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Functional characterization of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 effectors in *Nicotiana benthamiana*

Presented by

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Declaration

I, **Celiwe Innocentia Nxumalo,** declare that the dissertation, which I hereby submit for MSc. Microbiology at the University of Pretoria, is my own work and has not previously been submitted by for a degree at this or other tertiary institution.

Celiwe Innocentia Nxumalo

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Summary

Phytophthora parasitica is one of the most destructive oomycetes, classified within the kingdom Stramenopila. During its infection process, *P. parasitica* secretes hundreds of RxLR effector proteins into the cytoplasmic region, as a major virulence factor. However, RxLR effectors are under pressure to evolve for different infection strategy. Despite the loss and gain of RxLR effectors, comparative genome analyses of *Phytophthora* spp. revealed that some RxLR effectors are conserved (CRE) across pathogen strains or species. A remarkable progress has been made in understanding the function of RxLR effector proteins including CRE from other *Phytophthora* spp., however, little is known about CRE from *P. parasitica*. Therefore, understanding the biological function of *P. parasitica* CRE in host-pathogen interaction is a promising route for breeding resistance in plants.

Using *in silico* analyses, a study conducted in our lab revealed that there are approximately 158 to 356 of RxLR effectors proteins that are secreted by *P. parasitica* isolates. In *P. parasitica* INRA 310, 71 CRE effectors were shown to be highly conserved within *Phytophthora* spp. Among the 71 CRE, PpRxLR1 and PpRxLR6 effector proteins were selected for further functional characterization as they were shown to be highly conserved and upregulated during early stages of infection.

Like other *Phytophthora* spp., *P. parasitica* exhibit a hemibiotrophic life cycle, therefore, the first objective was to determine how does the life cycle of *P. parasitica* changes, and at what phase is PpRxLR1 and PpRxLR6 expressed. To achieve this *N. benthamiana* leaves were inoculated with *P. parasitica* zoospores at 0, 6, 12, 24, 36, 48, 60 and 72 hrs. The results obtained showed that leaves were green and healthy from 0 to 24 hrs, turned yellow with water soaking lesion at 36 and 48 hrs, followed by necrosis lesion at 60 and 72 hrs. The qPCR results correlated well with the phenotypic changes of leaves, as the biotrophic phase maker was differentially expressed starting from 0 to 24 hrs indicating a biotrophic phase. The decline in expression of the biotrophic phase marker (at 36 hpi), coincided with the induction, followed by increasing expression of the necrotrophic phase marker as well as yellowing of leaves. The necrotrophic growth like symptoms was observed at 60 and 72 hpi where there was high expression of the necrotrophic phase marker. We also observed that both effectors were expressed during the biotrophic phase with PpRxLR6 expressed early

(24 hpi), and PpRxLR1 (48 hpi) expressed later than PpRxLR6 during infection. Our results suggest that both effectors might play a role in the virulence mechanism of *P. parasitica* as they were highly expressed during the biotrophic phase.

The interaction of *Phytophthora* RxLR effectors with their host plants has gained attention as they are seen as a promising route to combat crop losses. The second objective was to determine whether PpRxLR1 and PpRxLR6 play a role in the virulence of *P. parasitica* and, if this was the case, what are the modes of action. The results indicated that PpRxLR1 promote colonization of *P. parasitica, P. cinnamomi* and *P. sojae,* PpRxLR6 only promote the colonization of *P. parasitica.* This suggests that the function of PpRxLR1 is conserved among *Phytophthora* spp*.,* while the function of PpRxLR6 is conserved within *P*. *parasitica.* The mechanisms of PpRxLR1 and PpRxLR6 may include inducing ROS, callose, SA, ET, JA and MPK3/6 during biotrophic phase which later trigger plant cell death. However, their targets in the induced plant response need to be evaluated.

Table of contents

List of abbreviation

ACC:	Aminocyclopropane-1-carboxylic acid
ACS:	Aminocyclopropane synthase
ASA:	Ascorbate
AVR:	Avirulence genes
BK1:	BOTYIS-INDUCED KINASE 1
Cals:	Callose synthase
CAT:	Catalase
CBEL:	Cellulose binding elicitor lectin
CO_2:	Carbon dioxide
CRN:	Crinkler effector proteins
CTR1:	CONSTITUTIVE TRIPLE RESPONSE 1
CWDEs:	Cell wall degrading enzymes
DDHB:	2, 3 dihydro-2, 3-benzoate
EBF1:	BINDING F-BOX PROTEIN 1
ERF1:	Ethylene response factor 1
ERF1:	ETHYLENE RESPONSE FACTOR 1
ET:	Ethylene
ETI:	Effector triggered immunity
Flg22:	Flagellin 22 (22-amino –acid peptide)
g:	grams
GFP:	Empty vector
GPX:	Glutathione peroxidase
GR:	Glutathione reductase
GSH:	Glutathione
H_2O_2:	Hydrogen peroxide
hpa:	Hours post agroinfiltration
hpi:	Hours post inoculation
HR:	Hypersensitive response
IC:	Isochorismate
ICS:	Isochorismate synthase

INF1:	Elicitors of *Phytophthora infestans*
JA:	Jasmonic acid
MAPKs:	Mitogen activated protein kinases
MTA:	5′-methylthioadenosine
NbLOX:	Lipoxygenase
NLP:	Necrosis and ethylene inducing peptide like proteins
NPP1:	Necrosis-inducing *Phytophthora* protein 1
NPR1:	NONEXPRESSER OF PATHOGENESIS RELATED GENES1
O_2:	Superoxide
OH:	Hydroxyl radical
PAL:	Phenylalanine lyse
PAMPs:	Pathogen associated molecular patterns
PCD:	Programmed cell death
PcF:	*Phytophthora cactorum-Fragaria*
PcHmp1:	*P. infestans* Haustorial membrane protein 1
PcNpp1:	Nep1-like Protein 1
PRRs:	Pathogen recognition receptors
PTI:	Pathogen associated molecular pattern –triggered immunity
R genes:	Resistance genes
ROS:	Reactive oxygen species
RT-PCR:	Reverse transcription polymerase chain reaction
RxLR:	Arginine-any amino acid-Leucirine-Arginine
SA:	Salicylic acid
SAM:	S-adenosylmethionine
SAR:	Systemic acquired resistance
SCR:	Small cysteine-rich secretory protein
SOD:	Superoxide dismutase

List of figures

List of table

Chapter one

Literature review: Plant pathogen interaction

1.1 Background and overview of *Phytophthora* species

Oomycetes, —the water molds'' represent a group of heterotrophic eukaryotes classified in the kingdom Stramenopila along with algae, diatoms, and other planktons (Latijnhouwers *et al.,* 2003). Most oomycetes are aquatic and are important decomposers in these ecosystems, but some have evolved to parasitize terrestrial plants (though these still rely on water) (Sperschneider *et al.,* 2015). Lin and Aronson (1970) defined them as having a cellulose-based cell wall and a vast network of filaments that allow for nutrient uptake. Because of their fungus-like shape, physiology, and plant-attacking techniques, they were once classed as fungi. The evolutionary separation of fungi and oomycetes, on the other hand, is reflected in significant differences in biochemistry, cell structure, and development (Kamoun *et al.,* 2015, Meng *et al.,* 2014). In this group, the genus *Phytophthora*, the —plant destroyer‖ have taken the research community by storm, as they are responsible for a vast array of destructive diseases of plants important to agriculture, forestry, ornamental of recreational plants and natural ecosystems (Moy *et al.,* 2004). There are over 150 species of *Phytophthora* and the number is increasing yearly (Kroon *et al.,* 2012).

1.2 Disease symptoms and host plants of *Phytophthora* spp.

Phytophthora species normally attack the root system and stem base of a plant, but they may also infect the aerial parts of a plant (Gilardi *et al.,* 2015). From a global perspective, they cause more than 66 % of all root diseases and more than 90 % collar rots of woody plants (Jung *et al.,* 2017). *Phytophthora*-infected plant roots and stems are discoloured, necrotic, and dark brown (Gilardi *et al.,* 2015). When the stem has been infected or when *Phytophthora* has invaded the stem base, foliar symptoms are observed as a result of problems with the uptake of water and nutrients (Gevens *et al.,* 2008). When the foliar turns yellow, leaves fall off, and the plant wilts and dies (Jung *et al.,* 2004).

Some *Phytophthora* spp. are economically important than others in terms of host range and distribution (Kroon *et al.,* 2012). Important *Phytophthora* are presented in **Table 1.1** below.

Table 1.1: *Phytophthora* host plants and diseases that have been identified over the past century

Phytophthora spp.	**Host plants**	**Diseases**	**References**
P. agathidicida	Kauri trees	Collar-rot	Bradshaw *et al.,* 2020
P. brassicae	Cabbage	Club-root	Schlaepp *et al.,* 2010
P. cactorum	Strawberry	Root rot	Li *et al.,* 2011
P. capsici	Cucumber, squash	Root rot	Hausbeck and Lamour, 2004
P. cinnamomi	Cinnamon and fruit tree	Root-rot	Hardham and Blackman, 2018
P. citricola	Citrus trees	Root rot and stem canker	Schena *et al.,* 2006
P. fragarie	Strawberries	Red root-rot	Bonants *et al.,* 1997
P. infestans	Potato and tomato	Late blight	Majeed *et al*., 2017
P. kernoviae	Beech and rhododendron	Bleeding canker	Brasier *et al.,* 2005
P. lateralis	Cedar trees	Root-rot	Green *et al.,* 2013
P. megakaryota	Cocoa	Black pod	Ali *et al.,* 2017
P. multivora	Forest trees	Dieback	Scott *et al.,* 2009
P. palmivora	Coconuts	Fruits rot	Migliorini *et al.,* 2019
P. parasitica	Tobacco, citrus	Black shank	Gallup and Shew, 2010
P. ramorum	Oak trees	Sudden oak death	Petersen *et al.,* 2019
P. sojae	Soybean	Root-rot	Tyler *et al.,* 2007

Phytophthora parasitica, also known as *P. nicotianae Breda de Haan*, is unique among *Phytophthora* spp. since it causes severe losses to a large variety of host plants all over the world (Gallup *et al.,* 2010). Since its first description on tobacco in 1896, it has been documented on 255 genera in 90 families, covering both herbaceous and woody hosts (Biasi *et al.,* 2016). It is particularly severe in the United States, Brazil, and South Africa (Dos Santos, 2016, Maseko and Coutinho, 2002). *Phytophthora parasitica* has been implicated in quality and yield losses in citrus and tobacco-growing areas in South Africa, and it is the most common *Phytophthora* pathogen (76%) (Maseko and Coutinho, 2002, Meitz-Hopkins *et al.,* 2014). The black shank of tobacco is one of the most serious disease caused by *P. parasitica*, and it can be found in all tobacco-growing areas around the world (Biasi *et al.,* 2016). The losses can be as high as 100%, and in some cases, the disease can cause so much damage that tobacco can no longer be produced on the affected farms (Panabieres *et al.,* 2020). Despite significant improvements in *Phytophthora* control, *Phytophthora* species continue to pose a major danger to agriculture and are difficult species to control globally. As a result, a collaborative effort is required to find and develop novel control strategies.

1.3 The biology and life cycle of *Phytophthora parasitica*

Phytophthora parasitica is a hemibiotrophic pathogen, exhibit two phases: an early asymptomatic biotrophic phase that allows the pathogen to establish a foothold in the host, and a late necrotrophic phase characterized by tissue breakdown and disease progression (Wi *et al.,* 2012). This means that the pathogen develops various, though not seemingly contradictory, pathogenic methods across infection cycles, from suppressing plant basal defences to the ultimate death of the host (Kelley *et al.,* 2010). However, the duration of both biotrophic and necrotrophic phase, depends on the *Phytophthora* pathogen. For instance, *P. infestans* has a long infection cycle, while *P. capsici* has a short infection cycle (Jupe *et al.,* 2013, Zuluaga *et al.,* 2016).

The sexually and asexually cycle of *Phytophthora parasitica*

Phytophthora parasitica reproduce both sexually and asexually (Dahlin, 2016). Sexual reproduction is an efficient and vital means for diseases to generate genetic variety, potentially resulting in a huge number of different genotypes and pathotypes that allow pathogens to adapt to unfavourable conditions (Andersson, 2007, Judelson

et al., 2007, Lamour *et al.,* 2012, Hausbeck and Lamour*,* 2004). *Phytophthora parasitica* is mainly heterothallic, producing oospores with A1 and A2 mating types. Male antheridia and female oogonia are generated on each thallus when an A1 strain detects an A2 and vice versa. Septa separate the antheridia and oogonia from the mycelium (Judelson, 2007). The oogonial incept of the opposing mating type is attached to an antheridium, and the oogonial incept grows through the antheridium (Erwin *et al.,* 1996). The male surrounds the female in this process, as opposed to paragynous attachment (the male is beside the female), in which the antheridium clings to the side of an oogonium, close to the oogonium's attachment site to the mycelium. A meiotic or reductional division of the diploid genome occurs in the antheridia and oogonia, resulting in the formation of haploid nuclei (Dahlin, 2016). *Phytophthora* spp. do not produce uninucleate gametes, a feature that distinguishes them from real fungi (Bhat *et al.,* 1993, Schardl and Craven*,* 2003, Turner*,* 2005). A fertilization tube transports one antheridial nucleus to the oogonium, where it combines with one oogonial nucleus. Infected plant tissue produces an oospore, which is a thick-walled structure (Meng *et al.,* 2014). Oospores are discharged into the soil as the plant dies and the plant tissue decays, where they can persist for many years (Akinsanmi *et al.,* 2017). Oospores can germinate while the soil is wet with water, but only on a very rare basis. Depending on environmental factors such as moisture and temperature, germinating oospores develop sporangia or zoosporangia, which release zoospores (Andersson, 2007). When a host plant is present near developing oospores, the zoospores respond to chemical stimuli in exudates from the host plant's roots by swimming towards the root tips. They then infect host plants, starting new infection cycles (Dahlin, 2016).

In the asexual cycle, multinucleate sporangia are generated on specialized hyphal structures (sporangiophores) (Judelson *et al.,* 2008). The hyphae are hyaline and aseptate, with hyphal swellings on occasion (Wang *et al.,* 2020). Depending on the availability of water and suitable temperatures, sporangia either germinate directly or create uninucleate, wall-less zoospores with two flagella that allow them to swim and reach host tissues by a variety of attraction mechanisms including chemotaxis and electrotaxis (Lamour *et al.,* 2012). For most *Phytophthora* spp*.,* zoospores are thought to be the primary infective agents that cause plant diseases (Chepsergon *et al.,* 2020). They are also effective disseminators since they can easily be spread

in soil water, irrigation water, and hydroponic solutions. Following host recognition, zoospores form cysts with a cell wall, and these germinate to create germ tubes that penetrate plant cells (Meng *et al.,* 2014). Before penetration, an appressorium forms at the end of a germ tube tip; a penetration hypha emerges from the appressorium, invades the epidermal plant cell, and the hyphae grow intracellularly in the mesophyll (Judelson *et al.,* 2008). Thereafter, haustoria-like structures which are specialized feeding structures that infiltrate the mesophyll cells to extract nutrients to sustain the mycelium's growth emerge. The initial signs of infection arise soon after infection, usually in the form of water-soaked lesions that spread quickly (Dahlin, 2016). This causes the leaves and stems to deteriorate, and eventually the entire plant collapses (Dahlin, 2016). New sporangiophores emerge at the borders of the lesions after three to five days, resulting in a large number of sporangia on the surface of infected plants (Lamour *et al.,* 2012). Furthermore, if the environment is unfavourable, thick walled chlamydospores are formed from the hyphae. The chlamydospores can live for years in the soil and are used as the principal inoculum in the field (Akinsanmi *et al.,* 2017).

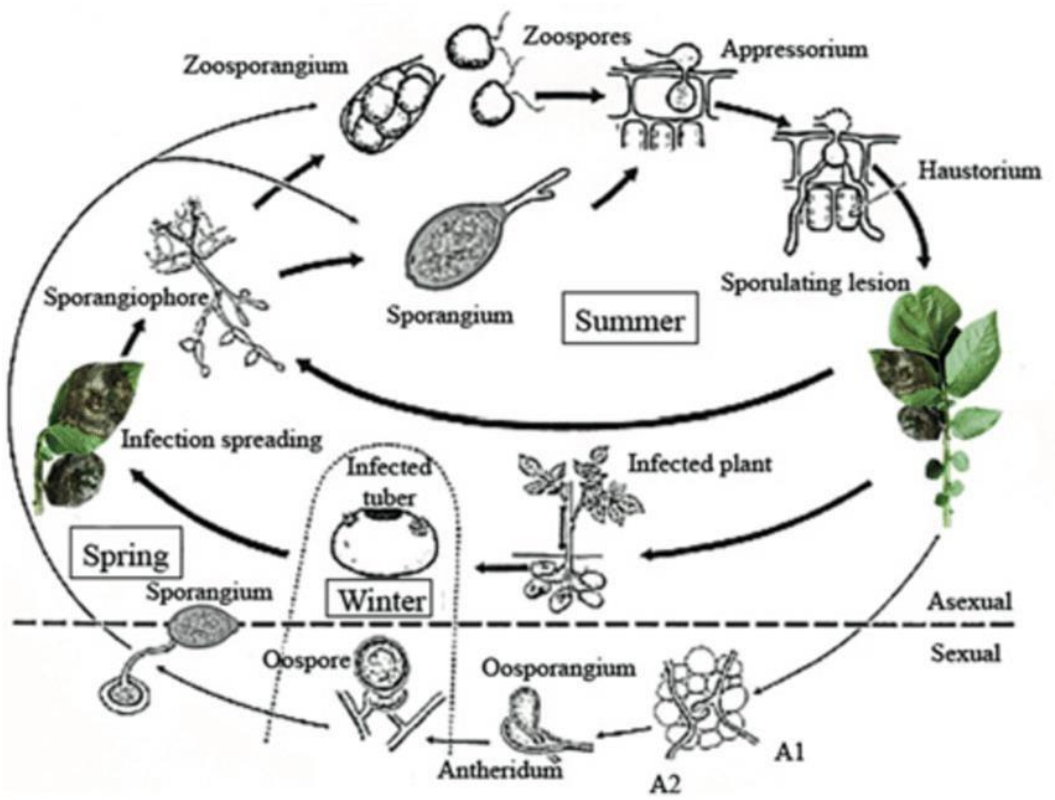

Figure 1.1: Diagrammatic representation of *Phytophthora parasitica* sexual and asexual cycle. The sexual cycle of *Phytophthora parasitica* start with the production of oospores to necrosis of the plant.Asexual cycle start with attachment of zoospores to necrosis of the plant (Dahlin, 2016).

1.4 *Phytophthora* virulence mechanisms

Many researchers have recently focused on *Phytophthora* effector proteins, which are thought to play an important role in virulence (Akino *et al.,* 2014, Boevink *et al.,* 2020, Bozkurt *et al.,* 2012, Chepsergon *et al.,* 2020, Dong and Ma, 2021, Li *et al.,* 2018, Varden *et al.,* 2017, Wang *et al.,* 2019). Effector proteins are small molecules that are secreted by pathogens to manipulate host cell structure and function, thereby promoting colonization and infection (Kazan and Lyons, 2014). They are divided into two groups based on their subcellular localization; apoplastic and cytoplasmic effectors (Wang *et al.,* 2019). The effector proteins include: cell wall degrading enzymes, elicitors, and cell –entering (Crinkler and RxLR) effectors (Boevink *et al.,* 2020, Li *et al.,* 2018)

1.4.1 Cell wall degrading enzymes

The plant's first line of defence against invading pathogens is the cell wall (Davis and Hahlbrock, 1987, Dörmann *et al.,* 2014, Krzesłowska, 2011, Rashid, 2016, Underwood, 2012). Plant tissues, especially the apoplastic space, are rich in polysaccharides and glycoproteins (Rashid, 2016). *Phytophthora* spp. secretes several carbohydrates, lipids and protein-active enzymes that degrade plant cell wall polymers (Hardham and Blackman, 2010). These enzymes include pectinases, polygalacturonases, glucanases, cellulases, xyloglucanases, lipases and proteases (Rashid, 2016). Blackman *et al.,* 2014 reported that *P. parasitica* contains 431 cell wall degrading enzymes (CWDEs). Their transcriptomic data provide strong evidence that CWDEs are highly upregulated during early infection and that they target the main categories of plant cell wall components. In addition, *Phytophthora* spp. secretes enzyme inhibitors, including proteinases that play important roles in pathogenesis (Kay *et al.,* 2011). *Phytophthora infestans*, for example, produces two serine protease inhibitors, EPI1 and EPI10, which block the tomato protease P69B, which is responsible for pathogen-induced protease activity in the apoplast (Tian *et al.,* 2007).

1.4.2 Elicitors

Studies have shown that *Phytophthora* spp. produce several extracellular toxins that trigger plant cell death (Denes *et al.,* 2015, Dong *et al.,* 2012, Kamoun *et al.,* 2006, Feng *et al.,* 2014, Nicastro *et al.,* 2009, Orsomando *et al.,* 2011, Wawra *et al.,* 2012). These extracellular toxins include necrosis and ethylene inducing peptide like proteins (NPLs), *Phytophthora cactorum-Fragaria* protein (PcF) and small cysteine- rich secretory protein (SCR) (Chen *et al.,* 2016, Orsomando *et al.,* 2011). The necrosis-inducing *Phytophthora* protein 1 (NPP1) domain is a semi-conserved domain that normally follows an N-terminal secretion signal peptide. Notable examples include *P. sojae* (PsNLP1), which was shown to trigger cell death on soybean, *Arabidopsis*, and *N. benthamiana* (Dong *et al.,* 2012). *Phytophthora parasitica,* NPP1, has been demonstrated to promote cell death on parsley by causing reactive oxygen species and mitogen-activated protein kinase activation (Attard *et al.,* 2008). *Phytophthora cactorum-Fragaria* protein is a 52-residues acidic protein presenting three disulphide bonds (Nicastro *et al.,* 2009). *Phytophthora cactorum-Fragaria* protein homologues predicted proteins include: SCR74 and

SCR91 encoded by polymorphic genes from *P. infestans*, PcSCR82 from *P. capsici* which was reported to induce necrotic activity in pepper and tomato plant (Lin *et al.,* 2020, Zhang *et al.,* 2021). In members of the Solanaceae family, SCR96 from *P. cactorum* was discovered to generate reactive oxygen species (ROS) and disease resistance (Chen *et al.,* 2016). Other elicitors include the CBEL glycoprotein from *P. nicotianae*, which binds crystalline cellulose and stimulates a variety of defense responses in tobacco, including signaling pathways for salicylic acid (SA) and jasmonate (JA), as well as participating in callose deposition (Khatib *et al.,* 2004). Although *Phytophthora* spp. elicitors are known for causing plant cell death, however studies have shown that they also induce a broad-spectrum of plant responses during the biotrophic phase to promote cell death (Du *et al.,* 2015, Takemoto and Mizuno, 2016, Van Poppel *et al.,* 2008 Vidhyasekaran, 2014,).

1.4.3 Cytoplasmic crinkler effectors

Crinkler (CRN) effector proteins are named for their crinkling and necrosis inducing activity, when overexpressed during transient expression (Stassen and Van den Ackerveken, 2011). Cytoplasmic CRN effectors were first reported in *Phytophthora infestans*, but they have since been revealed in other plant pathogenic oomycetes such *P. sojae, P. parasitica, P. ramorum, Hyaloperonospora arabidopsidis, Bremia lactucae, and Pythium ultimum* (Ai *et al.,* 2021, Anderson *et al.,* 2015, Everthart *et al.,* 2014, Maximo *et al.,* 2019, Raffaele *et al.,* 2010, Torto *et al.,* 2003). CRN effectors are among the most highly expressed pathogen genes both prior to and during infection (Schornack *et al.,* 2009). Crinkler effectors have a modular structure similar to RxLR effectors, with a signal peptide and a 50-amino-acid Leu-Xaa-Leu-Phe-Leu-Leu-Ala-Lys (LXFLAK) motif that facilitates translocation into the host cell. The C-terminal section starts with a tri-peptide signature (Asp-Trp-Leu, DWL) and ends with a tri-peptide signature that is well conserved HVLVxxP (His-Val-Leu-Val-Val-Xaa-Xaa-Pro) motif (Schornack *et al.,* 2009, Tyler, 2009, Raffaele *et al.,* 2010). Crinkler effectors have been shown to localize to, target, and concentrate in the nucleus of the host. Only a few CRN effectors have been shown to trigger cell death, whereas the majority suppress cell death caused by PAMPs or other effectors (Jiang *et al.,* 2012, Wawra *et al.,* 2012). For example, PpCRN7 and PpCRN20 from *P. parasitica* suppress cell death in *N. benthamiana* (Dias *et al.,* 2019, Maximo *et al.,* 2019). *Phytophthora sojae* PsCRN115 suppresses plant cell death by

interaction with and effecting stability of plant catalases, the essential enzymes of scavenging reactive oxygen species, while *P. sojae* PsCRN63 was shown to induce cell death (Rajput *et al.,* 2014, Zhang *et al.,* 2015).

1.4.4 Cytoplasmic RxLR effectors

Aside from the Crinkler effectors, *Phytophthora* genomes encode another type of host-translocated effectors, the RxLR effectors. The RxLR effectors have an N- terminal signal peptide followed by a conserved Arg-any amino acid-Leu-Arg (RxLR) motif (Ai *et al.,* 2020, Birch *et al.,* 2009, Morgan and Kamoun, 2007, Oh *et al.,* 2010). The RxLR motif appears within 30 residues of a secretion signal cleavage site and is usually followed by the less conserved Asp-Glu-Glu-Arg (dEER) motif (Huang *et al.,* 2019). The RxLR-dEER motif is hypothesized to have a role in translocation of effector proteins from the haustorium (Wang *et al.,* 2017). Additional conserved motifs named W, Y, and L motifs have been discovered in the C terminus of several RxLR effectors (Birch *et al.,* 2008, Win *et al.,* 2012, Mesarich *et al.,* 2015, Wood *et al.,* 2020). Furthermore, these motifs have been found to be important in pathogenicity (Boevink *et al.,* 2020, Jiang *et al.,* 2012). The RxLR motif's mechanisms are thought to be comparable to those of the "pexel" translocation motif identified in *Plasmodium falciparum*, the malaria parasite (Wawra *et al.,* 2012). These findings suggest that plant and animal eukaryotes may use comparable strategies to secrete effector proteins into their hosts. Furthermore, recent research has shown that RxLR effectors promotes infection through targeting a number of plant regulatory proteins (Ali *et al.,* 2020, Deb *et al.,* 2018, Du *et al.,* 2021, Evangelisti *et al.,* 2013, Lin *et al.,* 2021, Murphy *et al.,* 2018, Ren *et al.,* 2019, Wang *et al.,* 2019, Yang *et al.,* 2019, Yu *et al.,* 2012, Zheng *et al.,* 2018). For example, in the presence of Ca2+, *P. infestans* SFI5 interacts with calmodulin to suppress ROS accumulation and mitogen-activated protein kinases (Zheng *et al.,* 2018). *Phytophthora sojae* destabilizes soybean Type2 GmACSs to suppress ethylene biosynthesis, and *Peronophythora litchii* PiAvhh142, triggers oxidative burst, callose deposition, and hormone signalling pathways (Situ *et al.,* 2020, Yang and Hong *et al.,* 2018).

In *Phytophthora*, RxLR effectors are a broad family of virulence proteins (Huang *et al.,* 2019, Wood *et al.,* 2020, Wang *et al.,* 2019). Sequenced *Phytophthora* genomes have been shown to encode RxLR effectors ranging from approximately 40 to 550 amino acid (Du *et al.,* 2018, Zhang *et al.,* 2019). These RxLR genes were shown to be

rapidly evolving due to the continuous arms race for survival between *Phytophthora* pathogens and host plants (Jiang *et al.,* 2008, Zhang *et al.,* 2019). Comparative genome analyses of *Phytophthora* spp. demonstrate that some RxLR effectors are substantially conserved among species, despite the loss and gain of RxLR effectors (Ai *et al.,* 2020, Deb *et al.,* 2018). Conserved RxLR effectors also known as ―core‖ RxLR effectors (CREs), are RxLR effector proteins that are conserved across pathogen strains or species and have the potential to perform a virulence role (Chepsergon *et al.,* 2021, Xiong *et al.,* 2014). Using bioinformatics pipeline, some of the CREs have been identified from *Phytophthora* spp., however, only few have been characterized. In *P. sojae,* 42 CREs (including PsAvh73, Psavh23 and PsPSR2) were reported to suppress PAMP, while Psavh241 was shown to induce soybean cell death (Deb *et al.,* 2018, Kong *et al.,* 2017, Zhang *et al.,* 2019). Five CREs have been identified in *P. infestans*, including AVR2, which has been reported to avoid detection by the host immunity system, AVR3a, which manipulates plant immunity by stabilizing host E3 ligase, and AVRblb2, which prevents host protease secretion (Yang *et al.,* 2020, Yin *et al.,* 2017). AVRblb1 and AVrvnt1 induce resistance in potato by encoding R genes Rpi-blb and Rpi-vnt1 respectively (Andriani *et al.,* 2021). *Phytophthora brassicae* RxLR24 (CRE) has been demonstrated to interact with RABA-type GTPases in the host to limit vesicle-mediated antimicrobial protein production (Schlaeppi *et al.,* 2010, Tomczynska *et al.,* 2018). Conserved RxLR effectors from other *Phytophthora* spp. have been studied, but nothing is known about CREs from *P. parasitica*, with the exception of *P. parasitica* isolate IAC 01-95, which was shown to secrete 172 CREs (Dalio *et al.,* 2018). Among the identified effectors three effectors were functionally characterized. PpRxLR2 has been demonstrated to totally reduce INF-1-induced cell death, but PpRxLR3 and PpRxLR5 suppress *N. benthamiana* immunity to a lesser extent (Dalio *et al.,* 2018). Understanding the biological functions of *P. parasitica* CREs RXLR effectors in the host–pathogen interaction could reveal mechanisms underlying *Phytophthora* pathogenicity, which would be useful for creating long-lasting plant resistance.

1.5 Plant defense

Plants and phytopathogens have been in an arms competition for millions of years. As a result, plants have developed two levels of protection to protect themselves from invading pathogens (Zhang *et al.,* 2019). Pattern recognition receptors (PRRs)

mediate the initial layer of defense system, which allows for the identification of conserved pathogen or microbial-associated molecular patterns (PAMPs / MAMPs) – triggered immunity (PTI) (Hann *et al.,* 2014). A bacterial flagellum peptide with 22 conserved amino acids (flg22), bacterial elongation factor Tu (EF-Tu), Nep1-like proteins (NLPs), cellulose-binding elicitor lectins (CBELs), INF1, and the glycoside hydrolase protein PsXEG1 have all been identified as PAMPs/MAMPs from bacterial, fungal, and oomycetes pathogens (Imran and Yun*,* 2020, Jeblick *et al.,* 2020, Lu *et al.,* 2020, Thor *et al.,* 2020, Wen *et al.,* 2021). Recognition of PAMPs / MAMPs by the plant PRRs results in a complicated signal transduction network including induction or suppression of mitogen-activated protein kinases (MAPK) cascades, generation of reactive oxygen species, callose deposition and hormone signalling (Situ *et al.,* 2020). Effector-triggered immunity (ETI) is the second line of defense, it is mediated by disease resistance (R) (nucleotide-binding leucine-rich repeat (NB-LRR) proteins that recognise avirulence (AVR) proteins (Bigeared *et al.,* 2015). The recognition of Avr proteins by R proteins results into a hypersensitive response (HR), which is a type of programmed cell death (PCD) linked to plant pathogen resistance at the infection site and inhibits pathogens from infecting the plant further (Bigeared *et al.,* 2015). Several *Avr* genes have been identified in *Phytophthora* spp*.,* for example, *Avrblb1*, *Avrblb2*, *Avr2*, *Avr3a* and *Avr4* have been identified in *P. infestans, Avr1b*, *Avr1a/3a*, *Avr3b*, *Avr3c*, *Avr4/6*, and *Avr5* from *P. sojae* (Dorrance*,* 2018, Guevara *et al.,* 2005, Lin *et al.,* 2021, Na *et al.,* 2013, Yin *et al.,* 2017).

1.5.1 Manipulation of reactive oxygen species

Reactive oxygen species (ROS) are a group of free radicals, derived from superoxide (O^{-}_{2}) hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Garg and Manchanda, 2009). Intracellular ROS are found in organelles such chloroplasts, peroxisomes, and mitochondria, whereas apoplastic ROS are formed by localized NADPH oxidases, cell wall peroxidases, and amine oxidases in the plasma membrane (Noctor *et al.,* 2018). Drought, salt, and disease infection increase the production of ROS in plants (Huang *et al.,* 2019). It is important for cells to manage the accumulation of ROS in order to avoid oxidative damage, rather than to fully eradicate ROS accumulation (Camejo *et al.,* 2016, Mittler*,* 2017). Excess ROS is scavenged or detoxified by an effective antioxidative system that includes both

enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and glutathione reductase are examples of enzymatic antioxidants (GR). Non- enzymatic antioxidants include ascorbate (ASA), glutathione (GSH), carotenoids, tocopherols, and phenolics (Akram *et al.,* 2017, Khalaf *et al.,* 2008, Sharma and Ahmad, 2014).

One of the early events triggered during plant-pathogen interaction is the accumulation of ROS (Torres, 2010, Wojtaszek, 1997). They function as direct antimicrobial compounds, as well as local and systemic secondary messengers, triggering further immune responses such gene expression and stomatal closure (Torres *et al.,* 2006, Bolwell and Wojtaszek, 1997). For example, hydrogen peroxide built up, has been found to be critical in the fight against biotrophic *Colletotrichum gloeosporioides* in cowpea (Silva *et al.,* 2019). Although ROS correlates with successful disease resistance responses, ROS build up favours necrotrophic and hemibiotrophic infections. For example, hemibiotrophic such as *P. sojae* PsCRN115 induce ROS in *N. benthamiana* by targeting respiratory burst oxidase (RbohA and RbohB), *P. capsici* effector targets ACD11 binding partners to induce ROS accumulation in *Arabidopsis* (Li *et al.,* 2019, Zhang *et al.,* 2015). Since *Phytophthora spp.* are hemibiotrophic pathogens, they have to keep the host cell alive during the biotrophic, therefore, some effectors are secreted to suppress the accumulation of ROS to maintain the biotrophic phase. *Phytophthora sojae* PsCRN70 and PsCRN63, for example, have been found to suppress ROS during the biotrophic phase (Zhang *et al.,* 2015). Thus, ROS is produced as part of a complex network of signals that responds to pathogen attack, sometimes with opposite effects, in different pathogens.

Reactive oxygen species have been reported to act as downstream regulators of other plant defences (Li *et al.,* 2019). Callose has been identified as another component of plant defense that is influenced by ROS build up, however little is known about how these two defense mechanisms interact (Kong *et al.,* 2013). Reactive oxygen species and SA work together to drive HR and the building of systemic defences. Salicylic acid down regulates ROS-scavenging systems to establish synergistic connections, which can lead to increased total ROS levels (Herrera—vasquiez *et al*., 2015). Furthermore, high levels of ROS cause a redox imbalance in the cell, favouring monomerization and migration of NONEXPRESSOR

OF PATHOGENESIS-RELATED GENES1 to the nuclei, where it induces PATHOGENESIS-RELATED (PR) gene expression in collaboration with TGACG-binding TGACG (Yun and Chen, 2011).

1.5.2 Manipulation of callose deposition

Callose, is a cell wall polymer that exist in all multicellular green algae and higher plants (Chen and Kim, 2009, Ellinger and Voigot, 2014). Callose deposition creates a primary barrier that prevents pathogen penetration into host cells, inhibits the secretion of virulence effectors and reduces the availability of nutrients to the pathogen thereby contributing to disease resistance (Luna *et al.,* 2011, Voigot, 2014, Wang *et al.,* 2022). Flagellin (Flg22) and the bacterial elongation factor EF-Tu (Elf18) have been identified as callose-induced PAMPs (Wang *et al.,* 2020). Callose is deposited in the plasmodesmata (PD) upon pathogen recognition (Voigot, 2014). The PD act as transport pathways to the vascular system and have a variety of roles in coordination at the cellular and organism level (Chen and Kim, 2009). Nutrients, metabolites, hormones, and other signaling molecules are among the substances transported across the PD channels (Hofmann *et al.,* 2010). The amount of callose in the plasmodesmatal neck zone is critical for cellular molecules transfer from cell to cell. Callose levels over a certain threshold diminish or even close plasmodesmatal channels, whereas callose levels below that threshold open channels (Wu *et al.,* 2018). Two groups of enzymes control the amount of callose in the plasmodesmata: callose synthase (CalS), which synthesizes callose, and 1, 3-glucanases, which degrades callose (De Storme and Geelen, 2014). In addition, the master regulator of SA (NPR1) was reported to regulate plasmodesmata closure and callose deposition in *Arabidopsis* (Huang *et al.,* 2019, Tomczyynska *et al.,* 2020). Regardless of how plasmodesmata closure and callose deposition are controlled, *Phytophthora* spp. have evolved to manipulate the deposition of callose. For example, *P. brassicae* RxLR3 localises to PD and physically interacts with CalS to inhibit callose deposition (Tomcyznska *et al.,* 2020).

1.5.3 Manipulation of salicylic acid

Salicylic acid is a key component of systemic and local acquired resistance (SAR) against biotrophic infections. *Pseudomonas syringe*, for example, is inhibited by SA, which colonizes between cells by creating nutrient-absorbing structures while keeping the host alive (Yang *et al.,* 2015). The isochorismate pathway and the

phenylpropanoid pathway are two separate branches of the SA biosynthesis, but both require the chemical chorismate generated from the shikimate system (Chen *et al.,* 2009).

Chorismate is transformed to SA in the chloroplast via two processes catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate in the isochorismate (IC) pathway (Sendon *et al.,* 2011). The phenylalanine ammonia lyase (PAL) route begins with the conversion of chorismate to prephenate catalyzed by chorismate mutase, followed by phenylalanine synthesis. The conversion of phenylalanine to cinnamate is then catalyzed by PAL, which can later be turned to SA via a sequence of enzymes (Kumar, 2014). The ICS system is the most significant in *Arabidopsis*, whereas the PAL pathway appears to be more important in rice (Chen *et al.,* 2009, Jiang *et al.,* 2010). It is possible that both mechanisms contribute equally in some cases, as it is in soybean (Khan *et al.,* 2003).

Salicylic acid is detected by two types of receptors during plant-pathogen interactions: NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 and NPR3/NPR4, both of which have a high affinity for SA (Zheng *et al.,* 2015). NPR1 is a transcriptional activator that enhances the production of defense genes activated by SA and pathogen resistance (Haung *et al.,* 2020). Through redox shifts, SA enhances the reduction of the oligomeric form of NPR1 in the cytoplasm to a monomeric form during plant–pathogen interactions. Monomeric NPR1 is then moved to the nucleus, where it promotes SA-responsive gene expression. When the SA level is low, NPR3 and NPR4 act as redundant transcriptional co-repressors, preventing the activation of defense-gene expression (Kumar, 2014). When SA levels are high, SA inhibits NPR3/transcriptional NPR4's repression activity, allowing SA-responsive genes to be expressed. A collection of redundant bZIP transcription factors, including TGA2, TGA5, and TGA6, which interact with both NPR1 and NPR3/NPR4, help NPR1 and NPR3/NPR4 regulate defense genes (Di *et al.,* 2017). *Phytophthora* spp., on the other hand, has devised strategies to reduce SA in order to suppress host immunity. This is corroborated by research on *Phytophthora* spp., such as *P. infestans* PexRD24, which was discovered to interact with three protein phosphatase 1 catalytic (PP1c) isoforms and drives their re-localization from the nucleolus to the nucleoplasm, which lowers SA build up (Boevink *et al.,* 2016). *Phytophthora sojae* PsIsc1 reduces SA levels by converting isochorismate to 2,3-

dihydro-2,3-dihydroxybenzoate (DDHB) and pyruvate, preventing isochorismate from being used in SA production (Toruno *et al.,* 2016). *Phytophthora sojae* PsAvh163 and *H. arabidopsidis* HaRxL62 and HaRxL96 effectors were also found to reduce SA levels (Anderson *et al.,* 2015). Salicylic acid and Jasmonic acid pathways have been demonstrated to be antagonistic in studies (Kunkel and Brooks, 2002, Leon-Reyes *et al.,* 2009, Van der Does *et al.,* 2013). The SA-signalling component nonexpressor of PATHOGENESIS-RELATED GENE 1, as well as the downstream transcription factors TGAs and WRKYs, play a role in this antagonism at the molecular level (Takahashi *et al.,* 2004). Pathogens, on the other hand, tend to take advantage of this crosstalk. *Hyaloperonospora arabidopsidis* HaRxL44 has been demonstrated to disrupt mediator function by degrading MED19, altering the balance of defense transcription from SA-responsive defense to JA/ET signaling, and increasing vulnerability to biotrophs by attenuating SA-dependent gene expression (Cambrode, 2020, Luo *et al.,* 2019).

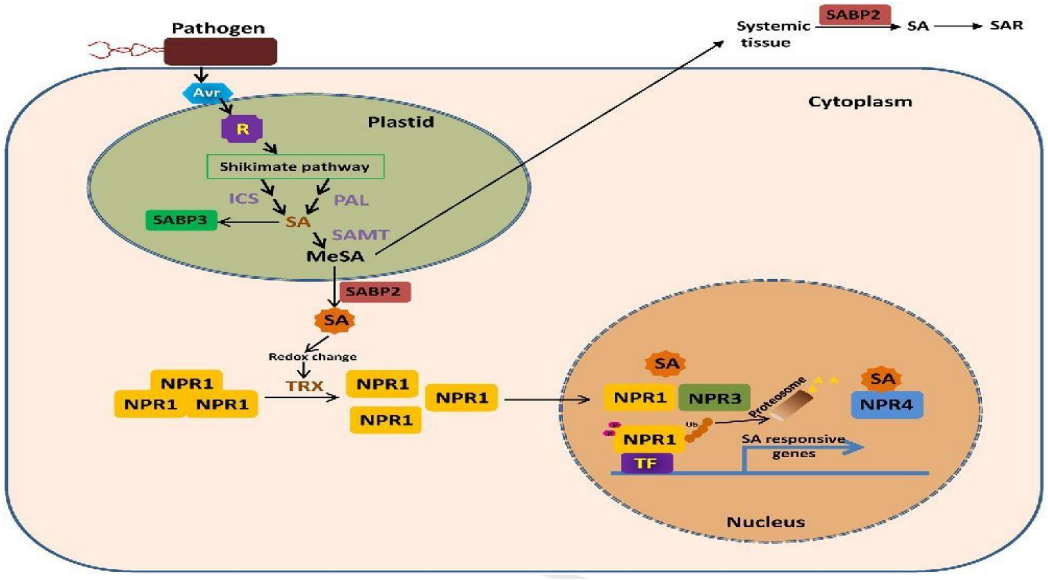

Figure 1.2: The biosynthesis of salicylic acid through isochorismate and phenylpropanoid pathway. Regulation of salicylic acid/ interaction of NONEXPRESSOR PATHOGENESIS-RELATED GENES1 (NPR1) and NPR3/NPR4 upon pathogen attack (Kumar, 2014).

1.5.4 Manipulation of ethylene

Ethylene is a gaseous phytohormone that plays an important function in plant growth and physiological responses (Wang and Ecker, 2002). The first committed step in the production of ethylene, is the conversion of S-adenosylmethionine (SAM) to aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) (Pech *et al.,* 2010). In addition, ACS also creates 5′-methylthioadenosine (MTA) during this stage, which is then converted to methionine via a modified methionine cycle (Guilfoyle *et al.,* 2015).

The methyl group is saved for another cycle of ethylene synthesis using this salvage mechanism. As a result, ethylene can be manufactured indefinitely without requiring a growing supply of methionine. At the same time, the methionine's sulfur group is preserved (Schaller and Binder, 2017). Finally, ACC is oxidized by ACC oxidase to produce ethylene, CO_2, and cyanide, which is detoxified to cyanoalanine synthase to avoid toxicity from accumulated cyanide during high rates of ethylene production (Pattyn *et al.,* 2021, Schaller and Binder, 2017, Xu and Zhang, 2015). The process for ethylene production is highly controlled, with overlapping transcriptional and post-transcriptional regulatory sites for the enzymes involved (Wang, 2002).

Ethylene is detected by endoplasmic reticulum (ER)-localized receptors, which operate as negative regulators of the ET signaling pathway after it accumulates (Sharma *et al.,* 2019). Because the Raf-like kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) associated with the receptors is inactivated when ET binds, the ER-localized EIN2 becomes dephosphorylated. The C-terminal domain (CEND) of dephosphorylated EIN2 penetrates the nucleus and sends signals to the EIN3 transcription factor (Dubois *et al.,* 2018). ETHYLENE RESPONSE FACTOR 1 (ERF1) and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59)
are two ET-responsive transcription factors that EIN3 directly activates, amplifying and eliciting the ET response (Schaller and Binder, 2017). Ethylene also stabilizes EIN3 by removing two F-box proteins, EIN3 BINDING F-BOX PROTEIN 1 (EBF1) and EBF2, which in the absence of ET target EIN3 for proteasomal destruction. EIN3 induces the production of EBF1 and EBF2, creating a negative feedback loop for the ET signaling pathway (Pech *et al.,* 2010). Furthermore, when necrotrophic pathogens attack, the expression of PDF1.2 via ERF1 and ORA59 may be controlled by a synergistic interaction between ET and JA (Binder, 2020,

Phukan *et al.,* 2017, Sharma *et al.,* 2019, Dubois *et al.,* 2018). Ethylene activates resistance to necrotrophic pathogens such as *Botrytis cinerea* and *P. irregulare,* in addition to its involvement in plant development (Liu *et al.,* 2014). Some necrotrophic and hemibiotrophic pathogens have devised techniques to regulate ET. For example, *P. sojae* PsAvh238, interacts with soybean ACSs, the rate-limiting enzyme, resulting in ethylene synthesis inhibition (Yang *et al.,* 2019). Other hemibiotrophic pathogens have been shown to induce ET, but their targets have yet to be identified.

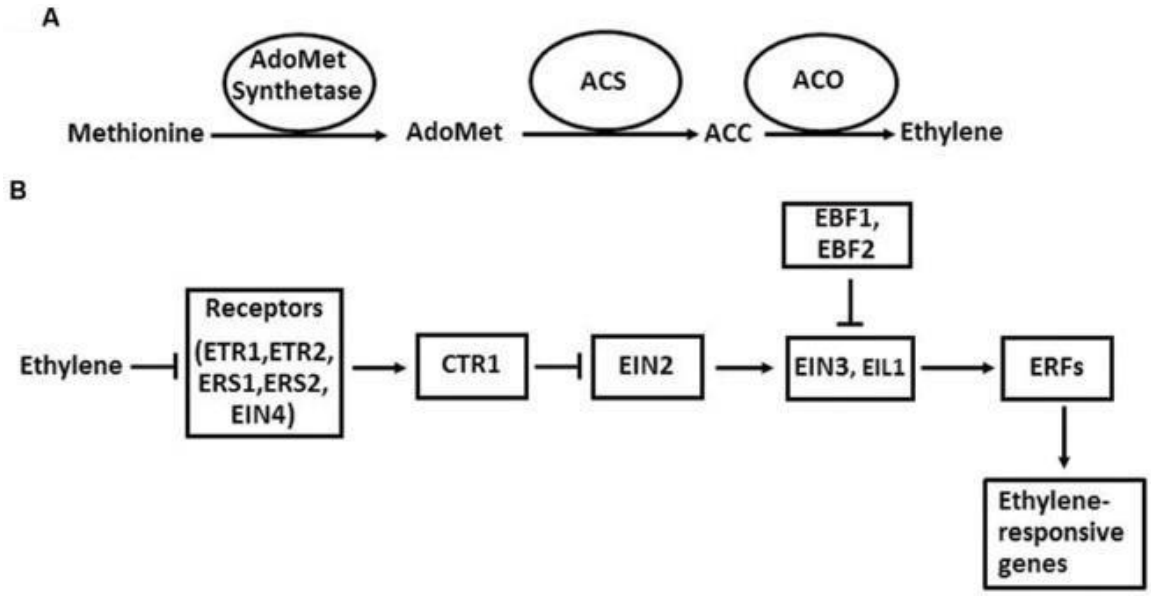

Figure 1.3: The biosynthesis and regulation of ethylene genes. (a) Represents the biosynthesis and steps that maintain continuous production of ethylene. (b) Represents regulation of ethylene after accumulation and interaction of the receptor genes (Song and Liu, 2015).

1.5.5 Manipulation of jasmonic acid

Another key plant hormone, jasmonic acid, governs growth, development, and activates resistance to necrotrophic pathogens (Mueller, 1997, Raun *et al.,* 2019, Zhang *et al.,* 2019). Its production begins with the fatty acid linolenic acid, which is converted to a bioactive 7-Iso-jasmonoyl-I-isoleucine (JA-lle) by lipoxygenase (13-LOX) (Halim *et al.,* 2006). As it is catalyzed by the enzyme allene oxide cyclase, this allene oxide rearranges to generate 12-oxophytodienoic acid, which then undergoes a series of -oxidations to form 7-iso-jasmonic acid. This iso-jasmonic acid isomerizes

to jasmonic acid in the absence of allene oxide cyclase (Tamaoki *et al.,* 2013; Wang *et al.,* 2020, Wasternack and Hause, 2013).

A collection of proteins known as the JASMONATE ZIM-DOMAIN proteins (JAZs) suppresses JA signaling (Ali and Baek, 2020, Zhang *et al.,* 2019). JASMONATE ZIM-DOMAIN protein interacts with JA-responsive transcription factors in the nucleus and represses their activities (Yang *et al.,* 2019). Development of a receptor complex containing JAZs, the F-box protein COI1, and inositol pentakisphosphate is aided by high levels of JA-Ile (Wasternack and Hause, 2013). The JA-Ile accumulates in response to stress and binds to the F-box protein COI1, allowing the development of the COI1-JAZs complex, which leads to ubiquitination and eventual degradation of JAZ repressors by the 26S proteasome (Li *et al.,* 2019). The MYC-branch and the ERF-branch are two different branches of the JA signaling pathway downstream of COI1-JAZ perception. The MYC-branch is in charge of wound- and insect-induced JA signaling pathway. MYC2, MYC3, and MYC4 are leucine zipper transcription factors with a basic helix-loop-helix structure that control this branch (Yang *et al.,* 2019). JASMONATE ZIM-DOMAIN proteins interact with MYC protein in a competitive manner, preventing it from interacting with the MED25 subunit of the transcriptional Mediator complex (An *et al.,* 2017). Mediator is a multiprotein complex (MED component) that acts as a signal integrator, relaying information from DNA- binding TFs to the transcriptional machinery of RNA polymerase II (Pol II) (Samanta and Thakur, 2015). When JAZs are removed, the MYC-branch is activated, resulting in the activation of a large number of JA-responsive genes, including the JA marker gene VSP2, the JA synthesis gene LOX2, and the JA signaling repressor JAZ genes (Li *et al.,* 2019). During a necrotrophic pathogen attack, the ERF-branch is activated (Wang *et al.,* 2020). The ET signaling pathway and transcription factors ORA59 and ERF1, which directly trigger the expression of ERF-branch marker genes like PDF1.2, are synergistically regulated by this branch. The ORA59 and ERF1 bind to the GCC-box motif via the ERF domain. However, it is unknown whether JAZ repressors interact with ERFs directly or indirectly (Meldau *et al.,* 2012).

Regardless of how JA is regulated, *Phytophthora* spp. has evolved to manipulate JA regulation. For example, the previously identified *P. infestans* effector PexRD24 reduces not only SA but also JA (Boevink *et al.,* 2016). *Phytophthora sojae* PsCRN70 suppress JA by interfering with the marker gene lipoxygenase (Rajput *et*

al., 2014). Jasmonic acid signaling has also been demonstrated to affect plant growth and development via inducing the MAP kinase pathway, calcium channel, and many other processes that interact with signaling molecules including ethylene, salicylic acid, and abscisic acid (Creelman and Mullet*,* 1995, Meldau *et al.,* 2012).

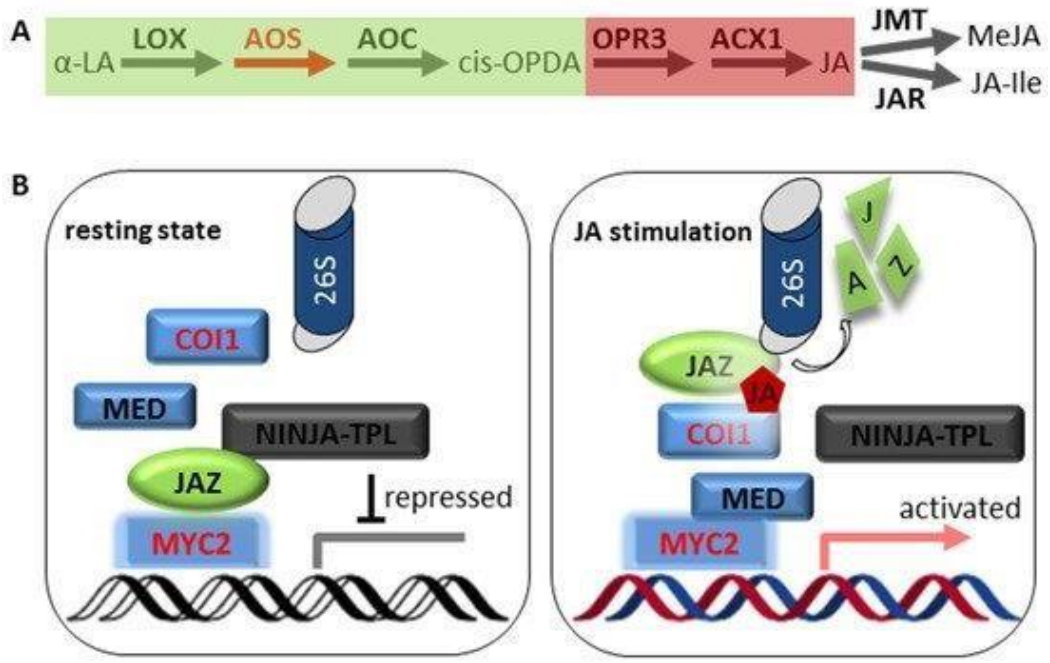

Figure 1.4: The biosynthesis and regulation of jasmonic acid. (a) Biosynthesis of jasmonic acid. (b) Regulation of jasmonic acid during pathogen attack (Li *et al.,* 2019).

1.5.6 Manipulation of mitogen activated protein kinases

Mitogen activated protein kinase cascades are one of the most frequently used mechanisms by which cells function and are found in all eukaryotes, including plants, fungi, and mammals (Bigeard and Hirt, 2018, Cristina *et al.,* 2010, Raja *et al.,* 2017). They play a role in a variety of cellular activities such as growth, development, and stress responses (Hettenhausen *et al.,* 2015, Medau *et al,* 2012). Three types of kinases are involved in MAPK signal transduction: mitogen activated protein (MAP) kinase kinase kinases (MAPKKKs), MAP kinase kinases (MKKs), and MAP kinases (MAPKs) (Colcombet and Hirt, 2008). The activation of MAPKKK by stimulation of plasma membrane receptors is the initial signal transduction step, by phosphorylating two serine or threonine residues in the S/T-X5-S/T (X is any amino acid) motif, the MAPKKK activates a downstream MAPKK. When active, the MAPKK

acts as a dual-specificity kinase, phosphorylating a MAPK on threonine and tyrosine residues in an activation loop's T-X-Y motif. When the terminal kinase is engaged, it can phosphorylate particular downstream effector proteins, causing cellular responses to be initiated (Hirt, 2000, Nakagami *et al.,* 2005, Pedley and Martin, 2005, Pitzschke *et al.,* 2009, Sinha *et al.,* 2011, Zhang *et al.,* 2006).

Mitogen activated protein kinase transcription is closely controlled by both negative and positive regulators to ensure that they are activated at the appropriate moment (Raman *et al.,* 2007). *Phytophthora* spp*.,* on the other hand, has developed mechanism to manipulate MAPK transcription for their own gain. *Phytophthora infestans* PexRD2, for example, inhibits the activity of the MAPKKK kinase domain, a positive regulator of plant cell death (King *et al.,* 2014). To reduce plant immunity, *P. infestans* RxLR effectors PITG20303 and PITG20300 target and stabilize a plant resistance negative regulator called StMKK1 (Chen *et al.,* 2021).

Mitogen activated protein kinases are involved in the regulation of defense genes such as SA, JA, and ET. On *Arabidopsis*, MPK6 was shown to govern ET biosynthesis via controlling the transcription of ACS, a rate liming enzyme (Lopez- Bucio *et al.,* 2014). Mitogen activated protein kinases like AtMPK4 and AtMPK6 are engaged in both JA and SA signaling (Yi *et al.,* 2015). In tomatoes SlMPK6-1 and SlMPK6-2 are positive regulators of JA biosynthesis and signaling pathways (Meldau *et al.,* 2012). In addition, some MAPKs may suppress plant response such as cell death, ROS and callose. For example, HopAI1, a conserved effector from *Pseudomonas syringe pv. tomato,* dephosphorylates MPK3 and MPK6 and hence suppresses flg22-induced gene expression, oxidative burst, and callose deposition in *Arabidopsis*, increasing disease susceptibility (Dahale *et al.,* 2021). *Phytophthora sojae* Avh331 inhibits the formation of reactive oxygen species and callose via interacting with the MAPKs RK1, WRKY22, and WRKY29 (Cheng *et al.,* 2012).

1.6 *Nicotiana benthamiana* as host plant in plant-pathogen interactions research

A variety of plants are used in plant- pathogen interactions such as, *A. thaliana,* tobacco *(Nicotiana tabacum*), tomato (*Solanum lycopersicum*), po-tato (*Solanum tuberosum*) and *N*. *benthamiana* (Hsu *et al.,* 2012). However*, N. benthamiana* is the commonly used host plant in plant-pathogen interactions and other areas of biology (Bally *et al.,* 2018). Plant virologists initially adopted the species due to its

susceptibility to a wide range of viral pathogens. This property led to its early use in studies of virus-induced gene silencing and the underlying mechanisms of RNA interference (Nguyen *et al.,* 2010). Because *N. benthamiana* has large leaves, researchers have used either designed viruses or syringe-infiltration of *Agrobacterium tumefaciens* to transiently express proteins (Bombarely *et al.,* 2012). These techniques make it easier to immunoprecipitate proteins, see where proteins are located within cells, and check for post-translational changes (Bally *et al.,* 2018). Additionally, *N. benthamiana* shares a lot of genetic similarities with other agriculturally important Solanaceous plants, including tomato, potato, pepper, and petunia, which are *Phytophthora* host plants (Goodwin *et al.,* 2008). As a result, functional characterization of *Phytophthora* RxLR effector proteins in *N. benthamiana* will almost certainly reveal a cadre of genes that play similar roles in agriculturally important crops. Despite its many benefits, it is an allotetraploid plant with a big genome (3.1 Gb) that makes it challenging to modify the plant's genome and generate mutants for plant biology and gene functional studies (Hsu *et al.,* 2012). Additionally, there are relatively few genomics resources available for it (Goodwin *et al.,* 2008).

1.7 Rationale of the study

Novel plant disease control strategies are in high demand in agriculture, food and forestry to combat high crop losses caused by *Phytophthora* species. The rapid appearance of resistance among *Phytophthora* species and side effect of chemical treatments do necessitate a search for alternative methods. Remarkable progress has been made in understanding *Phytophthora* RxLR effectors (including conserved RxLR effectors) as they play a major role in virulence. However, little is known about core RxLR effectors from *P. parasitica*. Our laboratory has identified a set of 71 effectors that are conserved amongst *P. parasitica* isolates (Chepsergon *et al.,* 2022). Many of these effectors are yet to be functionally characterised. Understanding the functions of *P. parasitica* core RxLR effectors in host- plant interactions could be beneficial for developing efficient and long lasting resistance in plants.

Therefore, based on this rationale, the objectives of the study are to;

- Determine the expression of two of the 71 *P. parasitica* core effectors, namely, PpRxLR1 and PpRxLR6 effector proteins using qPCR.
- Evaluate the role of *P. parasitica* PpRxLR1 and PpRxLR6 in *N. benthamiana* immunity (by determining ROS, callose deposition and hormonal signalling pathways and MAPKs).

1.8 References

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Chapter two

The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in *Nicotiana benthamiana*

2.1 Introduction

To combat pathogens, plants have developed multi-layered immune systems for self-defence (Zhang *et al.,* 2019). Defense responses can be initiated at the host cell surface by pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) (Hann *et al.,* 2014). The perception of PAMPs activates a complicated signal transduction network, including reactive oxygen species (ROS), callose deposition, plant hormones and mitogen-activated protein kinase (MAPK) cascades (Situ *et al.,* 2020). When PTI is suppressed by a pathogen to establish disease, a second layer of resistance (effector-triggered immunity) can be activated following direct or indirect detection of effectors by resistance (R) proteins (Bigeared *et al.,* 2015).

Phytophthora spp. are among the most damaging phytopathogens (Chang *et al.,* 2015). They not only continues to cost modern agriculture billions of dollars annually but also impacts subsistence farming in developing countries (Kamoun *et al.,* 2015, Lu *et al.,* 2020, Meng *et al.,* 2014). As hemibiotrophic pathogens, *Phytophthora* spp., initially requires a living host cells, then later in the infection process they induce cell death, generating disease lesions that may increase pathogen colonization (Lamour *et al.,* 2012, Meng *et al.,* 2014, Wi *et al.,* 2012). This may be achieved by secreting effector proteins which act outside or inside plant cells to target and perturb signalling, regulatory or mechanistic processes associated with defence (Bonnet *et al.,* 2007, Maximo *et al.,* 2019, Olivia *et al,* 2010). Among them, RxLR effectors are large superfamily of virulence proteins in *Phytophthora* (Birch *et al*, 2009, Dong *et al.,* 2021, Huang *et al.,* 2019, Wood *et al.,* 2020, Wang *et al.,* 2019). For example, *P. brassicae* RxLR3, suppress callose by interacting with CalS (Tomcyznska *et al.,* 2020). *Phytophthora infestans* PexRD24 lowers SA accumulation (Boevink *et al.,* 2016). *Phytophthora infestans* PexRD2, inhibits the activity of the MAPKKK kinase domain, a positive regulator of plant cell death (King *et al.,* 2014).

Phytophthora RxLR effectors are under pressure to evolve rapidly for different infection strategy or adapt to their host (Jiang *et al.,* 2008, Zhang *et al.,* 2019).

However, comparative genome analysis of *Phytophthora* spp. reveals that several RxLR effectors are conserved (CRE) across pathogen strains or species, suggesting that they may play virulence function (Chepsergon *et al.,* 2021, Jiang and Tyler*,* 2012). In *P. parasitica* 172 CRE effectors have been identified, however, only PpRxLR2, PpRxLR3 and PpRxLR5 were characterized (Dalio *et al.,* 2018). As a result, identifying and characterizing CRE from *P. parasitica* is critical.

In this study, we demonstrate that *P. parasitica* PpRxLR1 and PpRxLR6 induce plant defense responses, including ROS, callose deposition, phytohormones and MAPKs. In addition, they promote colonization of *P. parasitica*. The work provides a critical foundation for further dissection of the roles of *P. parasitica* PpRxLR1 and PpRxLR6 effectors in interaction with plant immunity.

2.2 Materials and Methods

2.2.1 Culture media and growth conditions

Phytophthora parasitica INRA 310 was grown on 10% (w/v) V8 agar medium in the dark at 25 ^{o} C to produce mycelium. Sporangia were induced by adding soil solution into plates containing mycelia and exposed to light. The plates with sporangia were incubated at 4 ^{o} C to release zoospores. *Nicotiana benthamiana* plants were grown in Phytotron with conditions 24 ^{o} C and 40 % - 80 % humidity for eight weeks. The eight weeks old *N. benthamiana* leaves were all harvested at the same time, inoculated with 10^5 µl of zoospores suspension (the detachment method) and placed in the dark (Dalio *et al.,* 2018). The phenotypic changes of the leaves (The phenotyphic changes can be easly observed on leaves compared to the roots) were recorded at 0, 6, 12, 24, 36, 48, 60 and 72 hours. The experiments were performed using three biological replicates.

2.2.2 Total RNA extraction

Nicotiana benthamiana leaves inoculated in section 2.21 were weighed (adjusted to 100 mg) and grounded in liquid nitrogen. Total RNA were extracted using RNase plant mini kit (Qiagen, Germantown, USA) according to manufactures' instructions. Genomic DNA contamination was removed with DNAse I (Thermo Fisher Scientific, Vilnius, Lithuania) according to manufactures' instructions.

2.2.3 Determination of total RNA quality

The concentration and purity of each extracted total RNA sample was evaluated using spectrophotometric analysis (NanoDrop ND -1000; NanoDrop technologies, Wilmington, DE) at a ratio of 230/260 nm. The 28/18s ratio was determined with Agilent 2100 Bioanalyzer (Agilent Technologies, California, Santa clara), shown in figure S2.1 (Garcia-Andrade *et al.,* 2011).

2.2.4 cDNA synthesis

Total extracted RNA for individual time course and replicates were used to generate first strand cDNA using SuperScript^{TM} Reverse Transcriptase kit according to manufacturer's instructions (Thermo Fisher Scientific, Vilnius, Lithuania). The synthesized cDNA concentrations were measured with qubit fluorimeter (Thermo Fisher Scientific, Vilnius, Lithuania) and adjusted to 20 µL for individual time course so that they all have the same concentration (Garcia-Andrade *et al.,* 2011).

2.2.5 Quantitative real-time PCR (qRT-PCR)

To determine expression of biotrophic (PpHmp1), necrotrophic (PpNPP1), PpRxLR1 and PpRxLR6 maker genes, the synthesized cDNA for individual time course and replicates were used for RT-qPCR analysis. The RT-qPCR primers were synthesized by Inqaba Biotech^{TM} (Pretoria, South Africa) (Table 2.3).The annealing temperatures and sizes were confirmed with normal PCR (Figure S2.3). The RT-qPCR was performed on Quantstudio 5 Real-Time PCR system (Thermo Fisher Scientific Vilnius, Lithuania). A mixture of 10 µL containing 5 µL SYBR green master mix, 0.3 µL forward primer, 0.7 µL reverse primer, 2.5 µL template (20ng) and 1.5 µL H_2O was use for RT-PCR analysis. The RT-qPCR cycling were set as follows: denaturation at 95 ^{o} C for 3 minutes then 35 cycles of 95 ^{o} C for 15 seconds, 49 ^{o} C (for all the primers) for 30 seconds, 72 ^{o} C for 1 minute and extension for 5 minutes at 72 ^{o} C .The relative fold differences of gene expression were calculated according to the $2^{-\Delta\Delta Ct}$ method using ubiquitin gene as an internal references.

2.2.6 Detection of reactive oxygen species (ROS)

Nicotiana benthamiana leaves were infiltrated with *A. tumefaciens* GV3101 expressing PpRxLR1, PpRxLR6 effectors and GFP (negative control). Infiltrated leaves were harvested after 36 hours post inoculation. Harvested leaves were stained with diaminobenzidie-HCL solution (1 mg/ml, pH 3, 8) overnight in the dark and distained with 96% (v/v) ethanol. Images were taken from whole leaves with a camera and on light microscope with the magnification of 10x (UL, Tokyo Japan). The accumulation of ROS was quantified with image J software (Hann *et al.,* 2014).

2.2.7 Detection of callose deposition

To determine callose deposition, *N. benthamiana* leaves infiltrated with *A. tumefaciens* GV3101 expressing PpRxLR1 and PpRxLR6 effectors and GFP (negative control) were harvested after 36 hours post inoculation. The chlorophyll of the harvested leaves was cleared with 95 % (v/v) ethanol. Completely distained leaves were stained with 0, 01 % (w/v) aniline blue in a solution of 150 mM, K_2HPO_4, pH 9,5 for 30 min. Callose deposits were visualized in a fluorescence microscope using DAPI filter (The excitation wavelength for aniline blue was 370 nm and emission maximum 509 nm)(Leica Microsystems, Wetzlar, Germany). The number of callose deposits was quantified with image J software (Jiang and Tyler, 2012).

2.2.8 Phytohormones and MAPKs analysis

To evaluate whether the effectors alter phytohormones and MAPKs in planta, *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 (concentration 0.4 mM) expressing PpRxLR1 and PpRxLR6 effectors and GFP (negative control) respectively. Infiltrated leaves were harvested from 0, 12, 24 and 36 hours post inoculation. The collected leaves were grounded in liquid nitrogen. Total RNA were extracted using RNase plant mini kit (Qiagen, USA), then remove genomic DNA with DNAse I (Thermo Fisher Scientific) and used the RNA to generate first strand DNA using SuperScript^{TM} Reverse Transcriptase (Thermo Fisher Scientific Vilnius, Lithuania) according to manufacturer's instructions (Figure S2.2). The RT-qPCR were performed on Quantstudio 5 Real- Time PCR system (Thermo Fisher Scientific, Vilnius, Lithuania) as previously described with slight modification (annealing temperature 47 ^{o} C for the markers, except NbPR1 which anneal at 49 ^{o} C). The expression kinetics of the effector genes were assessed using the salicylic acid (SA) –dependent defence pathways marker genes NbPR1 and NbPR2, jasmonic acid (JA)-dependent defence pathways marker gene NbLOX, ethylene (ET) –dependent defence pathways marker gene NbERF1, mitogen activated protein kinases MPK3 and MPK6 (Table 2.1). The relative fold differences of gene expression were calculated according to the $2^{-\Delta\Delta Ct}$ method using actin gene as an internal reference (Garcia-Andrade *et al.,* 2011).

2.2.9 Host susceptibility

Nicotiana benthamiana leaves were infiltrated with *A. tumefaciens* GV3101 expressing PpRxLR1 and PpRxLR6 effectors and GFP (negative control). The leaves were harvested after 2 days of infiltration. Mycelia discs of *P. parasitica*, *P. cinnamomi* and *P. sojae* were transferred to detached *N. benthamiana* leaves. Inoculated leaves were kept a 25 °C in a moisture box and inspected daily for disease symptoms. For the evaluation of disease severity, photographs were taken with camera and infected areas were measured using Image J (Chang, 2015).

Table 2.1: Candidate reference gene and target gene primer sequences for qPCR

Gene	Primer pairs (5'-3')	Source
Actin	F: CTTGAAACAGCAAAGACCAGC R: GGAATCTCTCAGCACCAATG	Chen *et al.,* 2018
NbPR1	F: CCGCCTTCCCTCAACTCAAC R: GCACAACCAAGACGTACTGAG	Situ *et al.,* 2020
NbPR2	F: CATCACAGGGTTCGTTTAGGA R: GGGTTCTTGTTGTTCTCATCA	Situ *et al.,* 2020
NbLOX	F: CCTTAAGAGGAGATGGAACT R: TCTAAGCTCATAAGCAATGG	Situ *et al.,* 2020
NbERF1	F: GGCGAATTTTCCGGGAGACT R: GGCTCCGATTTTACTTCGCC	Situ *et al.,* 2020
MPK3	F: TGACGTTTGACCCCAACAGA R: CTGTTCCTCATCCAGAGGCTG	Galleti *et al.,* 2011
MPK6	F: CCGACAGTGCATCCTTTAGCT R: TGGGCCAATGCGTCTAAAAC	Galleti *et al.,* 2011
UBC	F: CCACTTAGAGCACGCTAGGA R: TACCGACTGTCCTTCGTTCA	Dailo *et al.,* 2018
PcHmp1	F: GTCGCTCTAAATGCGGCTAC R: TGACGATCTGCAACTGGAAG	Zuluaga *et al.*, 2016
PcNpp1	F: AAACCGCAGATCCACATCTC R: AGACGCCATTGTACGTAGCC	Zuluaga *et al.*, 2016
PpRxLR1	F: AGAAAGGGAATGCGTCTGG R: CTCACAGCGGATTTCACAAG	In this study
PpRxLR6	F: ACGGAAGGTTTTTGTGCATC R: GGGTTATTCAAAGGGGGGA	In this study

2.2.10 Statistical analysis

In this study, experiments were performed in triplicate. The analysis of variance (ANOVA) was used for statistical analysis, followed by a Tukey-Kramer post hoc test with a p-value cut-off of less than 0.05.For RT-qPCR analysis, the mean Cq value of the internal control was subtracted from the mean Cq of the target gene (Cq mean target gene – Cq endogenous control) to normalize gene expression.Following that, the fold changes were computed using the 2-Ct technique (Livak and Schmittgen, 2001).

2.3 Results

2.3.1 *Phytophthora parasitica* changes its life cycle from biotrophic to necrotrophic phase in *Nicotiana benthamiana* leaves

Hemibiotrophic pathogens change their life cycle during infection, which can be observed on the leaves (phenotypic) and through quantification with qPCR. To determine the changes in life cycle of *P. parasitica* through phenotypic changes in the host, *N. benthamiana* leaves were inoculated with *P. parasitica* zoospores (concentration was adjusted to 10^5 µL) at different time points ranging from 0, 6, 12, 24, 36, 48, 48, 60 and 72 hours. The time points were selected based on the life cycle of *Phytophthora* spp. so as to include both the biotrophic and necrotrophic phase. After inoculation we observed the phenotype of the leaves from 0-72 hours post-inoculation hpi. The leaves where green and healthy from 0 to 24 hpi, turned yellow between 36 to 48 hpi. Necrotrophic growth with considerable cell death-like symptoms was observed at 60 and 72 hpi (figure 2.1). The results indicated that *P. parasitica* is indeed a hemibiotrophic pathogen, switches from non-damaging (0-24 hpi) to a damaging mode (36-72 hpi) (figure 2.1).

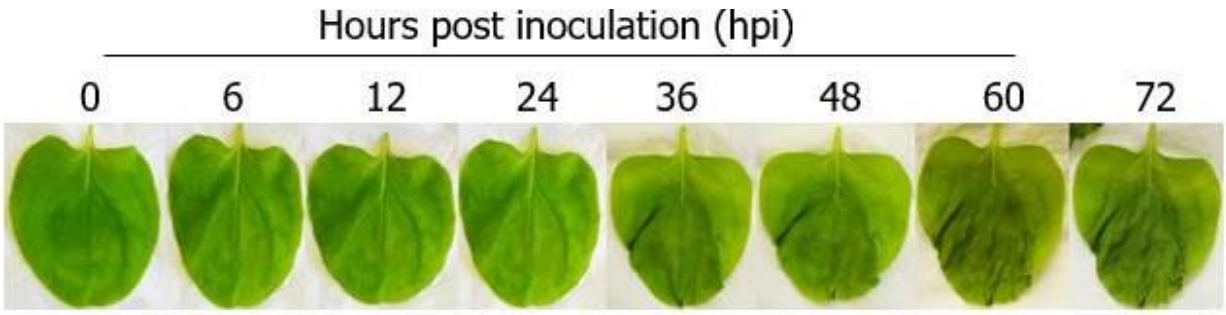

Figure 2.1: Phenotypic changes of *Nicotiana benthamiana* leaves as a result of the switch of *Phytophthora parasitica* from biotrophic to necrotrophic phase. The leaves where green and healthy from 0 to 24 hpi, turned yellow between 36 to 48 hpi. At 60 and 72 hours post-inoculation, necrotrophic growth with severe cell death-like symptoms was observed. Photographs were taken at the time points indicated.

2.3.2 qPCR reveals early expression of the biotrophic phase marker gene in contrast to the late expression of the necrotrophic phase marker gene

To understand the infection process of *P. parasitica* in more detail, we inoculated *N. benthamiana* leaves with *P. parasitica* zoospores (concentration was adjusted to 10^5) at different time points ranging from 0, 6, 12, 24, 36, 48, 48, 60 and 72 hours. After inoculation, RNA was extracted and used to synthesize cDNA explained in section 2.2.4. The expression of PcHmp1 and PcNpp1 marker genes for biotrophic and necrotrophic phase, respectively was analysed using real time qPCR analysis. The data obtained were normalized to the expression of ubiquitin gene, which shows a constant expression across different life stages. The qRT-PCR results showed that the biotrophic phase marker gene (PcHmp1) was differential ($p<0.05$) expressed starting from 0h to 12 hpi, and then the highest expression peak appeared at 24 hpi and rapidly declined at 36 hpi. The relative expression of the marker gene was barely detectable at 48 hpi and 60 hpi. On the contrary, the expression of the necrotrophic phase marker (PcNpp1) was expressed starting from 24 hpi to 48 hpi and increased steadily reaching a peak at 72 hpi. The qPCR results correlated well with phenotypic results shown in Figure 2.1, as the leaves were green and healthy from 0- 24 hpi on the phenotypic results and on qPCR there was an expression starting from 0h and reach a peak at 24 phi both results indicating a biotrophic phase. The decline in expression of the biotrophic phase marker (at 36 hpi) in Figure 2.2a, coincided with the induction, followed by increasing expression of the necrotrophic phase marker (Figure 2.2b) as

well as yellowing of leaves (Figure 2.1).The necrotrophic growth like symptoms were observed at 60 and 72 hpi where there was high expression of necrotrophic phase marker. This result suggests that during the expression of biotrophic phase there are no visible symptoms; however, visible symptoms appeared when the expression of the necrotrophic phase starts.

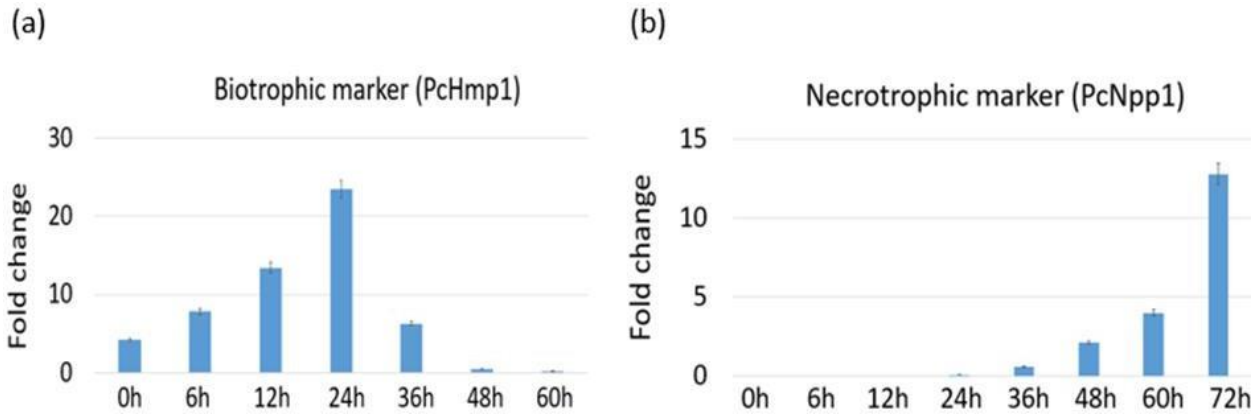

Figure 2.2: Reverse transcription polymerase chain reaction analysis of *Phytophthora parasitica* life cycle. (a) Biotrophic phase marker gene (PcHmp1) was differential expressed starting from 0 hpi to 12 hpi, reach a peak at 24 hpi, declined at 36hpi and barely detectable at 48 hpi and 60 hpi. (b) Necrotrophic phase marker (PcNpp1) was expressed starting from 24 hpi to 48 hpi and increased steadily reaching a peak at 72 hpi.The RNA samples were prepared from *Nicotiana benthamiana* plants (5 weeks old) inoculated with zoospores of *Phytophthora parasitica*.

2.3.3 The expression of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in *N. benthamiana* leaves.

To validate the expression profile of *P. parasitica* PpRxLR1 and PpRxLR6 during infection, the cDNA synthesized on section 2.3.2 were used for qPCR. *Phytophthora parasitica* PpRxLR1 and PpRxLR6 marker genes were used for qPCR analysis. The data were normalized to the expression of ubiquitin gene, which shows constant expression across different life stages. During infection of *N. benthamiana* leaves with *P. parasitica* zoospores, PpRxLR1 transcripts was differentially ($p<0.05$) expressed from 24 to 36hpi, reach a peak at 48hpi, and started declining at 60 and 72 hpi. Whereas, PpRxLR6 was expressed from 0 to12hpi, steadily reach a peak at 24hpi and started declining from at 36 and 48 hpi. No transcripts of PpRxLR6 could be detected

at 60 and 72 hpi. The expression of both effectors during the early stages of infection suggests that they might be important in the virulence of *P. parasitica.*

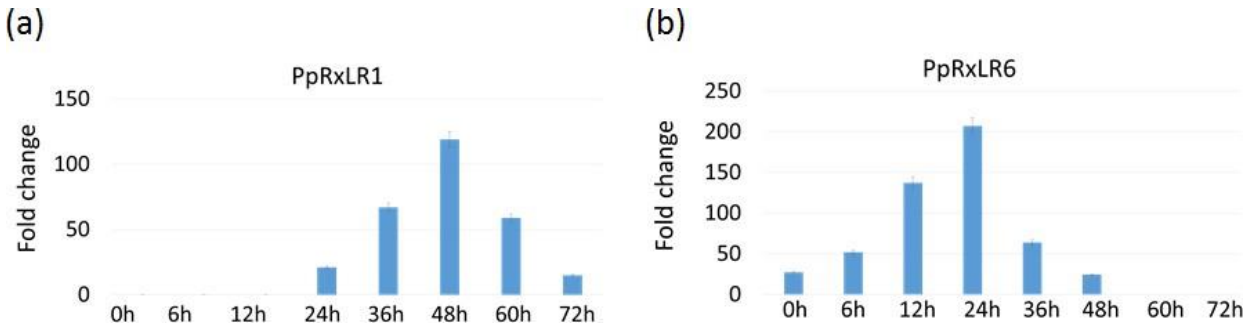

Figure 2.3: Reverse transcription polymerase chain reaction analysis of *Phytophthora parasitica* core effectors PpRxLR1 and PpRxLR6 during infection. The RNA samples were prepared from *Nicotiana benthamiana* plants (5 weeks old) inoculated with zoospores of *Phytophthora parasitica* at eight different time points.(a) PpRxLR1. (b) PpRxLR6.

2.3.4 The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in reactive oxygen species (ROS) accumulation

To understand the role of *P. parasitica* PpRxLR1 and PpRxLR6 during infection, we tested whether the transient expression of PpRxLR1 and PpRxLR6 affects H_2O_2 accumulation in *N. benthamiana* leaves via agro-infiltration with GFP as a negative control. The accumulation of brown polymer in figure 2.4 show that PpRxLR1 and PpRxLR6 induce ROS accumulation compared with the negative control (GFP) at 36 hours post agro-infiltration (hpa). High accumulation of ROS was observed on the side of leaves infiltrated with PpRxLR1 compared to PpRxLR6 ($p<0.05$). A study that was done in our lab revealed that *P. parasitica* PpRxLR1 and PpRxLR6 induce cell death in *N. benthamiana*. Since H_2O_2 is a critical trigger of plant cell death we anticipate that both effectors promote cell death in *N. benthamiana* by inducing ROS accumulation.

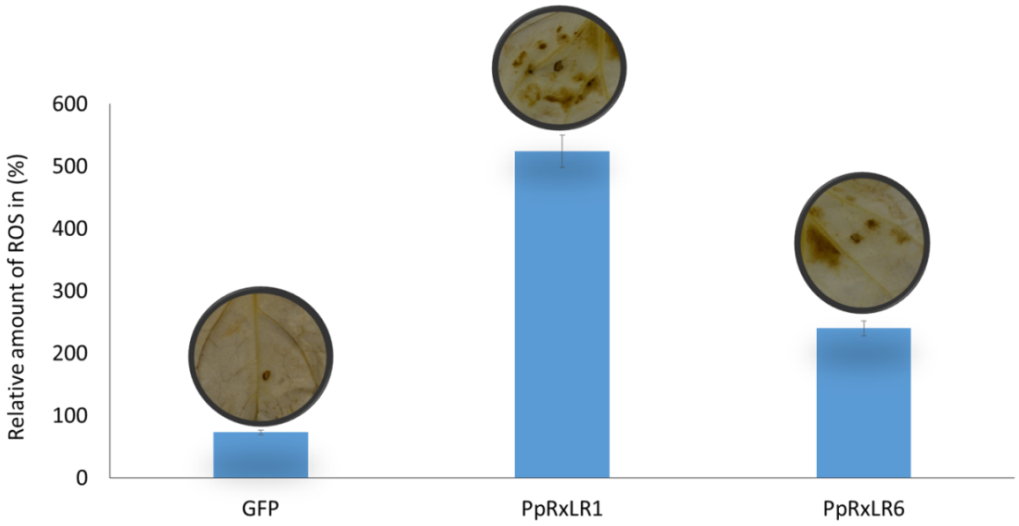

Figure 2.4: Relative ROS accumulation in *Nicotiana benthamiana* leaves. In leaves expressing PpRxLR1 and PpRxLR6 there was high accumulation of ROS compared to GFP at 36 hpa. Photographs were taken at 36 hpa and quantified with ImageJ software. Mean ± SE was derived from three independent biological repeats.

2.3.5 The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in callose deposition

To determine whether *P. parasitica* PpRxLR1 and PpRxLR6 interferes with callose deposition, *N. benthamiana* leaves were infiltrated with PpRxLR1, PpRxLR6 and GFP as negative control separately. As shown in figure 2.5, the ANOVA analysis show that PpRxLR1 and PpRxLR6 induce callose deposition, which is presented by the white pixels. However, there was high accumulation of callose in leaves expressing PpRxLR1 compared to PpRxLR6 at 36 hpa. *Phytophthora* effectors induce callose deposition directly by targeting the synthesis or indirectly by inducing other plant immunity that will act downstream of callose. Therefore, we anticipate that both effectors might induce callose directly or indirectly to promotes cell death.

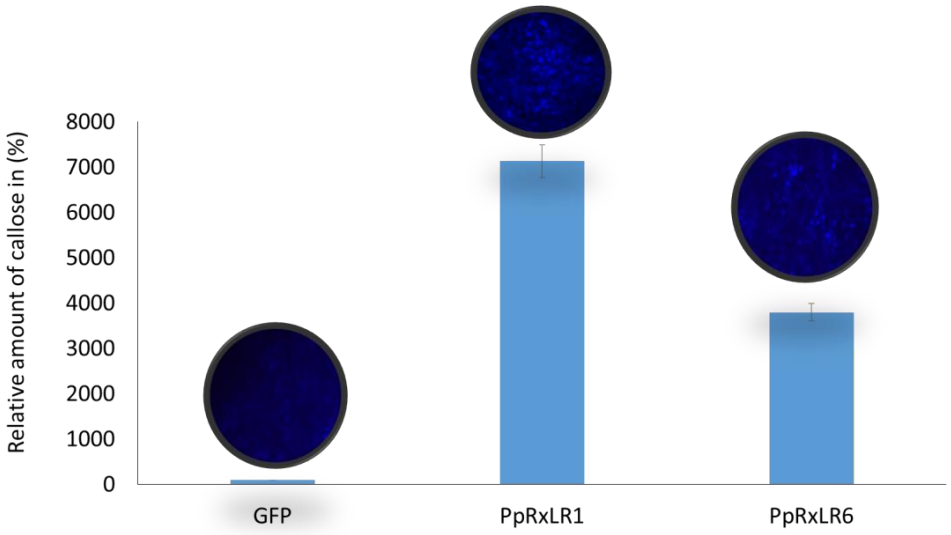

Figure 2.5: Callose deposition in *Nicotiana benthamiana* leaves. Callose deposition was induced in leaves expressing PpRxLR1 and PpRxLR6 compared to GFP at 36 hpa. Callose deposition was visualized with fluorescence microscope (wavelength 370nm and emission maximum 509nm) and quantified with ImageJ software. Mean ± SE was derived from three independent biological repeats.

2.3.6 The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in phytohormones and MAPKs.

To increase our understanding on the role of PpRxLR1 and PpRxLR6 in plant defense responses, transcriptional levels of defence-related genes were measured using real time qRT-PCR analysis. Defence-related genes were measured at various time points (0, 12, 24, and 36 hr) after agro-infiltration of *N. benthamiana* leaves with PpRxLR1, PpRxLR6 or GFP. The NbPR1, NbPR2, NbLOX, NbERF1, MPK3 and MPK6 are marker genes for salicylate (first two), ethylene, jasmonate, mitogen activated protein (last two), respectively. Data were normalized to the expression of actin gene, which shows a constant expression across different life stages. The expression levels of these defence-related genes (SA, ET, JA, MPK3 & MPK6) followed the same trend for leaves expressing both effectors. In figure 2.6 SA (NbPR1, NbPR2), ET (NbERF1), JA (NbLOX), MPK3 and MPK6 marker genes were differentially expressed ($p<0.05$) starting from 0 hpa, increased steadily at 24 hpa reaching a peak a 36 hpa compared to GFP which was constantly expressed. However, the expression for PpRxLR6 was high compared to PpRxLR1 for all the defense genes. The results suggest that both effectors induce defense genes during the biotrophic phase which might contributes to cell death.

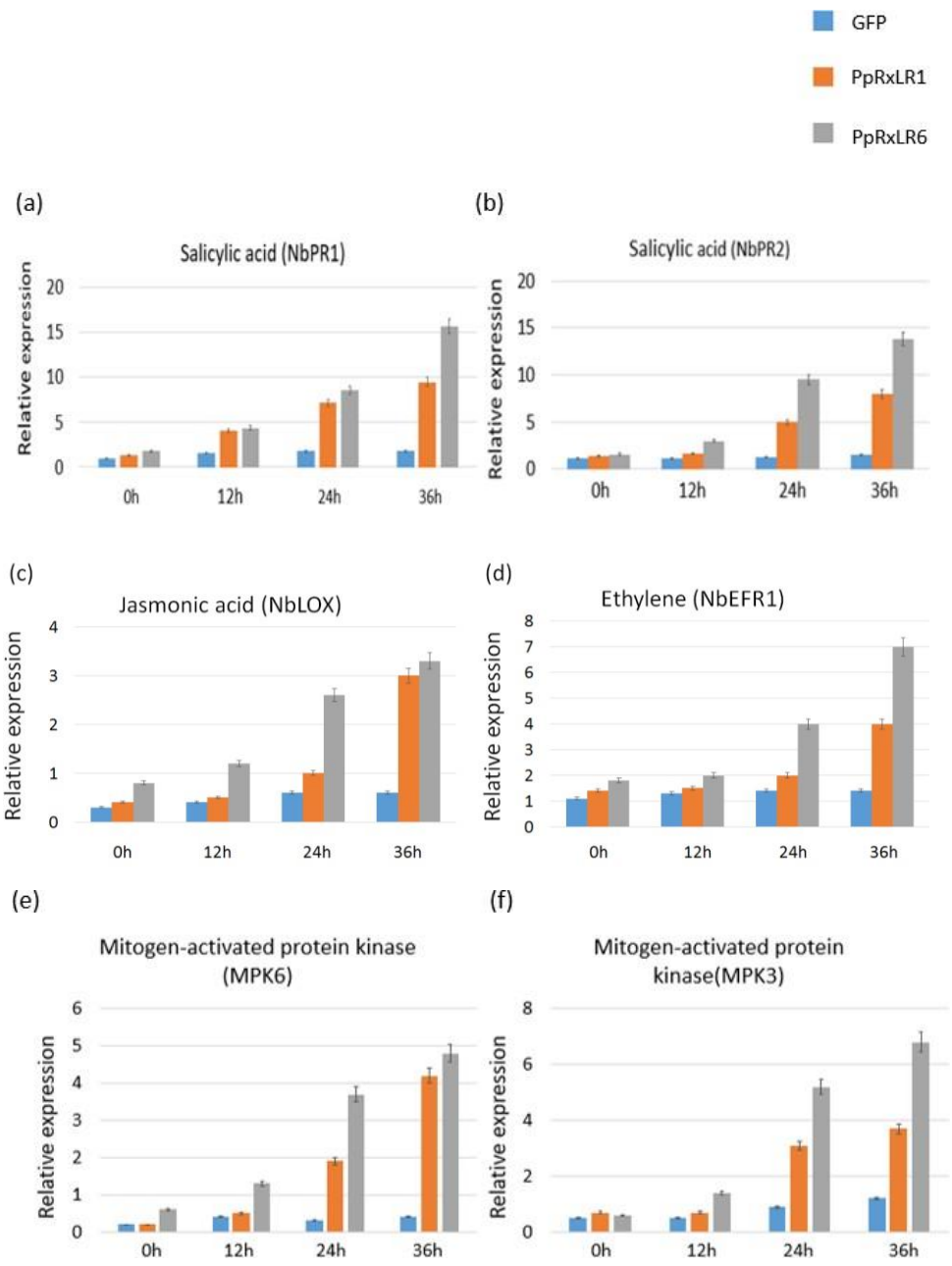

Figure 2.6: Relative expression of defence-related genes in *Nicotiana benthamiana*. RNA samples were extracted from leaves expressing PpRxLR1, PpRxLR6 or GFP at 0 hpi, 12 hpi, 24 hpi and 36 hpi. All defense related genes were up regulated in leaves expressing PpRxLR1 and PpRxLR6 at 0 hpa, increase from 12 hpa to 24 hpa and reach a peak at 36 hpa. (a) The expression of salicylic acid NPR1. (b) Salicylic acid maker gene NPR2. (c) Expression of ethylene marker gene NbERF1. (d) The expression of jasmonic acid (NbLOX). (e) Expression of MPK3 gene. (f) Expression of the MPK6 gene.

2.3.7 The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in *Nicotiana benthamiana* susceptibility to other *Phytophthora* species

To elucidate roles of PpRxLR1 and PpRxLR6 in *N. benthamiana* in host susceptibility to *P. parasitica*, we performed agro-infiltration on *N. benthamiana* leaves, with one-half of the leaf expressing PpRxLR1 and GFP, PpRxLR6 and GFP respectively. To determine *N. benthamiana* susceptibility to other *Phytophthora spp*., we performed agro-infiltration on *N. benthamiana* leaves, with one-half of the leaf expressing PpRxLR1, PpRxLR6 on the other half and GFP at the bottom. We inoculated both sides of the leaves with *P. parasitica*, *P. cinnamomi* and *P. sojae* mycelia discs two days after infiltration and checked them daily for disease symptoms. Compared with GFP, PpRxLR1-pretreated leaves showed significantly high disease severity in terms of symptoms in *P. parasitica*, *P. cinnamomi* and *P. sojae* (shown in figure 2.7*)*. The results show that PpRxLR1 induce *P. parasitica, P. cinnamomi* and *P. sojae*. Interestingly, PpRxLR6 increases the disease progression of *P. parasitica*, however, reduces disease symptoms of both *P. cinnamomi* and *P. sojae*. Taken together, these results showed that PpRxLR1 enhanced susceptibility of *N. benthamiana* to *Phytophthora* spp., while PpRxLR6 promotes *P. parasitica* and reduceses *P. cinnamomi* and *P. sojae* susceptibility *N. benthamiana*.

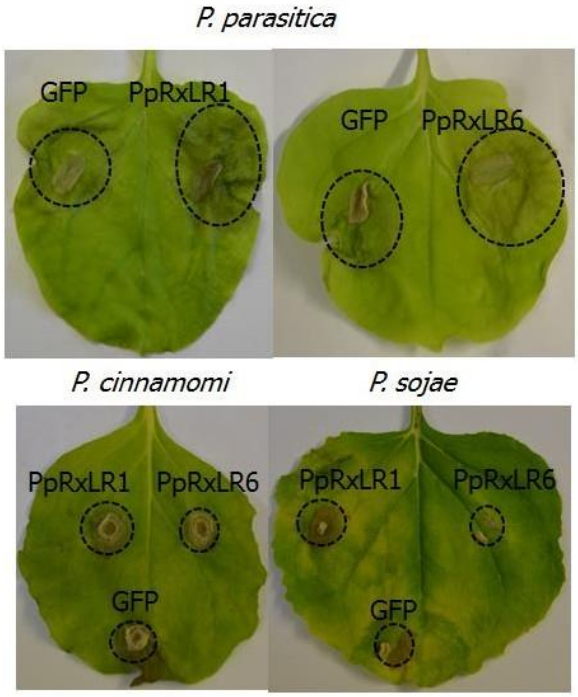

Figure 2.7: The role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in *Nicotiana benthamiana* susceptibility to *Phytophthora parasitica*, *Phytophthora cinammomi* and *Phytophthora sojae*. Two days after infiltration with PpRxLR1, PpRxLR6 or GFP on leaves of 5-week-old *N. benthamiana*, leaves were inoculated with mycelia discs of *Phytophthora parasitica*, *Phytophthora cinammomi* and *Phytophthora sojae.* Photographs were taken at 3 days post-inoculation (dpi).

2.4 Discussion

Hemibiotrophic pathogens must maintain the host cell alive during the biotrophic stage and later generate disease lesions that may increase pathogen colonization (Meng *et al.,* 2014). During the interaction of *P. parasitica* INRA 310 with *N. benthamiana*, *P. parasitica* switches it life cycle from non-damaging mode to parasitic growth which was observed from 36 to 72 hrs of post inoculation. Based on the phenotypic data and qPCR analysis, the duration of the biotrophic phase was short, with an early necrotrophic phase. In *A. thaliana* the necrotrophic symptoms were observed after 3 days, which correlate with our results. This suggests that the biotrophic phase of *P. parasitica* might be short with an early necrotrophic phase in other host plants. In addition, these results are similar to observations made on *P. capsici*, however, different with what was observed on *P. infestans* as it was shown to have a long biotrophic phase and late necrotrophic phase (Jupe *et al.,* 2013). Therefore, this suggests that the duration of the life cycles varies among this species. The

symptomless, biotrophic phase of *P. parasitica*, is a cause for concern under field conditions as this could make the disease essentially undetectable during early stages of infection. During the interaction of *P. parasitica* with its hosts, *P. parasitica* secretes hundreds of RxLR effector proteins into the cytoplasmic region to promote infection (Deb *et al.,* 2018). Using *in silico* analyses a study conducted in our lab revealed *P. parasitica* secretes 71 CRE. Among the 71 CRE, PpRxLR1 and PpRxLR6 effector proteins were selected for further functional characterization as they were shown to be highly conserved and expressed during early stages of infection *in silico*. PpRxLR1 and PpRxLR6 were shown to be expressed from 24 hrs and 0 hrs, respectively. Given the observed early expression of PpRxLR6, it is possible that it is expressed in the zoospores. RxLR effectors that are functionally significant are frequently expressed during infection stages, although just a few RxLR effectors are regarded to be crucial for pathogen pathogenicity, despite the fact that they provide the great majority of RxLR effector transcripts (Haung *et al.,* 2019). Both effectors mostly accumulated during the biotrophic phase, with PpRxLR1 being expressed in the late biotrophic phase and PpRxLR6 expressed in the earlier phase, suggesting that they might play a role in host colonization processes as well as the transition of *P. parasitica* from the biotrophic to necrotrophic phase. This observation correlates with the study that was done on *P. parasitica PpE4*, as it was shown to be highly expressed during the early stages of infection and contributes to the transition of the pathogen (Zhang *et al.,* 2020).

In the arms between plants and phytopathogens, plants have developed strategies to prevent pathogen colonization (Zhang *et al.,* 2019). The accumulation of ROS has been hypothesized as one of the first events triggered during plant-pathogen interactions, which controls and restricts pathogen growth (Haung *et al.,* 2019). Plants produce biphasic ROS during interactions with pathogens, with the first phase having a low amplitude transient and the second phase having a significantly higher and prolonged accumulation of ROS (Torres, 2010). The second phase of ROS build up has only been described during avirulent pathogenesis, which occurs before the HR and cell death that frequently accompany effective pathogen detection, resulting in incompatible interactions (Bigeared *et al.,* 2015). Although ROS correlates with successful disease resistance responses, ROS build up favours necrotrophic and hemibiotrophic pathogens (Li *et al.,* 2019, Zhang *et al.,* 2015). In this study, we observed strong accumulation of ROS in leaves expressing PpRxLR1 and PpRxLR6

compared to the control (GFP). Therefore, it is possible that *P. parasitica* PpRxLR1 and PpRxLR6 promote plant cell death or necrotrophic phase by inducing ROS during the biotrophic phase of infection to enable successful or transitioning of *P. parasitica* from biotrophic to necrotrophic phase. Since *P.parasitica* was not restricted by the accumulation of ROS, we anticipate that *P.parasitica* is not being recognized by the plant R-genes. In addition, *P.parasitica* might have mechanisms to overcome ROS accumulation.

Callose deposition is another defense strategy for plants that results in a primary barrier preventing pathogen penetration into the host cells, it inhibits the secretion of virulence effectors and reduces the availability of nutrients to the pathogen thereby contributing to disease resistance (Vatenet *et al.,* 2011). The cell-to-cell transport of biological substances such as nutrients, metabolites, hormones, and other signaling molecules is aided by callose deposition in the plasmodesmata callose levels over a certain threshold diminish or even close plasmodesmatal channels, whereas callose levels below that threshold open the channels (Voigot, 2014). In this study, we observed high accumulation of callose on leaves expressing PpRxLR1 and PpRxLR6 compared to the control (GFP). We anticipate that *P. parasitica* secrete these effectors' to induce callose which will later might results into trafficking of important molecules including metabolites, hormones and signaling molecules as additional strategy of transition the pathogen to necrotrophic phase. It is also possible that these effectors induce callose indirectly by inducing ROS, as callose deposition follow the same trend with ROS mentioned above. The results correlate with what was found on other *Phytophthora* spp. including *P. sojae* PsCRN63 which was shown to suppress both ROS and callose, while *P. sojae* PsCRN115 was shown to induce ROS and callose (Zhang *et al.,* 2015).

Salicylic acid (SA) is a key component of systemic and local acquired resistance (SAR) against biotrophic pathogens (Yang *et al.,* 2015). Ding *et al.,* 2018 reported that NPR1 is the master regular of SA, as loss of NPR1 results in diminished SA and higher vulnerability to pathogens. In this study, we wanted to know the role of PpRxLR1 and PpRxLR6 in the master regulator of SA. We observed high expression of SA (NbPR1 and NbPR2) on leaves expressing PpRxLR1 and PpRxLR6 compared to the control (GFP) at 36 hours post inoculation. We anticipate that *P. parasitica* PpRxLR1 and PpRxLR6 induce SA as another strategy to promote colonization, as high accumulation of SA down-regulates ROS-scavenging systems which results into

increased overall ROS levels (major trigger of necrosis). The accumulation of SA and ROS does not have an effect on the *P.parasitica*, suggesting that *P.parasitica* have mechanism not to be restricted by plant defense. However, at this point we are unable to tell the exact targets of the effectors on SA, as studies on SA have shown that *Phytophthora* spp. manipulate SA using different mechanisms including targeting the biosynthesis. *Phytophthora sojae* PsIsc1, for example, reduces SA levels by converting isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate (DDHB) and pyruvate, rendering isochorismate unavailable for SA production (Toruno *et al.,* 2016). In addition, some have been shown to take advantage of the antagonistic relationship between SA and JA (Li *et al.,* 2019). For example, the effector HaRxL44 from *H. arabidopsidis* interferes with mediator function by degrading MED19, altering the balance of defense transcription from SA-responsive defense to JA/ET signaling, and increasing vulnerability to biotrophs by suppressing SA-dependent gene expression (Combrode, 2020). However, in the present study both SA and JA hormones were induced, therefore we anticipate that the hormones induced by both effectors were not enough to trigger the antagonistic relationship.

Ethylene boosts resistance against necrotrophs like *Botrytis cinerea* (Li *et al.,* 2019). There are studies on the role of *Phytophthora* effectors on SA and JA (Anderson *et al.,* 2015, Boevink *et al.,* 2016, Meldau *et al.,* 2012, Toruno *et al.,* 2016). However, little is known about the role of *Phytophthora* effectors in ethylene, except *P. sojae* PsAvh238, which interacts with soybean ACSs, the rate limiting enzyme, resulting in suppression of ethylene synthesis (Na *et al.,* 2013). In this study we observed high expression of ET on leaves expressing PpRxLR1 and PpRxLR6 compared to the control (GFP) at 36 hrs post inoculation. We anticipate that *P. parasitica* induce ethylene directly or indirectly by inducing SA and JA, since they have been shown to have synergy relationship.

Jasmonic acid, is another important plant hormone that regulate plant responses to abiotic, biotic as well as plant growth and development. Jasmonic acid, is another target of *Phytophthora*, as it regulate other parts of plant immunity including MAP kinase cascade pathway, calcium channel, ethylene and salicylic acid (Meldau *et al.,* 2012). Our results suggest that PpRxLR1 and PpRxLR6 induce JA, however at this point we are unable to show whether these effectors induce JA directly which may include targeting the biosynthesis, as it was observed with *P. sojae* PsCRN70 which suppress JA by interfering with the marker gene lipoxygenase.Indirectly include

interacting with MAPKs and ET since both were shown to be induced (Boevink *et al.,* 2016).

Besides the defense genes mentioned above, plants recognition of pathogen lead to activation MAPKs (Cheng *et al.,* 2012). The role of MAPKs in plant defense have been well characterized in *Arabidopsis*, including MPK3, MPK4 and MPK6 (Lopez- Bucio *et al.,* 2014). The MAPKs have been shown to induce plant resistance to pathogen. In this study, we determined the role of *P. parasitica* PpRxLR1 and PpRxLR6 in MAPKs specifically MPK3 and MPK6. The expression of MPK3/6 were upregulated on the leaves inoculated with PpRxLR1 and PpRxLR6 compared to GFP. The results suggest the PpRxLR1 and PpRxLR6 uses different strategies to promote colonization, as MPK3/6 induce plant cell death.

Phytophthora parasitica PpRxLR1 and PpRxLR6 were shown to contribute to the susceptibility of *N. benthamiana* when challenged with *P. parasitica.* However, when they were challenged with *P. cinnamomi* and *P. sojae,* the results with PpRxLR1 were the same (induce susceptibility), however, with PpRxLR6 it was interesting to notes that *N. benthamiana* susceptibility to *P. cinammomi* and *P. sojae* was drastically reduced compared to PpRxLR1 and GFP. Our results suggest that the function for PpRxLR1 is conserved for *Phytophthora spp.,* while the function of PpRxLR6 is conserved only for *P. parasitica.* Increases in SA levels are associated with greater PR gene expression and improved disease resistance. According to Zhu *et al.,* 2014 applying SA to tobacco plants boosts their resistance to *tobacco mosaic virus* (TMV). Since SA was highly induced on PpRxLR6 compared to PpRxLR1 we anticipate that SA induced by PpRxLR6 was enough to trigger resistance, this correlate with *P. parasitica* OPEL effector which induce SA to increases the resistance against different pathogens (Haung *et al .,* 2019).

2.5 Conclusion

Phytophthora parasitica INRA310 switches from asymptomatic biotrophic phase to symptomatic necrotrophic phase. *Phytophthora parasitica* achieved colonization by secreting hundreds of RxLR effectors including PpRxLR1 and PpRxLR6 which were shown to be highly conserved *in silico*. Functional characterization in *N. benthamiana* revealed that *P. parasitica* PpRxLR1 and PpRxLR6 were expressed during the biotrophic phase.

The expression of this effectors induce *N. benthamiana* immunity during the biotrophic phase, including ROS, callose, phytohormones and MAPKs. In addition both effectors promotes the colonization of *P. parasitica,* however this still need to be confirmed by determining the pathogen load. Understanding the principal targets of the *P. parasitica* PpRxLR1 and PpRxLR6-induced pathways could lead to a better understanding of the mechanisms driving *P. parasitica* pathogenicity and breeding long-lasting resistance in plants.

2.6 Supporting data

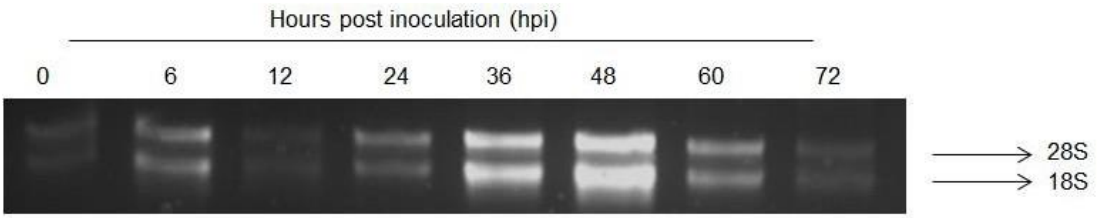

Figure S2.1: Total RNA extracted from *Nicotiana benthamiana* inoculated with *Phytophthora parasitica* zoospore at eight different time points. The samples were analysed on a 1.5% non-denaturing agarose gel in TBE stained with ethidium bromide (0.01µg/ml).

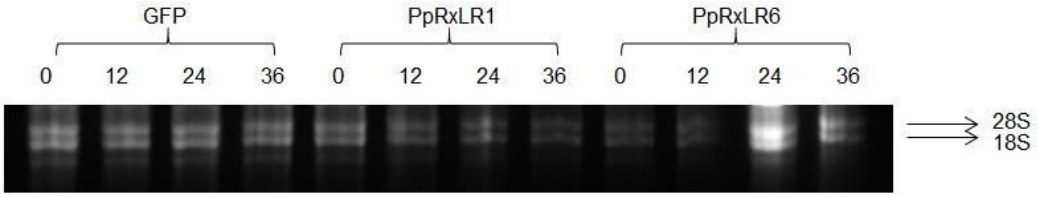

Figure S2.2: Total RNA extracted from *Nicotiana benthamiana* infiltrated with GFP, PpRxLR1 and PpRxLR6 respectively. The leaves were extracted from 0, 12, 24, and 36 hours. The samples were analysed on a 1.5% non-denaturing agarose gel in TBE stained with ethidium bromide (0.01µg/ml). Lane 1-4: GFP, lane 5-8: PpRxLR1, lane 9-12: PpRxLR6 .The expected ribosomal bands were in a ratio of 28S/18S.

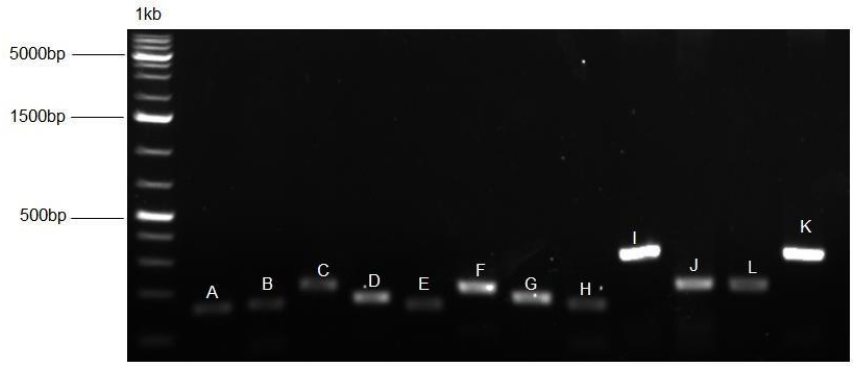

Figure S2.3: PCR verification of primers used for qPCR(1kb Ladder) (Thermo Fisher Scientific Vilnius, Lithuania).(A) actin,(B) Ubiquitin,(C) PcHmp1,(D) PcNpp1,(E) PpRxLR1(F) PpRxLR6,(G) NbPR1,(H) NbPR2,(I) NbLOX ,(J) NbERF1,(L) MPK3,(K) MPK6.

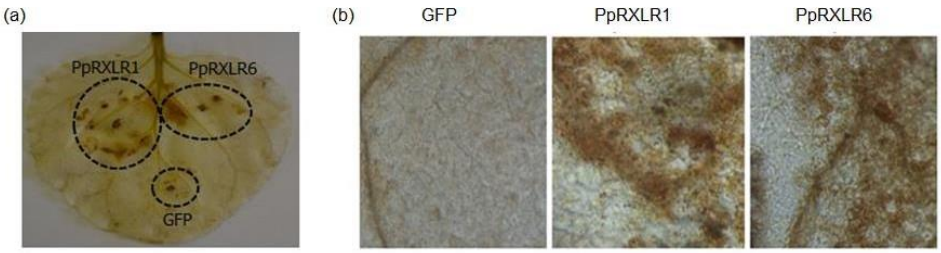

Figure S2.4: Relative Reactive oxygen species accumulation in *Nicotiana benthamiana* leaves. (a) Whole leave representing sites where ROS accumulated .Photographs were taken with a camera (b) Microscopic representation of ROS (Light microscope 10x).

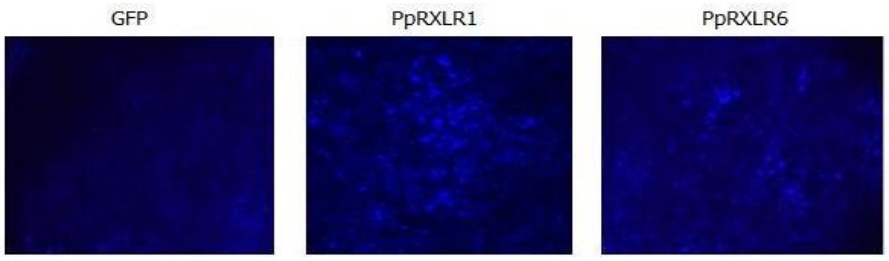

Figure S2.5 Callose deposition in *Nicotiana benthamiana* leaves expressing GFP, PpRxLR1 and PpRxLR6 respectively.

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Chapter three

Concluding remarks and future prospects

Phytophthora parasitica is a soil borne pathogen responsible for black shank of tobacco, its main host. Currently, there are no effective control strategies for *Phytophthora* spp., due to the rapid evolving nature of this species. However, the cytoplasmic effectors (RxLR) have gain attention in the research field as they play a major role in virulence. Comparative genome analysis of *Phytophthora* spp., revealed that some of the RxLR effectors are conserved (Deb *et al.,* 2018). These are referred to as conserved RxLR effectors (CRE). A large number of studies have been made in other *Phytophthora* CRE, however, little is known about CRE from *P. parasitica* (Dalio *et al.,* 2018, Kong *et al.,* 2017, Zhang *et al.,* 2019). Therefore, understanding the mechanisms of *P. parasitica* CRE proteins with its host would be benefiacial in breeding resistance plants against *Phytophthora* spp.

To understand the virulence mechanisms employed by *P. parasitica*, we inoculated *N. benthamiana* leaves with zoospores, observed the phenotypic changes and analyses the expression of the cDNA synthesized from inoculated leaves tobacco leaves. Comparison of the phenotypic changes and qPCR, revealed that *P. parasitica* is indeed a hemibiotrophic pathogen, switching from asymptomatic to symptomatic mode. The asymptomatic phase (biotrophic phase) of *P. parasitica* makes it difficult for diagnosis and when the first symptoms appeared the pathogen has already secured a foot hold in the host plant. The mechanisms that *Phytophthora spp.* use to transition from biotrophic to necrotrophic phase is not well understood, therefore, identifying the genes that are expressed during the biotrophic phase might uncover the mechanism. After establishing the lifestyle pattern, we endeavoured to ascertain the potential role of CREs in Promoting *P. parasitica* virulence. To achieve this, *P. parasitica* PpRxLR1 and PpRxLR6 were selected as they were shown to be highly conserved *in silico* (Chepsergon *et al.,* 2022). In this study, the qPCR revealed that both effectors are expressed during the biotrophic phase, suggesting their importance in *P. parasitica* virulence. Since both effectors were expressed early, we went further to determine the role of these effectors, specifically during the biotrophic phase. We infiltrated *N. benthamiana* leaves with PpRxLR1, PpRxLR6 and GFP as a

control. As depicted in figure 3.1, our study proposes a model where both effectors induce *N. benthamiana* immunity during the biotrophic phase (0h-36h), these include inducing ROS, callose, phytohormones and MAPKs. These results suggest that *P. parasitica* PpRxLR1 and PpRxLR6 promotes plant cell death by inducing ROS, callose, phytohormones and MAPKs. Since both effectors induce plant immunity, we went further to evaluate the role of these effectors in *N. benthamiana* susceptibility to other *Phytophthora spp*. Interestingly, PpRxLR1 promotes *N. benthamiana* susceptibility to *Phytophthora spp.,* while PpRxLR6 promotes susceptibility to *P. parasitica,* however, supresses *N. benthamiana* susceptibility to other *Phytophthora* spp. This suggests that the function of PpRxLR1 is conserved for *Phytophthora* spp, while the function for PpRxLR6 is conserved for *P .parasitica.* This is a major rationale to continue with mechanistic studies of PpRxLR1 and PpRxLR6 effector proteins.

To better understand the mechanism of PpRxLR1 and PpRxLR6, studies are needed to determine the pathways that are altered and whether the alteration result from direct manipulation of the putative target. Furthermore, the results obtained suggest that there could be substantial interest in the role of CRE in *Phytophthora* virulence. Consequently, it will be of great interest (as part of my future studies) to characterize more effectors from the 71 CREs identified *in silico*. Future work will include screening the remaining effectors (from the 71CREs) in *planta* and select one that might play a role in *P. parasitica* virulence. The characterization will include determining the expression, the role in plant response, its subcellular localization within the hot cell as well as protein–protein interaction.

Overall, the findings are significant to agriculture since the identified CREs from *P. parasitica* were present in most *P. parasitica* strains and other *Phytophthora* spp., indicating that *P. parasitica* cannot easily lose them. As a result, R-genes products that recognize such effectors are expected to be more durable and confer broad spectrum resistance against *Phytophthora* spp.

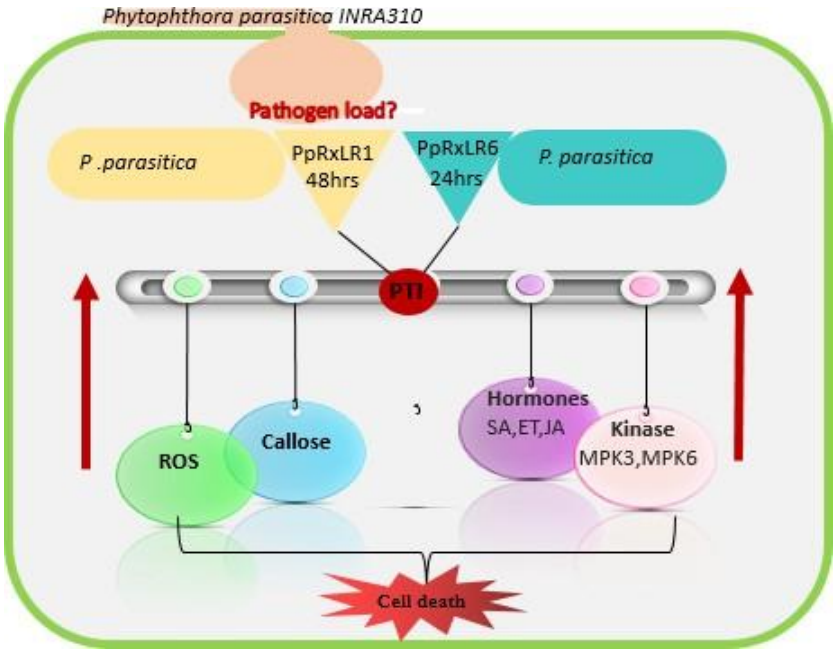

Figure 3.1: A summary representing the role of *Phytophthora parasitica* PpRxLR1 and PpRxLR6 in *Nicotiana benthamiana*. The model indicate that during interaction *P. parasitica* secretes 71 CREs including PpRxLR1 and PpRxLR6. When these effectors are expressed during the biotrophic phase, reactive oxygen species accumulate as an early response. The build-up of ROS induces the deposition of callose and SA. High levels of SA accumulation promote the expression of ET, which have a synergistic relationship with JA. Reactive oxygen species, callose and pytohomone accumulation leads to phosphorylation of the MPK3/6. The plant response's crosstalk results in redox imbalance in the cell, which leads to plant cell death. Furthermore, the induced plant response by both effectors favors *P. parasitica* colonization; however, this still needs to be evaluated by determining pathogen load. response.

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