4.3 Genotyping

4.3.1 Identification of homozygous GABI-Kat (GK) lines

GABI-Kat mutants are marked with a Sulphadiazine resistant gene; successfully transformed plants are therefore capable of germinating and growing in Sulphadiazine containing media. 16 sterile seeds were planted in 4x4 rows on Knop media containing of Sulphadiazine antibiotic and allowed to grow in the dark room.

4.3Expression check via RT-PCR

4.3.1 RNA Extraction

Plant material from 14 day old plants was collected in 2ml Eppendorf tubes and preserved in liquid nitrogen. Glass beads were added to samples and the samples were ground into a fine powder using the Retsch MM400 tissue lyser. RNA was extracted using the RNeasy® Plant Mini Kit according to the manufacturer's protocol. DNA traces were removed using the TURBO DNA-free™ Kit according to the manufacturer's protocol. RNA was then quantified using the Nanodrop 2000C Spectrophometer.

4.3.2 cDNA synthesis

cDNA was synthesized using random primers of the High Capacity cDNA Reverse Transcriptome Kit according to the manufacturer's manual indicated in table 4. PCR program for cDNA synthesis was run on protocol describes in table 5 ; 18S was used as an endogenous control as used in (Grundler et al., 2007) and the total reaction mixture was 20 µ.

Amplicon was then run on gel electrophoresis; mutants that truly lost function for the gene of interest showed no band.

Table 3: RT-PCR mix for cDNA synthesis

Chemical	Quantity
Nuclease free H_2O	4.2 µL
10x RE buffer	2 µL
10x RT random primer	2 µL
25x dNTPs Mix	0.8 µL
Multiscript Reverse transcriptase	1 µL
RNA	10 µL

Table 4: RT-PCR protocol for cDNA synthesis

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (Minutes)	10	120	5	∞

4.4 Nematode infection assay

4.4.1 Hatching Eggs

Hatching chambers were assembled and sterilized using the autoclave. Funnel was filled with 3mM $ZnCl_2$ to facilitate hatching of eggs. 300 brown cysts were picked from a sterile culture of mustard roots and suspended into the $ZnCl_2$. $ZnCl_2$ from funnels was streaked on LB media to find out if there was any microbial contamination. Funnels were covered with aluminum foil and sealed off with parafilm. Hatching chambers were left to stand for 4-5 days before harvesting J2s.

4.4.2 Infection Assay

J2s were released from the hatching funnels into a sieve and washed about 5 times with sterile water before inoculation. Infection assay was performed as described in (Siddique et al., 2015) as follows: optimization of sterile J2s was done by diluting juvenile until each 5µL water drop contained approximately 30 juveniles. Two drop of juvenile containing water were then applied to the upper and lower root segments of 12 day old plants bringing the total number of juveniles per plant to 60-70. Petri dishes were then sealed with parafilm and returned to dark room. Inoculation of plants was done under a clean bench. Females and males were counted at 12 dpi using a Leica binocular. Sexual dimorphism (See Figure 11) allowed for easy distinction between the males and females; females have a round to oval body shape filled with eggs while males regain vermiform habitus at J4 stage before emerging out of the molt. Males were marked with a x-shaped cross (X) and females with a solid dot (•) before counting and tabulation of each.

4.4.3 Size measurement of female nematode and syncytium

The first measurement of female sizes and syncytium was done at 14 dpi and the final was done at 28dpi on 30 randomly selected cysts and corresponding syncytium. Images were taken and processed by the Leica Camera software and analyzed using the LAS V.4.3 application.

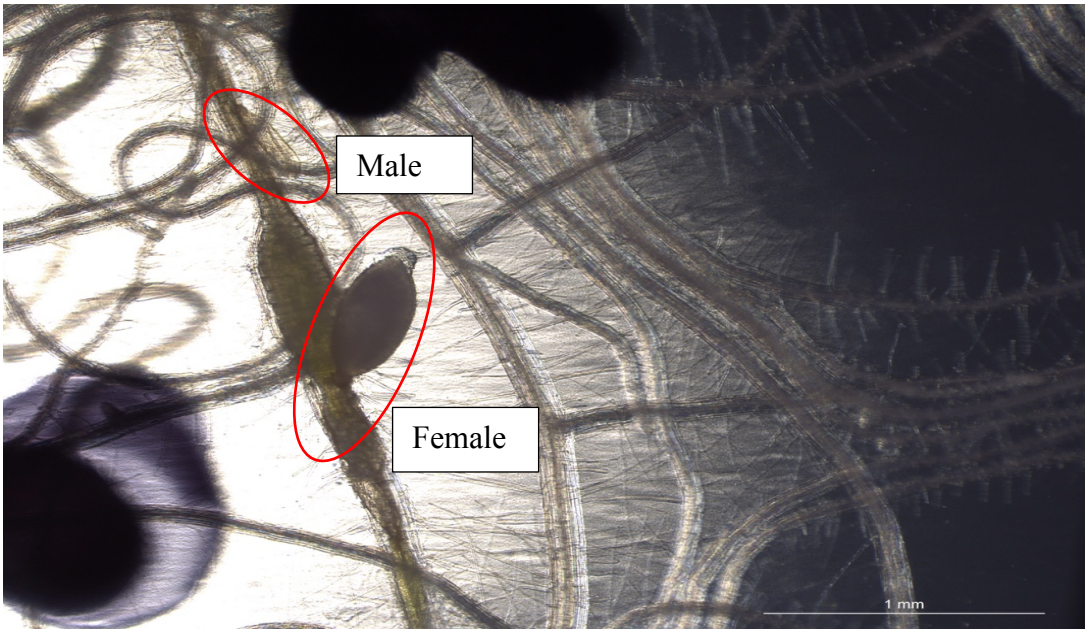


Figure 8: A light microscope image of a female and male H. schachtii at 14 dpi.

4.5 Root Scanning via WinRHIZO™ (Analysis of Washed Roots and Arabidopsis Seedlings)

Plants were loosened from agar by microwaving petri dish for 15 seconds. Using a blade and a pair of forceps, the root was detached from the shoot and carefully rinsed in lukewarm water to remove any remaining agar pieces. The intact root system was then transferred to a tray of water on the root scanner and spread out before scanning it using the WinRHIZO™ set up. Different parameters of the root morphology was generated after every scan and stored in a text file.

4.6 In-silico phylogenetic analysis

Gene ontology was performed using the Omics Box/Blast2go software and phylogenetic relationship established using the MEGAX application.

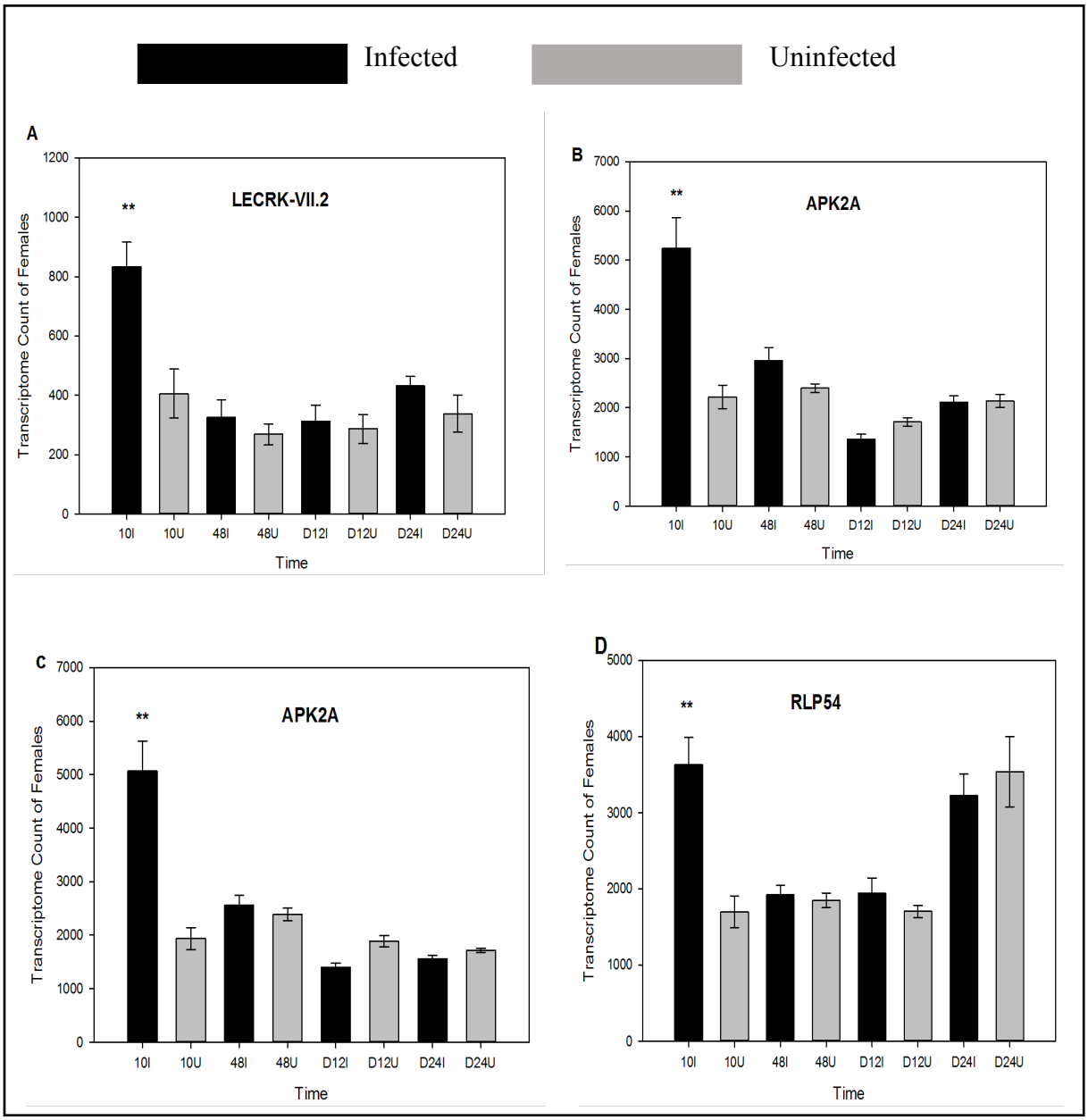
4.7 Data Analysis

SigmaPlot13 and Microsoft Excel were used for drawing graphs and obtaining significance values of the results.

5.RESULTS

5.1Differential Expression of Putative Genes

RNA-Seq analysis was performed previously to determine the expression levels of plant and nematode genes at different development stages and time periods post inoculation (unpublished data). Root samples with nematodes were collected at 10hpi, 48hpi, 12dpi and 24dpi and RNA extracted. RNA from an uninfected plant was used as the control. The expression level of the selected genes was mined in this transcriptome data and we found that a significant upregulation of plant genes at 10hpi for most of the Putative RR genes; . However, for three of the genes, SD2.5, CRK10 and LRR-TPK expression levels were not significantly higher than the uninfected control in RNA-Seq analysis (Fig 9.)



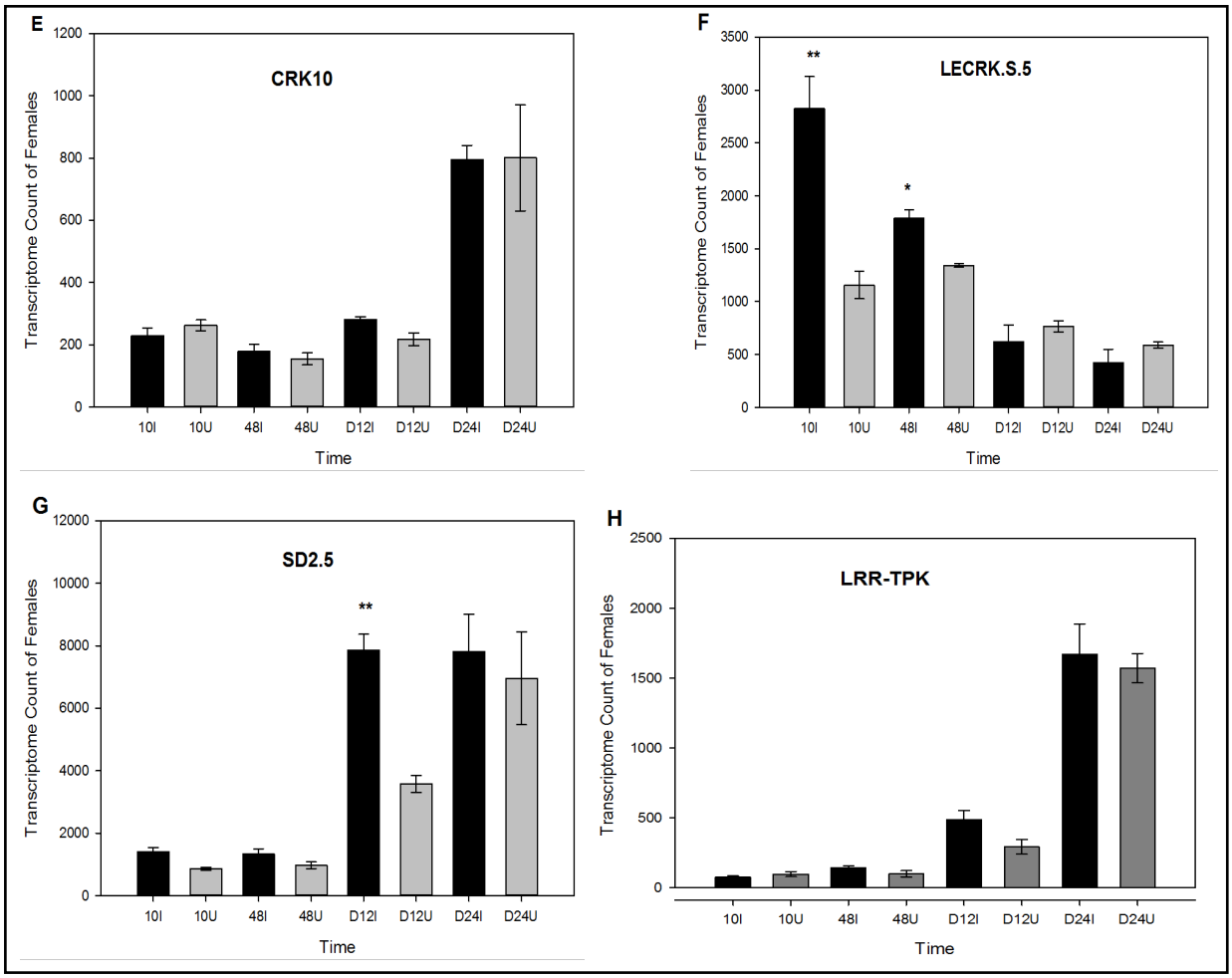


Figure 9: Differential expression of putative genes at different time periods post nematode inoculation.

5.2.1 Genotyping GABI-Kat (GK) lines

None of the seeds of the wild-type lines germinated, the heterozygous lines had some seeds germinating and growing while others failed to grow at all . 16 of 16 of all the seeds of the homozygous lines germinated and grew.

Figure 10: Sulphudizine Screening of Gabi-Kat Mutants.

5.2.2 Expression Check

True loss-of-function mutants did not show expression for gene of interest evidenced by no band (see Figure 11) while some lines showed reduced expression for gene of interest seen as faint band.

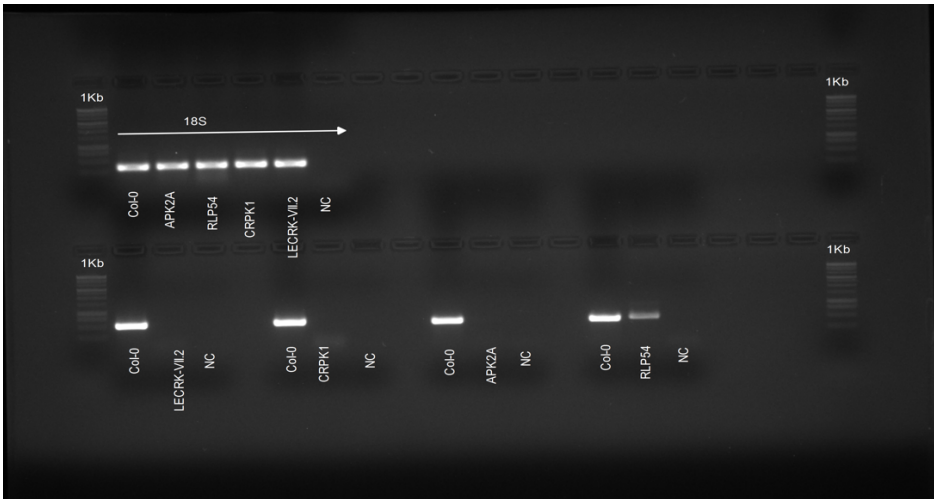


Figure 11: Gene Expression Check. 18S was used as an endogenous control.

5.2Infection Assay of Putative PRR Genes

Twelve-day-old Arabidopsis loss of function mutants were inoculated with infective stage J2 of *H. schachtii* to investigate the plant's immune response in the absence of putative PRR genes. Females and males were counted at 12 dpi; sexual dimorphism in cysts nematodes aided easy identification as females have swollen bodies and males regain the vermiform habitus.

LRR-TPK had a significantly higher number of nematodes ($P<0.01$) compared to the wildtype; Col-0. While the rest of the mutants had no significant difference in nematode numbers, SD2.5 had a much lower number of nematodes. However, it was also evident that some of these mutants; SD2.5 and LECRK.V.II had a root phenotype with significantly smaller root lengths and root surface areas at $P<0.03$. To normalize the root area, the plants were numbered and then scanned using the WinRHIZO™ to obtain the root length and surface area of each plant. The counted number of nematodes per plant was then divided by the root length of the corresponding plant in order to remove any variations associated with a root phenotype. Post root normalization, LRR-TPK retained its significantly higher number of nematodes while SD2.5 that previously had a lower number of nematodes than Col-0 attained a significantly higher number ($P<0.01$) as shown in Figure 12.

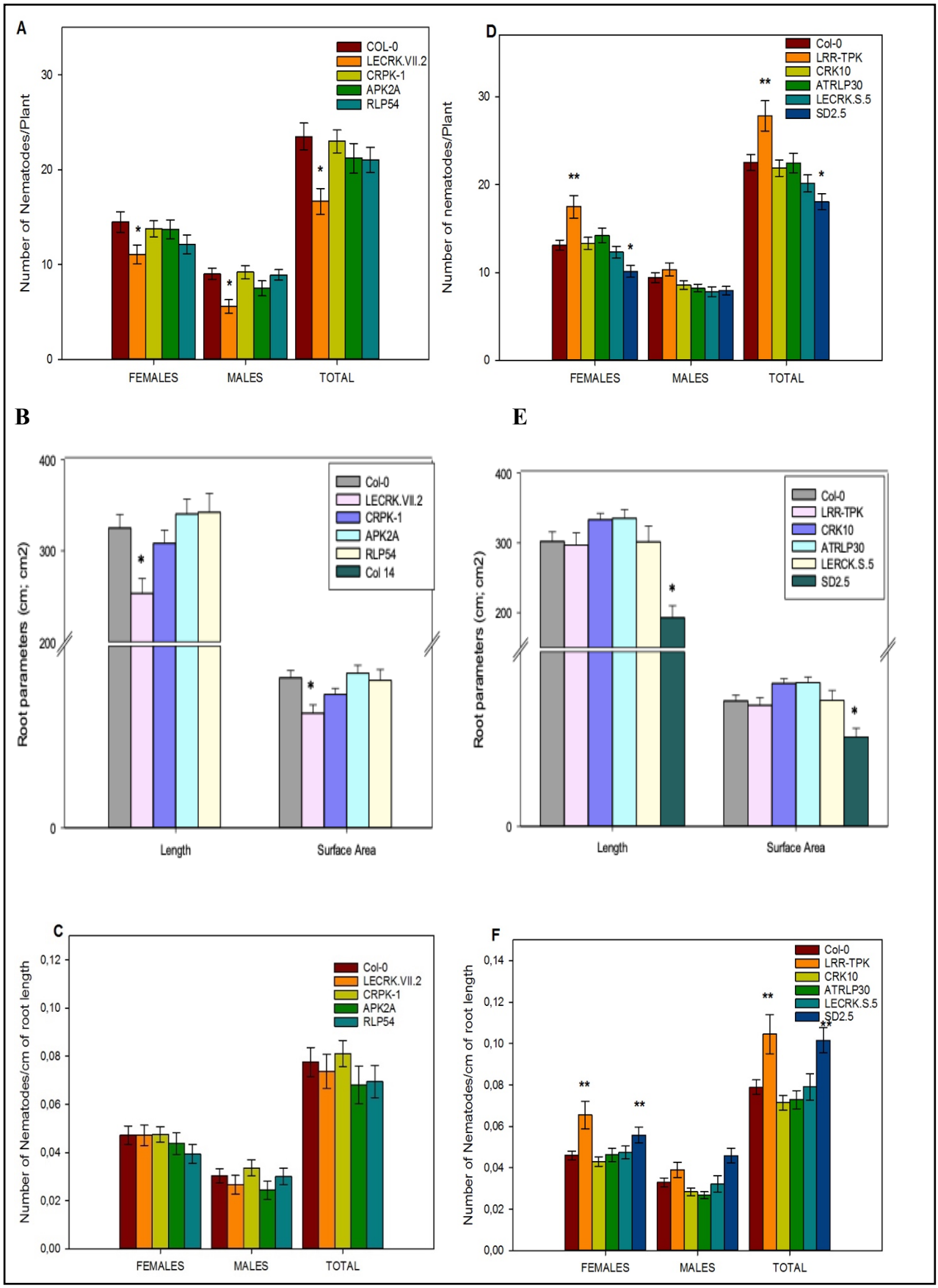


Figure 12: Infection assay of Mutants. A-C; Gaby-Kat lines. D-F; SAIL &SALK lines. B and E; measures of root parameters.

At 14 dpi, images of females nematodes and female-induced syncytium were taken. At P>0.05, there was no significant difference between female sizes of the wildtype and mutants as seen in Figures 13A and Figure 14A.

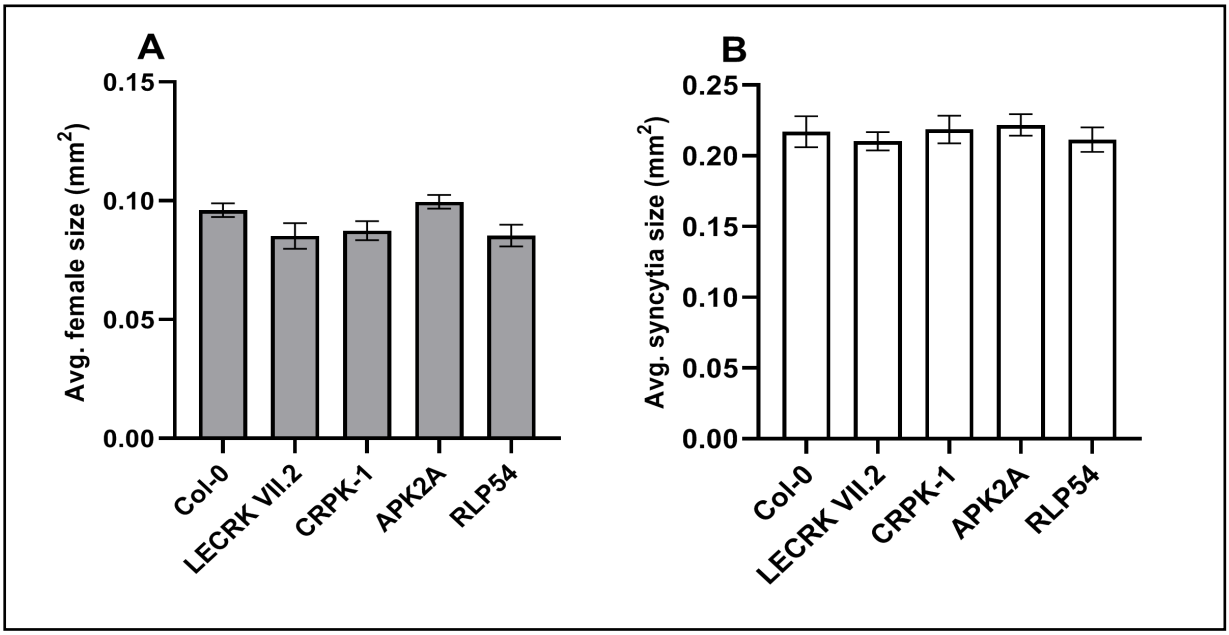


Figure 13: Average female size(A) and syncytium size(B) of Gabi-Kat Mutants

As with the size of females, a similar trend was observed with female-induced syncytia of the wildtype and the mutants with no significant difference in the size of syncytia as elaborated in Figures 13B and 14A.

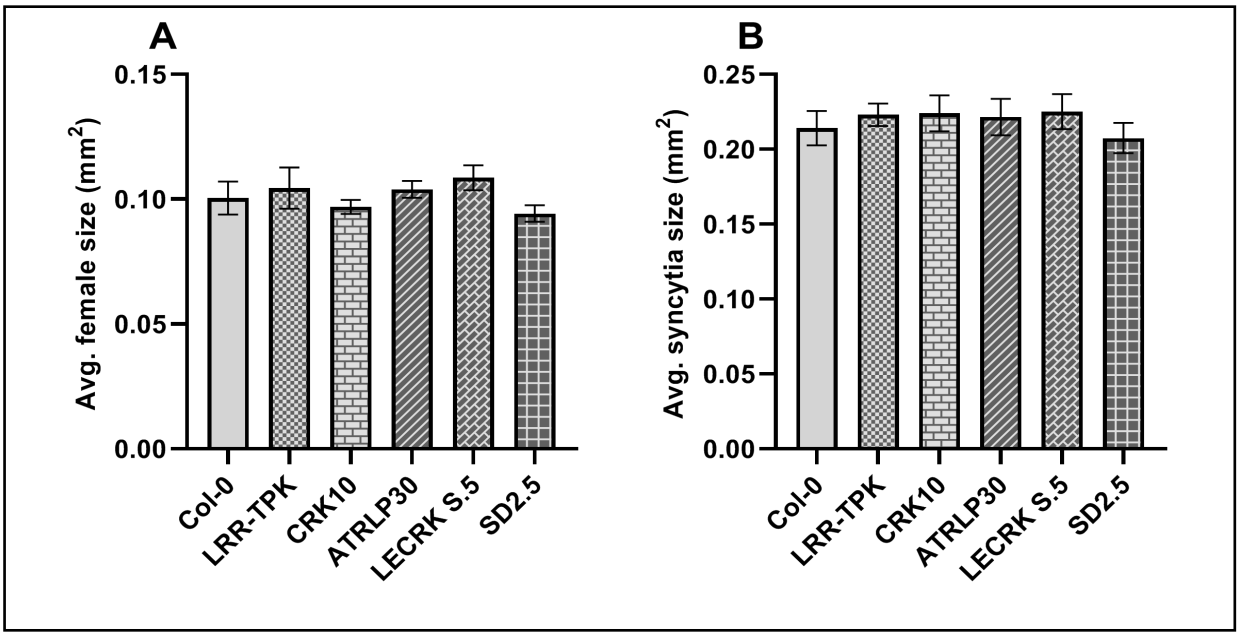


Figure 14: Average female size(A) and syncytium size(B) of SALK and SAIL Mutants

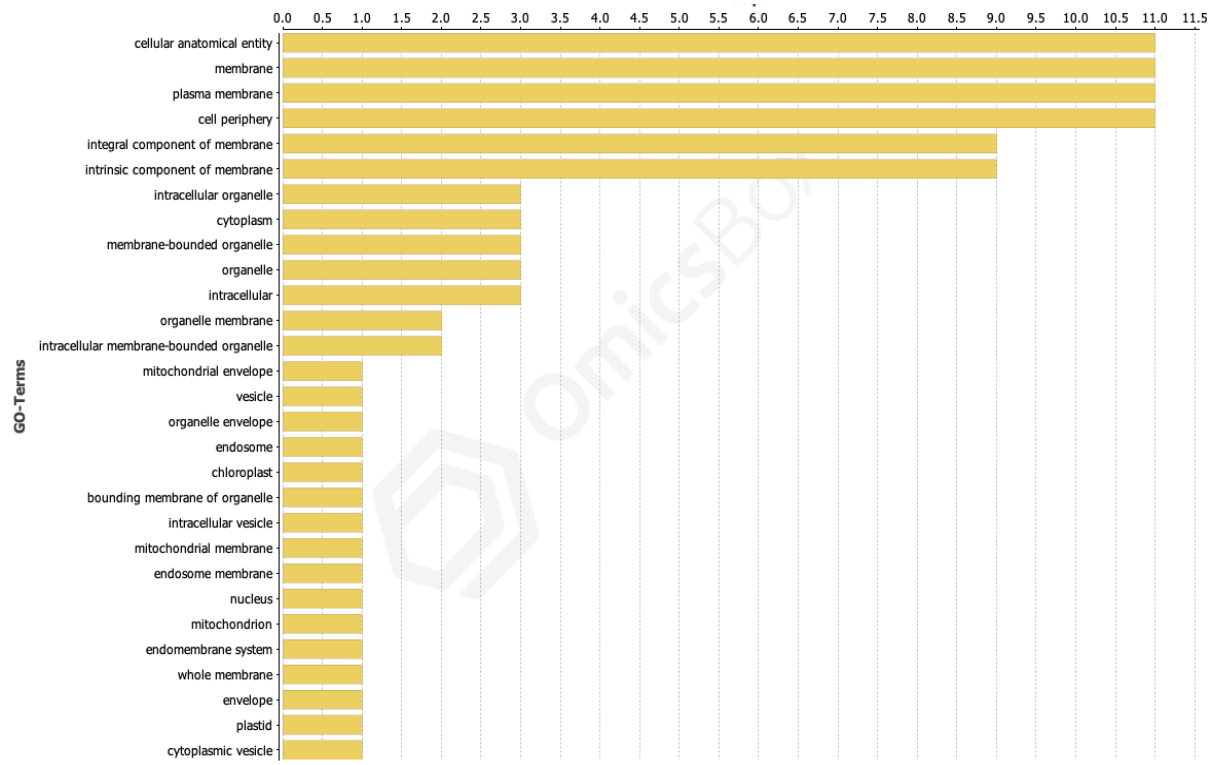
5.3 In-silico Study

5.3.1Characterization of Putative PRRs

Gene ontology and cell component analysis was performed by Omics Box/Blast2go . Sequences were Blasted, Mapped and Annotated and analyses indicated that all putative 9 PRR proteins alongside with BAK1 and NILR1 are located on the plasma membrane. BAK1 and NILR1 were used as reference proteins and/or positive controls.

Biological function analysis using the same program also indicated that at least 8 of the 11 sequences analyzed are involved with response to biotic stimulus and response to other organisms (See Fig 15).

A



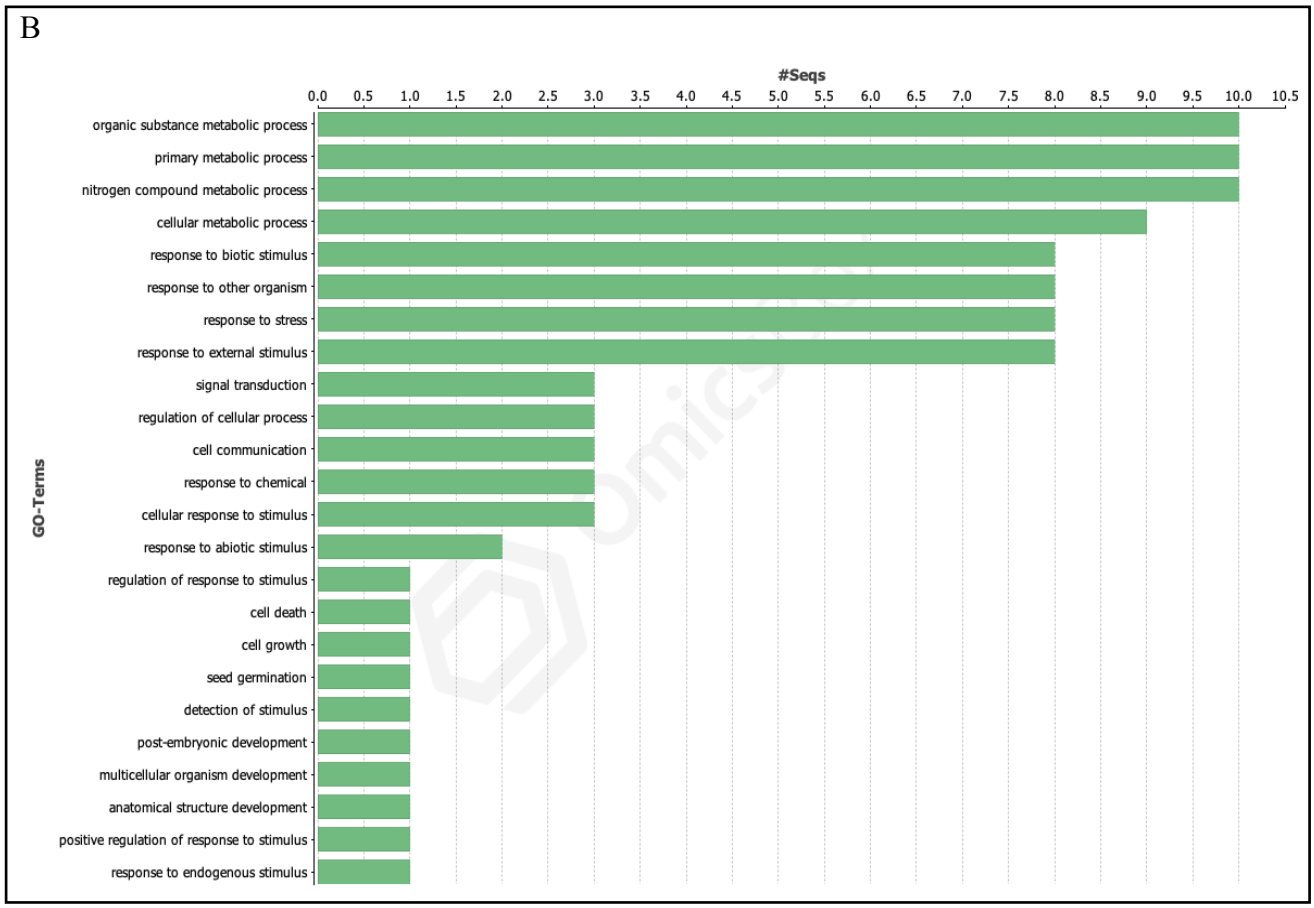


Figure 15: In-silico Analysis of Putative PRRs. A; Cellular Component Analysis. B; Biological function Analysis. Gene Ontology was performed using Omics Box/Blast2go.

5.3.2Phylogenetic Analysis

In line with the idea of gene homology and similarity of gene function, we tested the evolutionary relationship between the known nematode associated PRR; NILR1, its correspondent co-receptor BAK1 and the putative PRR genes. Nucleotide sequences were obtained from the NCBI Database, aligned with Seaview/MEGAX and phylogenetic trees drawn with the same. The Maximum likelihood method and the Tamura-Nei model were used for tree construction.

LECRK-S.5, LECRK-VII.2, SD2.5, APK2A and NILR1 had a degree of evolutionary relatedness with a shared ancestry at bootstrapping value of 71%. SD2.5 also showed evolutionary relatedness with BAK1 at bootstrapping value of 70% .

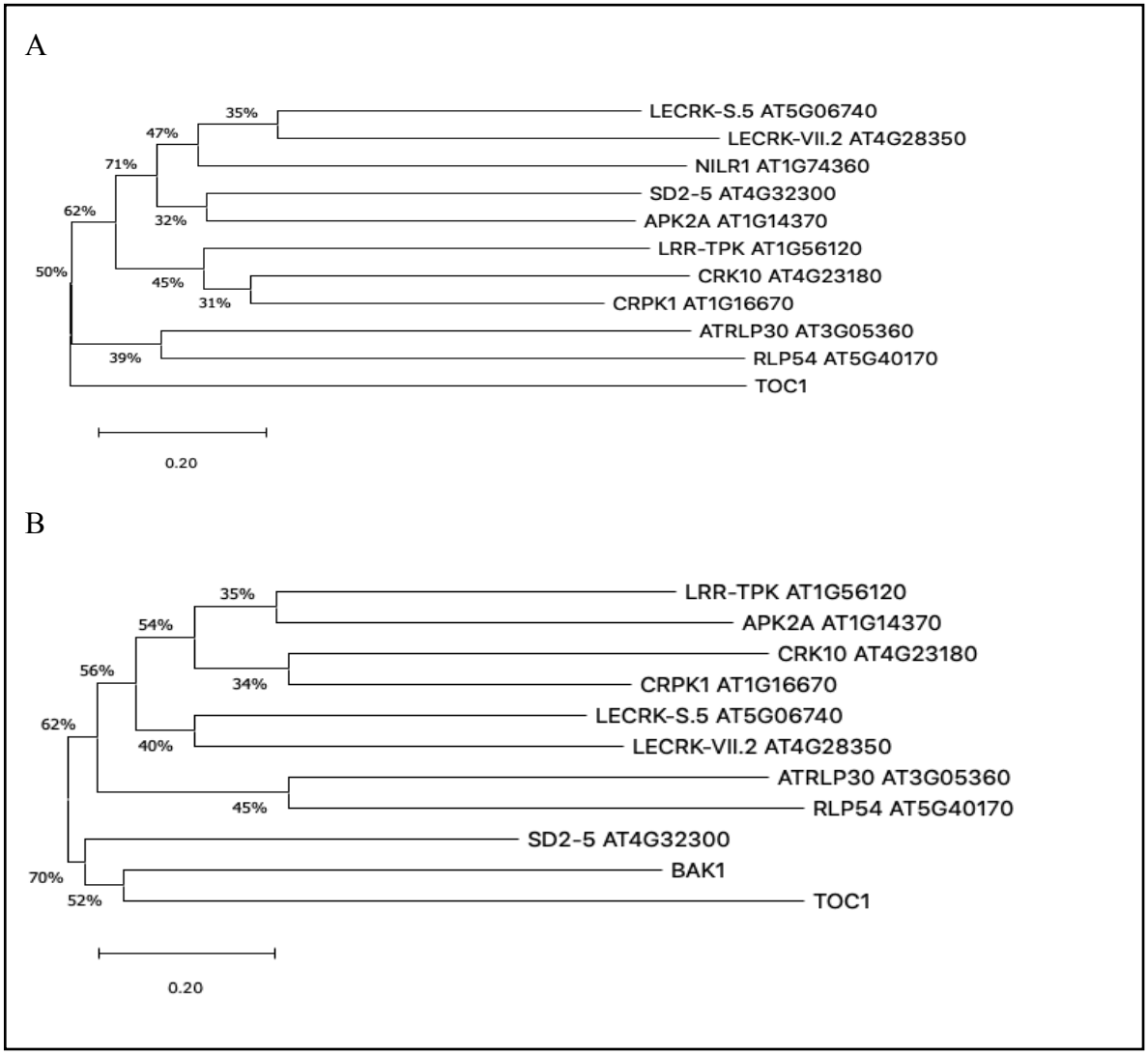


Figure 16: Phylogenetic analysis of Putative PRRs. DNA sequences were obtained from NCBI database. Maximum likelihood method and the Tamura-Nei model was used to establish phylogenic relationship. The Arabidopsis TOC1 gene was used as an outgroup.

5.3.3 Characterization of SD2.5 and LRR-TPK

The KEGG database provided motif alignment for the SD2.5 and the LRR-TPK proteins. The SD2.5 gene encodes an approximately 821 amino acid protein that is made up of a B-lectic extracellular domain which could be the putative ligand binding domain. The extracellular domain is followed by a S-locus glycoprotein domain, a transmembrane domain and two main cytosolic domains; the Tyr-Ser-Thr amino acid domain and the kinase domains.

On the other hand, the LRR-TPK gene encodes a protein that is made of about 1029 amino acids. Its domain arrangement from the N-C terminal comprises of an extracellular domain with

nine leucine-rich- repeats, a Malectin and Malectin-like domain, a Podoplanin domain, a Tyr-Ser-Thr amino acid domain and intracellular kinase domains (See figure 17)

BLAST analysis was performed on SD2.5 and LRR-TPK to identify homologues in other land plants. Besides other Arabidopsis species, plant species in Brassicaceae family had the top hits for shared identity for both genes with some having E. values as low as 0.0. Notably, a couple of food crop groups and species also seem to have a SD2.5 and LRR-TPK homologs, namely, *Brassica oleracea* both genes and *Arachis hypogea* for SD2.5 which are commonly known as the edible cabbage species and the ground nuts respectively.

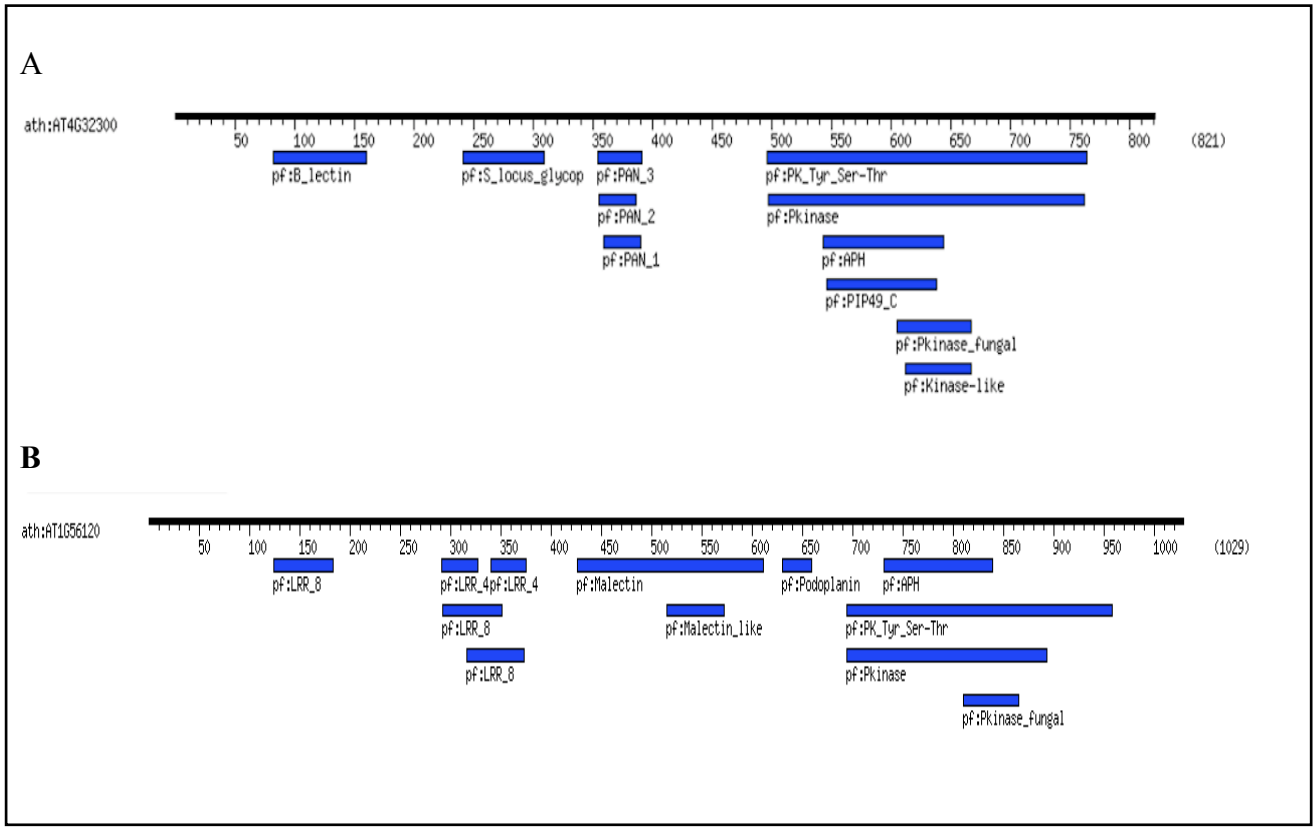


Figure 17: Protein motif. A; SD2.5 Protein and B; LRR-TPK protein domain arrangements. Information was acquired from the KEGG database.

6.DISCUSSION

6.1 Differential Gene Expression In Nematode-Infected plants

The struggle between plant parasitic nematodes and host plants also termed as "arm's races" (Jwa & Hwang, 2017) is aimed at survival of both organisms. Being obligate endoparasites, the cyst nematodes must device means of evading plant immune responses to ensure feeding and reproduction. In order to suppress pathogen attack and disease which could be fatal in severe case, the plant too mounts up its resistance to ensure survival: a phenomenon famously described as the Zig-Zag Pattern (Nekrasov et al., 2009). This interaction between plants and parasitic nematodes is characterized by release of molecules that will either facilitate parasitism in the nematodes or effect resistance to pathogen in the plant (Lu et al., 2009; Siddique et al., 2015; Vijayapalani et al., 2018).

The differential expression of genes in the plant upon nematode infection could therefore be rooted in this phenomenon. The upregulated genes could be involved in plant defense responses against the establishment of the nematode. A similar occurrence was reported in (Mendy et al., 2017), where the PRR (NILR1) associated with nematode perception and consequent immune responses against the same was upregulated within the first 10-48 hours of nematode inoculation. Nematodes have also been reported to manipulate their host's genetic machinery in order to upregulate plant genes involved in growth and development to ensure the establishment of nematode feeding sites (NFS). For example, Grunewald and colleagues reported that the auxin influx gene AUX1 is stimulated in Arabidopsis during initial feeding cell formation. This upregulation of AUX1 is to ensure the enlargement of NFS and is coupled with the downregulation of the auxin efflux gene PIN1 to ensure accumulation of auxin (Grunewald et al., 2009).

Additionally, host susceptibility genes are plant genes that are manipulated by the nematode for their establishment and survival in the plant and these are often upregulated upon infection. Radokovic and colleagues reported of a one HIPP27 (Heavy Metal-Associated IsoPRE-Nylated Plant Protein 27) gene that was strongly upregulated upon infection of Arabidopsis with *H. schachtii*. Further investigation revealed that the gene was not involved in basal resistance but rather used for syncytium formation evidenced by localization of the protein in the syncytium upon GUS staining and the significant reduction in susceptibility to the nematode in the loss-of-function mutants of HIPP27 (Radakovic et al., 2018). Some of the differential upregulation

could, therefore, be as a result of the nematode manipulating plant gene expression for its own benefit.

SD2.5, CRK10, and LRR-TPK, on the contrary, did not show any significant upregulation in the infected plants within the first hours post-inoculation in comparison to the uninfected control in the RNA-Seq analysis. A plausible explanation could be due to the different approaches used for the transcriptome analysis, i.e., microarray and RNA-Seq analysis. Also, these analyses were done on the small root segments containing the nematode infection sites, which were sampled by different sets of peoples. Moreover, this could be due to their down-regulation and/or modulation by nematode effectors, which could also point to their role in plant defense.

Pathogen effectors have been reported to suppress PTI and signaling associated molecules in a bid to halt plant immune responses and to successfully establish themselves in the plant (Irieda et al., 2019). One of the ways pathogen suppresses PTI is by targeting and degradation of molecules involved in intracellular signaling. An example of this mechanism is reported by Zhang and colleagues who deciphered that the *Pseudomonas syringae* effector AvrPphB attaches itself to and consequently degrades a membrane-bound- and intracellularly localized kinase proteins involved in signal transduction like RLCK PBS1 and PBS1-Like kinases of BIK1, PBL1 and PBL2 (Zhang et al., 2010) in a bid to interfere with downstream signaling and cognate immune responses. Therefore, it could be that SD2.5, CRK10 or LRR-TPK are membrane-bound proteins associated with signal transduction like BIK1, and they could have been degraded and thereby modulated to lower levels by nematode effectors in a bid to suppress a PTI response within the first 10hpi.

6.2 Infection Assay and Susceptibility to Nematodes

We observed differential root phenotyping in some of the loss-of-function mutants. This could be associated with the fact that some transmembrane and intracellular receptors are not only involved in responses to biotic stress, but they also play an important role in plant growth and development. Taking BAK1 for incidence , this LRR co-receptor has been reported to associate with the brassinosteroid-insensitive 1 (BRI1) receptor which is an important hormone in plant growth and development. Upon brassinosteroid perception, BRI1 is activated and it forms a heterocomplex with BAK1 characterized with the sequential transphosphorylation of both

molecules, thereby activating the BR signaling pathway (Zhang et al., 2020). It is, therefore, probable that either LECRK.VII.2 or SD2.5 are involved in a growth and development pathway in the plant and knocking them out resulted into a growth defect that manifested itself as shorter root lengths and reduced root surface area in comparison to the wildtype, Col-0.

The idea of performing an infection assay on mutants of putative PRR genes was to evaluate the plant's immune response to the pathogen of interest in the absence of these genes. The hypothesis therefore is that if these genes are truly involved in pathogen resistance, the mutant genotype should then be more susceptible to the pathogen than the wild type. The infection assay we carried out had both the ideal results for this hypothesis and the contrary. Results from two genotypes; SD2.5 and LRR-TPK fitted the hypothesis and had a significantly higher number of nematodes than the wildtype genotype. From these results, we could suggest that these two genes are probably involved in the plant's immune response to nematodes since their absence made the plants more susceptible. Similar results have been reported by numerous authors where knocking out and silencing of genes involved in immune responses led to increased susceptibility of plant to a pathogen. For example, Lozano-Torres and colleges reported that the immune activity of the membrane bound Cf2 PRR that is also capable of recognizing effectors by indirect perception through surveillance of perturbations and modifications in the guardee protein $Rcr3^{pim}$ is halted when this protein is knocked out. Cf-2 loss-of-function mutants were more susceptible to *G. rostochiensis* infection in tomato as opposed to the wildtype pointing to its importance in resistance to this pathogen (Lozano-torres et al., 2012). However, failure to record significant susceptibility in the other loss-of-function mutants should not automatically disregard their role in the plant's immune pathway. Some plant defense related protein has been identified to belong to a family that has similar and/or related proteins that can perform the same function in the absence of the other. For example, the mutants of the RLCK PBS1 were still able to confer resistance to *P. syringae* because the plant employed another PBS1-like kinase BIK1 for signal transduction in the absence of the former (J. Zhang et al., 2010).

Molecular functional analysis of SD2.5 (Fig. 18) indicated that it is involved in Calmodulin binding. Calmodulin (CaM) has been reported to be associated with modulation of cytosolic calcium (Ca^{2+}) levels through regulation of calcium channels and calcium pumps like CNGCs and ACAs respectively (Cheval et al., 2013). Calcium influx is one of the first immune

responses upon pathogen perception and it is responsible for activation of proteins that trigger production of ROS within the first 1-3 minutes of ligand recognition (Cristina et al., 2010; Hettenhausen et al., 2013). Ca^{2+} also plays a pivotal role in initiation of downstream immune signaling like MAPK signal cascades which consequently activate transcription factors towards immune responses like HR and defense related phytohormones like SA, JA and Ethylene (Aslam et al., 2008). Proof of evidence for the role of CaM and Calmodulin-Like Proteins (CML) in calcium modulation and thereby immune responses against pathogens has been provided by a couple of authors. For example, transient expression of CaM1 in *Capsicum annuum* led to local resistance to *Xanthomona campestris* (Choi, Lee, & Hwang, 2009), virus-induced silencing of CaM13 in *N. benthiana* led to enhanced susceptibility to the tobacco mosaic virus; *Ralstonia solanacearum* (Takabatake et al., 2007) and overexpression of the Calmodulin-Like Protein 43 (CML43) gene in Arabidopsis led to an increased aggressive HR to avirulent Pst(avrRpt2) (Chiasson et al., 2005).

While we cannot yet deduce how CaM associates with SD2.5 and if this association indeed has an impact on Ca^{2+} signaling and a subsequent ripple effect on the plants immune response, Ranf and colleges reported of another gene in the B-type Lectin RLKs class otherwise known as 'SD-RLKS'; SD1-29 which could shade more light on this presently oblivious area. *Sd1-25* mutants showed reduced accumulation of ligand induced Ca^{2+} levels and subsequently a lowered Pseudomonas liposaccharide-triggered release of ROS. Activation of MPK3 and MPK6 was also impaired in the mutants and expression of PTI response genes such as *FRK1* and *GST1* were also greatly diminished (Ranf et al., 2015)
Therefore, piecing together the molecular function of SD2.5 in calmodulin binding, the role of calmodulin in calcium signaling and the work done by Ranf and colleges on another member of a gene family that SD2.5 belongs to, it is probable that the calcium signaling in the SD2.5 loss-of-function mutant was impaired. This thereby rendered the plant more susceptible to nematode infection since it was unable to effect immune responses that primarily depend on Ca^{2+} .

At present, there is not much literature about LRR-TPK and its role in plant resistance against pathogens, however, molecular function analysis of LRR-TPK showed that it has a shared molecular function of protein binding with NILR1 (see Fig 18). While this is a very broad functional categorization , we could suggest that this protein binding is in-line with either

perception of proteineous ligands or association with protein coreceptors like BAK1. For instance, Mendy et al., (2017) reported that the NILR1 PRR; a leucine-rich repeat receptor kinase perceives a proteineous nematode elicitor and also recruits the BAK1 protein for ligand perception and signal transduction (Mendy et al., 2017). Additionally, the protein motif similarity between NILR1 and LRR-TPK (see Figure 17) can be used to argue a probable similarity in function between these two genes since both were upregulated in the microarray experiment (Table 1) in response to the plant's exposure to nematode elicitors. The loss-of-function of LRR-TPK could therefore have hindered nematode perception and along with it PTI immune responses that follow ligand perception accounting for the increased susceptibility in LRR-TPK loss-of-function mutants.

6.3 Evolutionary Relationship Between Putative PRRs, NILR1 and BAK1

Gene homology (orthologs and paralogs) is a result of events of gene duplications which leads to formation of gene families or stand-alone genes often having common ancestry. While some gene orthologues may have similar functions, others are not functionally related regardless of having the same ancestry. An example of the former incidence is the Mi-1 and Mi-9 orthologues which both confer resistance to Meloidogyne spp (Jablonska et al., 2007). Contrarywise, the bHLH transcription factors are an example of plant genes that have a very high sequence identity and thereby suggesting their common ancestry but perform different functions (Armisén, Lecharny, & Aubourg, 2008). With regard to assessing confidence of phylogenetic analysis and consequently establishing homologies, Hillis & Bull (1993) empirically suggest that phylogenetic relationships can be trusted from bootstrapping values of at least 70% (Hillis & Bull, 1993). This implies that SD2.5 could be phylogenetically related to NILR1 and/or BAK1 at 71% and 70% respectively indicating a probable relatedness in functionality between these genes too. On the other hand, the convergence theory explains that genes with different ancestral backgrounds can have similar or related functions (Ghani et al., 2018). Given the relatedness in biological function of the putative PRR genes to NILR1 and BAK1 (see Figure 15), some of the genes could therefore have a case of convergent evolution where they have shared functionality with no evolutionary similarity/shared ancestry between them and NILR1 or BAK1.

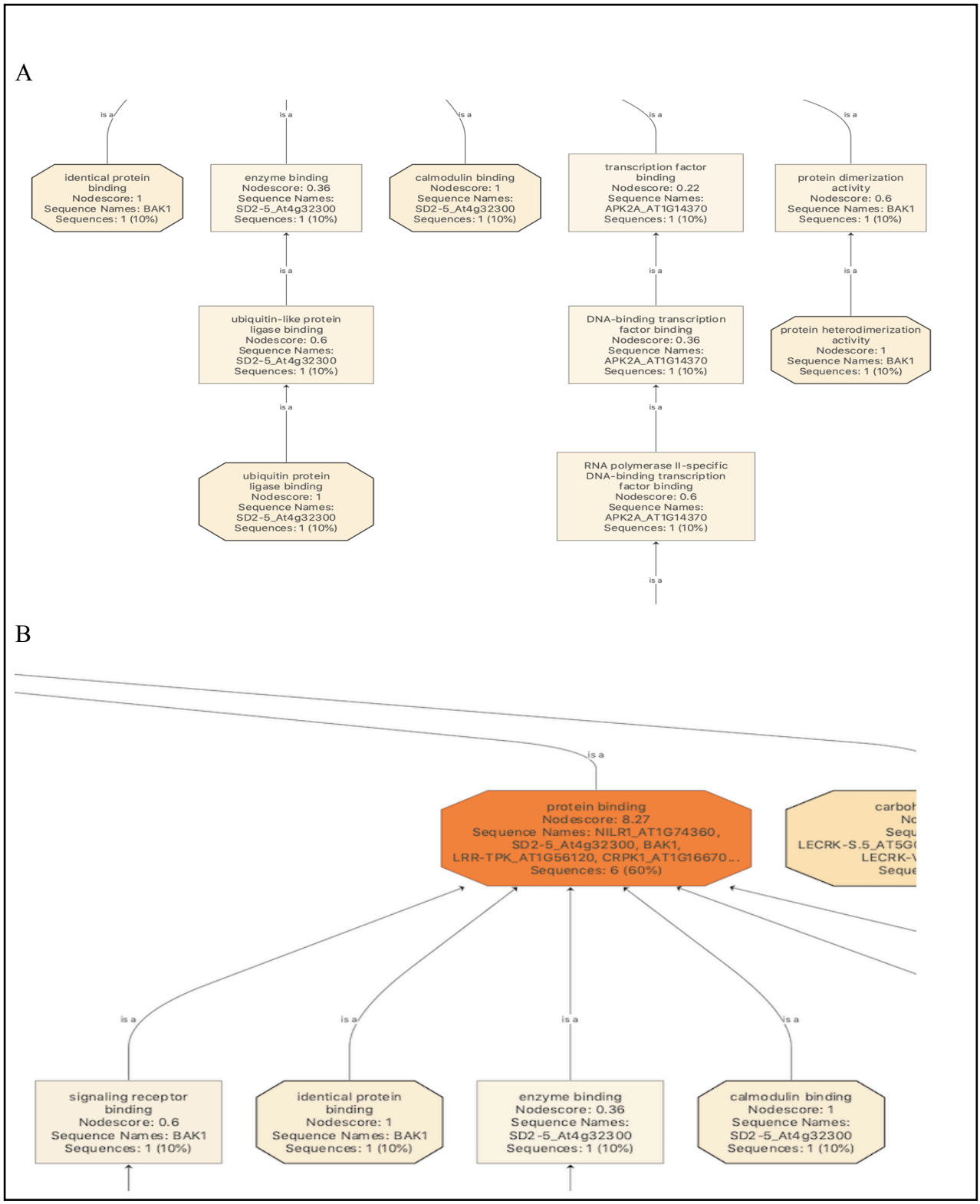


Figure 18: Molecular function of Putative PRR genes. A; Gene ontology reveals that one of the molecular function of SD2.5 is its association with calmodulin and B; LRR-TPK is involved in protein binding alongside NILR1. This analysis was run on the Omics Box/Blast2go software.

7. CONCLUSION

The increased susceptibility of SD2.5 and LRR-TPK loss-of-function mutants to *H. schachtii* is coupled with their biological function in response to biotic stress and their cellular location on the cell membrane point to their probable role as surface localized PRRs. Individually, the molecular association of SD2.5 with calmodulin, an important protein in plant defense signaling point to SD2.5 as a probable plant defense protein. Moreover, its B-Lectin extracellular domain suggests that it could have a ligand-binding property which fits the profile of a plant PRR. Additionally, the protein motif of LRR-TPK is very similar to the already identified and characterized nematode-associated PRR- NILR1 with a particular focus on the excellular leucine-rich repeat domains which could be involved in ligand binding and the intracellular kinase domains for probable signal transductions. The above evidence provides a hint for the probable involvement of these two putative PRRs in plant PTI responses. Nonetheless, majority of the protein characterization was done in-silico, therefore, laboratory confirmation of for instance cellular localization with methods like the use of GUS-reporter lines or fusing the protein with GFP is recommended.

In fact, time and technical limitations during the COVID-19 pandemic did not allow us to investigate other important PTI parameters like ROS burst and expression of PTI marker genes. Therefore, it is recommended that these should be investigated in the near future to come to more concrete and evidence-based conclusions on the role of these putative PRR genes in PTI immune responses to nematodes.

ACKNOWLEDGEMENT

I would like the extend my gratitude to my promoter, Prof. Dr. Florian M. W. Grundler for granting me the opportunity to work and learn from his laboratory and advising me through the thesis process. Secondly, the effort, time and encouragement invested by my supervisor Dr. M. Shamim Hasan into the successful completion of my thesis cannot go unnoticed and unappreciated. Without your guidance, my thesis journey would have been a close-to-impossible mission to accomplish especially with all the difficulties that presented themselves along the way due to the COVID-19 pandemic. I would also like to appreciate my family and friends, all my classmates and the colleagues and Technical Assistants (TA) in the MPM research group who helped me in various capacities- you all contributed to the success of this thesis one way or another. Last but not least, I would like to thank VLIR-UOS for awarding me the scholarship and ERASMUS+ for the additional funding during my German mobility that allowed me to pursue this master's degree and research without worrying about the financial burdens of living and studying away from home.

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APPENDICES

Table 5: Primer list for Expression Check.

Gene Locus	Gene Symbol	Primer
AT4G28350	LECRK-VII.2	FP: GCTTCATTGCTGGGGTCTCT RP: TTTGTCTCCCACCCTTCGTG
AT1G16670	CRPK1	FP: CCCGCCACCATAAGAAGACC RP: CTTGACTCAGCCGAGAGGAC
AT5G40170	RLP54	FP: CCCCGCTTTGGTCTTATCCT RP: TCCAAACAACACTCCGGGTC
AT3G05360	ATRLP30	FP: TGGTACATGGGCGAGAAAGG RP: ATTGCCGCTGCTATCCAGTT