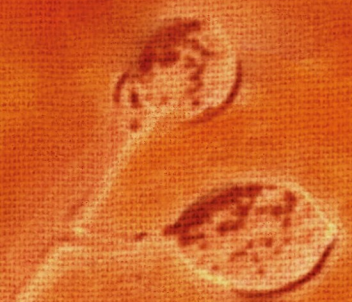
Phytophthora infestans

enzymes and their role in the interaction with its hosts

Charikleia Schoina



Propositions

1. Understanding *Phytophthora infestans* effector delivery mechanisms holds promise for the rational design of disease control strategies.
   (this thesis)

2. The three Plasmepsin V-like *Phytophthora infestans* proteases are highly homologous, but nevertheless have distinct functions.
   (this thesis)

3. Despite the major advances in genome sequencing technologies we are still unable to fully decipher the genomic complexity of organisms.

4. Advancements in -omics are unlikely to replace forward genetics for crop improvement.

5. Virulence factors are not necessarily secreted.

6. People's perception of 'healthy' diet is heavily determined by marketing hypes.

7. Only phytopathologists can see the beauty of a sick plant.

8. Wageningen is a great place to live but has its limitations when one wants to experience the real Dutch culture.

Propositions belonging to the thesis entitled
"*Phytophthora infestans* enzymes and their role in the interaction with its hosts"
Charikleia Schoina
Wageningen, 1 June 2018

Phytophthora infestans enzymes and their role in the interaction with its hosts

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Phytophthora infestans enzymes and their role in the interaction with its hosts

Charikleia Schoina

Thesis

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Chapter 1

General introduction

1

Introduction

Plants are constantly attacked by multiple microbes including fungi, bacteria, and oomycetes. During evolution, plants developed several layers of defense to ward off pathogens (Jones & Dangl, 2006, Dangl *et al.*, 2013, Cook *et al.*, 2015). In counter response, plant pathogens co-evolve to circumvent immune responses by producing a plethora of molecules that re-enable infection. To protect crop plants against adapting pathogens it is crucial to gain knowledge in their molecular arsenal and the mechanisms employed by them in order to promote infection.

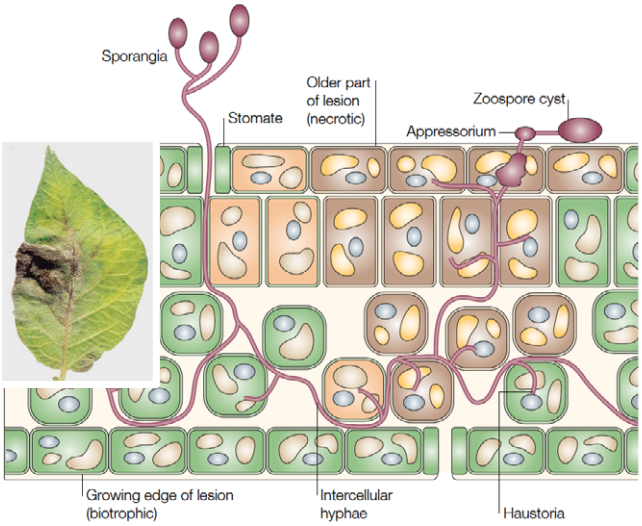
The central theme of this thesis is the oomycete *Phytophthora infestans*, the causal agent of late blight and one of the most devastating pathogens of potato. It focuses specifically on *P. infestans* enzymes in order to determine their role as putative pathogenicity factors. In this general introduction we first highlight the history of late blight and the life cycle of the pathogen. Subsequently, we elaborate on the current molecular toolbox that is available for studying oomycete pathogens and the practices that are used for controlling the late blight disease. We then provide background information on some major classes of enzymes and their potential roles in plant-pathogen interactions. The chapter ends with the scope of this thesis.

Potato and potato late blight

The domestication of potato (*Solanum tuberosum*) probably started at least 10,000 years ago around Lake Titicaca in modern-day Peru and Bolivia. Since 1400 BC, when the earliest farmers settled in the Andes, potato production has been of major importance for Andean societies. In the 16th century the Spanish conquered South-America and one of the treasures that they brought to Europe was the potato. By the late 1700s, potato cultivation was widespread in Europe and today potato is the third most important food crop worldwide. The first reports of potatoes being vulnerable to disease appeared in Belgium in June 1845. With an unprecedented speed a mysterious disease spread over Western Europe and wiped out the entire potato crop. By mid-October of that same year it had reached Ireland, a country where the socioeconomic structure forced the poor peasants to solely rely on potato for their daily food. This led to the Great Irish Famine, a disaster that caused a turning point in history. Apart from the one million people that died, another one and a half million settled as refugees in North America, resulting in the birth of "Irish America". At

Box 1. The disease cycle of the potato late blight pathogen *Phytophthora infestans.*

A typical late blight lesion has a necrotic center with heavy sporulation surrounded by a water-soaked zone. Outside these zones the pathogen continues to invade healthy cells and the lesion expands further. The schematic figure shows the vegetative life cycle of *P. infestans.* Infection starts when a spore lands on the leaf and germinates. The germ tube forms an appressorium and an emerging penetration peg enters the epidermal cell. From there, hyphae colonize the inner cell layers where they grow in between the plant cells and produce finger-like protrusions that penetrate the plant cell. These so-called haustoria are specialized structures that facilitate the delivery of effectors into plant cells and the uptake of nutrients from plant cells. *P. infestans* is a hemi-biotrophic pathogen that needs living plant tissue for the initial phases of colonization, the biotrophic phase. Gradually, the plant cells die and the leaf necrotizes. In this phase hyphae escape through the stomata and produce numerous spores named sporangia that easily detach and disperse by wind or water. A sporangium that finds a new host can either germinate directly and initiate a new cycle or, at lower temperatures, undergo cleavage resulting to release flagellated spores. These zoospores can swim for several hours but once they touch a solid surface they encyst and germinate to initiate new infections. Under favorable conditions the pathogen can complete its cycle from infection to sporulation in 4 days. In the field this cycle is repeated multiple times during one growing season resulting in billions of spores and a continuous increase of disease pressure. Besides leaves, also stems and tubers get infected and *P. infestans* can continue to flourish on the decaying plant material. If not managed properly, infected seed potatoes or waste on refuse piles are often the sources of inoculum for new infections in spring. An alternative route for surviving the winter is via oospores, resting spores that can survive in the soil for many years and are produced upon mating. Most *Phytophthora* spp. are homothallic which means they produce sexual spores without the need for a partner. *P. infestans* is heterothallic; isolates are either of the A1 or A2 mating type and sex organs only develop when isolates of opposite mating type sense the sex hormone produced by the mate. The schematic figure is reproduced from Judelson and Blanco (2005).



that time the concept that microbes could cause plant diseases was unknown. The sudden appearance of late blight was blamed on abiotic factors like hidden volcanoes, the steam machine, electricity or the wet summer and even on the devil. Reverend M. J. Berkeley, however, put forward the hypothesis that the mold flourishing on the potato foliage was the cause and not the consequence of the disease (Large, 1940).

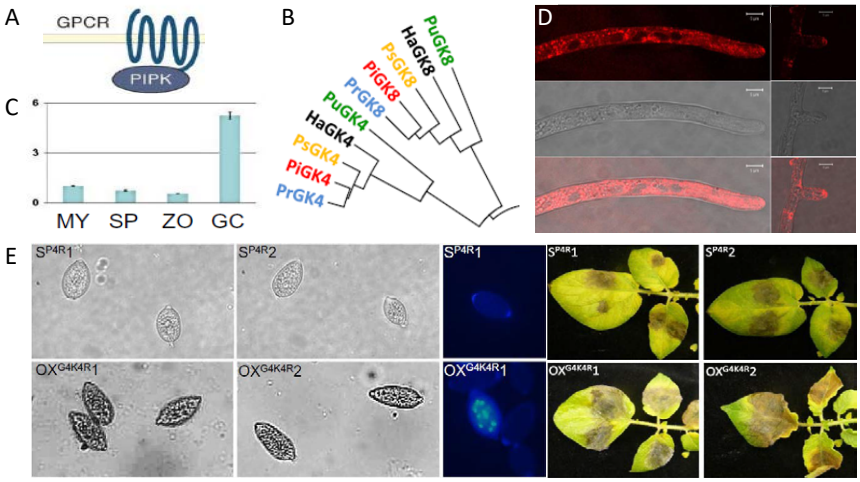
He succeeded in drawing a rather accurate picture of a fungus-like creature growing as mycelium inside a potato leaf and releasing spore-bearing hyphae through the stomata (**Box 1**). This creature was *Phytophthora infestans,* now widely known as the Irish potato famine pathogen and nowadays responsible for yearly economic losses of over 3 billion € worldwide (Fry, 2008). It belongs to the oomycetes, a group of diverse organisms that, similar to fungi, grow as mycelium and produce spores to propagate. Oomycetes are best known as plant pathogens – there are over a hundred *Phytophthora* spp. and many downy mildews and *Pythium* spp. – but the group also comprises animal and microbial pathogens as well as saprophytes (Kroon *et al.*, 2012, Kamoun *et al.*, 2015). As such, they occupy similar ecological niches as fungi. However, during evolution oomycetes evolved completely independently from fungi and this is reflected in differences in e.g. cell wall composition, actin cytoskeleton, biochemical and metabolic pathways, and mating systems (Judelson & Blanco, 2005).

The molecular toolbox to unravel *Phytophthora infestans*

In order to understand how *P. infestans* attacks and colonizes its hosts one needs to be able to investigate the molecular and cellular machinery of the organism and to gain insight into the type of components that the pathogen produces to cause disease. Most pathogenicity factors are secreted and often produced specifically or at higher levels during interaction with the host. Already in the early 1990s the first attempts were made to identify putative pathogenicity factors using differential gene expression profiling. At around the same time the first successful DNA transformation of *P. infestans* was reported and in the years to follow the molecular toolbox was expanded with protocols suitable for gene function analyses by a targeted 'knock-down' approach based on RNA interference or by overexpression (van West *et al.*, 1999). In all cases, phenotypic analyses of multiple independent transformants is required to demonstrate that an aberrant phenotype is consistently correlated with reduced or increased transcript levels of the target gene and not the result of disruption of another unrelated gene. In case the transgene carries a fluorescent tag, overexpression transformants can be analyzed by microscopy to visualize the subcellular location of the encoded protein. The transgene can also be modified with targeted deletions or potential 'gain- or loss-of-function' mutations or its anticipated function can be studied in other organisms enabling even more in-depth analyses at the biochemical or structural level. **Box 2** describes a case study of the functional analysis of a *P. infestans* gene using the various approaches described above. All these approaches have

Box 2. A case study of gene discovery and functional gene analyses in *Phytophthora infestans*.

Heterotrimeric G-proteins and phospholipids are key players in evolutionary conserved signaling networks in eukaryotes and have been shown to play a role in pathogenicity. A comprehensive inventory of *Phytophthora* genes involved in phospholipid signaling revealed that several indeed encode highly conserved proteins whereas others encode proteins with conserved catalytic domains but combined with other domains (Meijer & Govers, 2006). Examples are the GPCR-PIPKs (GKs), novel proteins that are composed of a G-protein coupled receptor (GPCR) domain fused to a phosphatidylinositol phosphate kinase (PIPK) domain (Bakthavatsalam *et al.*, 2006).



Based on this domain structure GKs are anticipated to link G-protein and phospholipid signaling (**A**). It could be that the GPCR, which normally transmits extracellular signals over the membrane to the heterotrimeric G-proteins, bypasses the G-proteins and activates directly the PIPK domain to phosphorylate either lipids or other proteins (van den Hoogen *et al.*, 2018). Phylogenetic analysis showed that each *Phytophthora* spp. has twelve GKs that are more highly conserved between species than between GKs within one species (Pu: *Pythium ultimum*, Ha: Hyaloperonospora *arabidopsidis*, Ps: *Phytophthora sojae*, Pi: *Phytophthora infestans*, Pr: *Phytophthora ramorum* (**B**). GKs are differently expressed during the life cycle. Shown in (**C**) is the expression of *PiGK4* in mycelium (MY), sporangia (SP), zoospores (ZO) and germinating cysts (GC). *PiGK4* is most highly expressed in germinated cysts whereas other GKs are higher expressed in zoospores (Hua *et al.*, 2013). Microscopic imaging of *P. infestans* transformants expressing PiGK4 with a fluorescent tag showed the subcellular location of PiGK4 (**D**). In other transformants the *PiGK4* expression was silenced (S) or overexpressed (OX). Phenotypic characterization demonstrated a role for PiGK4 in spore development, sporangial cleavage, hyphal elongation and virulence (**E**). In these transformants, the transgene is expressed in its own genetic background. Sporangia of the silenced lines $S^{PAR}1$ and $S^{PAR}2$ resemble the wild type phenotype whereas overexpressing lines $OX^{G4K4R}1$ and $OX^{G4K4R}2$ showed an aberrant morphology. Expression in heterologous hosts is another approach to study the gene of interest. For PiGK4, *Escherichia coli* was used as host organism to determine the orientation of the seven transmembrane domains that are characteristic for a canonical GPCR (Hua *et al.*, 2013). To test if the PIPK domain in PiGK4 has the anticipated enzyme activity, the yeast *Saccharomyces cerevisiae* was used as heterologous host. PiGK4 was able to restore a PIPK deficient yeast mutant in a similar way as the endogenous yeast PIPK gene. This case study is largely based on results from Hua *et al*, 2013.

been and are being used successfully. Nevertheless, manipulating genes in *P. infestans* remains a challenging task. Transformation efficiencies are low, primary transformants are often heterokaryons with only a subset of the nuclei carrying the transgene and transgenes are not always stable or expression is lost.

In recent years, a new genome editing technology known as CRISPR/Cas9 has become available. It is based on the adaptive immune system used by bacteria to fight against viral infections and characterized by clustered regularly interspaced short palindromic repeats (CRISPR) in the bacterial genome. The CRISPR/Cas9 system is based on the modification of DNA by the Cas9 endonuclease derived from *Streptococcus pyogenes*. This enables the targeted introduction, deletion or modification of a single gene in an organism. This system is a great asset to the molecular toolbox since it allows gene modifications within the organism in a controlled and precise manner (Bortesi & Fischer, 2015). Several studies have demonstrated success exploitation of CRISPR/Cas9 for targeted mutation or homologous recombination of one or more genes in different organisms including mammals (mice), insects (*Drosophila*) and plants (*Arabidopsis*, potato, rice, wheat and maize) (Miao *et al.*, 2013, Nekrasov *et al.*, 2013, Butler *et al.*, 2015, Jiang *et al.*, 2013, Wang *et al.*, 2014) as well as several microorganisms like e.g. the malaria pathogen *Plasmodium falciparum* and the yeast *Candida albicans* (Barakate & Stephens, 2016, Tian *et al.*, 2017). This method has also been successfully applied and established in the soybean root rot pathogen *Phytophthora sojae* (Fang & Tyler, 2015) and the cacao black pod pathogen *Phytophthora palmivora* (Gumtow *et al.*, 2017). However, in *P. infestans* all the attempts so far, have failed (van den Hoogen & Govers, 2018).

Genomics: expanding the toolbox

Because of the limitations in the toolbox for functional gene analysis, there was an enormous drive to exploit other means that would lead to insight into the mechanisms underlying pathogenicity. The genomics era created new opportunities and as early as 1999, when DNA sequencing was still costly and filamentous plant pathogens were hardly subjected to large scale sequencing, the first set of a thousand *P. infestans* Expressed Sequence Tags (ESTs) was published (Kamoun *et al.*, 1999). This already uncovered a gene repertoire far more complicated than was envisioned. One example is elicitin, a 10 kDa secreted protein highly abundant in culture medium and an elicitor of necrosis in tobacco that was a holy grail in plant-pathogen interaction research for many years. It turned out to be a member of an extensive family with other members also eliciting necrosis (Jiang *et al.*, 2006). Until today, their function in pathogenicity remains elusive. They bind sterols and since *Phytophthora* spp. cannot synthesize sterols themselves, they may have a role in snatching sterols from the environment. Silencing of the elicitin *inf1* in *P. infestans* did not change virulence on tomato and potato and no detrimental effects were observed (Kamoun *et al.*, 1997). Since elicitins are ubiquitous in *Phytophthora*, they have the characteristics of a

pathogen associated molecular pattern (PAMP). Recently a receptor that recognizes INF1, as well as other elicitins, was identified in a wild potato species (Du *et al.*, 2015). The receptor, named ELR, is a membrane-associated receptor-like protein with extracellular leucine-rich repeats, typical for a pattern recognition receptor (PRR), and enhances resistance to late blight when expressed in cultivated potato. Recently it was shown that ELR is able to physically interact with elicitins INF1, ParA1 and β-CRY (from *P. infestans*, *P. parasitica* and *P. cryptogea*, respectively), indicating that it is a true PRR (Domazakis E., PhD thesis).

A great leap forward was the completion of two *Phytophthora* genome projects (*P. sojae* and *P.* ramorum) in 2006 (Govers & Gijzen, 2006). For the first time the whole repertoire of potential pathogenicity genes could be mined. The usual suspects were the ones encoding hydrolases such as proteases, cutinases, lipases or pectinases, as well as protease inhibitors, protein toxins, and ABC transporters. Other genes were suspicious because of their evolutionary trajectory. This could be expansion in number in comparison to close relatives that are not pathogenic, acquisition specifically in *Phytophthora* or oomycetes as in the case of elicitins, or domain shuffling or fusion resulting in proteins that have uncommon domain compositions. Oomycetes have a relatively high proportion of such novel proteins and several of these are truly oomycete-specific (Seidl *et al.*, 2011). They may have evolved along with the metabolic, biochemical and structural features that are characteristic for oomycetes and as such they could be ideal targets for disease control.

Comparison of the genomes of these two species that attack different hosts revealed a large family of genes encoding highly divergent secreted proteins that share a common motif in the N-terminus (Jiang *et al.*, 2008). These proteins were coined RXLR effectors based on the amino acid composition of the shared motif. A few years later the *P. infestans* genome was sequenced and included in the comparison (Haas *et al.*, 2009). This genome of 240 Mb, at least twice the size of that of the other two species and much larger than that of most fungal plant pathogens, has a high repeat content of 74% and a typical bipartite organization with gene-dense regions or 'gene islands' where highly conserved genes are located, and gene-scarce regions or 'gene deserts'. The latter are full of repeats but scattered in these deserts are the RXLR effector genes that, as we know now, play a role in virulence and host specificity of these pathogens.

In the meantime, 37 oomycete genomes have been sequenced and the phylogenetic relationship among them has been determined, giving more insight into the evolutionary relationship of oomycete species as well as their different gene families (Kroon *et al.*, 2011, McCarthy & Fitzpatrick, 2017). In addition, several studies have focused on specific gene families encoding enzymes. For example, in *P.* infestans genes encoding cell wall-degrading enzymes (Ospina-Giraldo *et al.*, 2010), aspartic proteases, phospholipase D's (Kay *et al.*, 2011, Meijer *et al.*, 2011) and metalloproteases (Chapter 2) were mined and inventories were created. This allowed the thorough investigation of their characteristics in order to choose potential candidates for further analysis.

Controlling late blight disease

To prevent late blight infection, most farmers use chemical control. When the disease pressure is high they have to spray their crop once every week to be effective. Because of the adverse effects of chemicals on the environment and the emergence of fungicide resistant *Phytophthora* strains, there is an urgency to find alternatives. One approach is to aim for a novel generation of agrochemicals that are specifically targeted on oomycetes while another approach is to exploit the natural resistance in wild *Solanum* species for generating resistant late blight potato cultivars. Below we first elaborate on the molecular basis of resistance and subsequently we give examples of potential oomycete specific drug targets.

Already in the early twentieth century, breeders made their first attempts to cross late blight resistance traits into cultivated potato. Although they succeeded, the resistance did not hold longer than a few years. New *P. infestans* races emerged and some potato lines became susceptible to one race and others to another race of the pathogen (Fry, 2008). Genetic analyses confirmed that potato and *P. infestans* interact according to the 'gene-for-gene' model that was postulated in the 1940s to explain differential responses to pathogens within the same plant species. In retrospect, we now understand the reason for the rapid loss of resistance. Nearly all late blight resistance genes encode cytoplasmic 'nucleotide-binding leucine-rich repeat' (NLR) proteins that initiate a resistance response at the moment they encounter the presence of an RXLR effector. As described above, these effectors are encoded by genes located in the most dynamic regions of the genome. The RXLR motif functions as a host cell targeting motif, a kind of ZIP code that tells the protein where to go (Whisson *et al.*, 2007). By an unknown mechanism the numerous RXLR effectors are delivered into the host cell. Their function is to suppress host defense by manipulating the host cell machinery for the wellbeing of the pathogen and as such it is logic that resistance (R) proteins ring the alarm bell when they sense RXLR effectors nearby. The presence inside the plant cell of only one matching pair of R protein and RXLR effector is already sufficient for initiating a hypersensitive response culminating in localized cell death that arrests further growth of the pathogen. The key players in the classical 'gene-for-gene' model, are the RXLR effectors and the NLRs. The response of *P. infestans* to a newly introduced resistant cultivar is to rapidly evade recognition by the novel NLR and it does so in many different ways. For example, the matching RXLR gene can be deleted of modified by point mutations or frame shift mutations or its expression can be suppressed by gene silencing (Vleeshouwers *et al.*, 2011, Kasuga & Gijzen, 2013). In other cases, the effector is still produced but its activity is suppressed, likely by other RXLR effectors. Knowing how easily an RXLR effector can adapt has predictive value for the durability of the matching NLR gene. For example, already a long time ago breeders experienced that resistance derived from certain wild potato species is less durable than that from other wild potato species and we can now explain this by the flexibility of the

RXLR effectors matching the NLRs from those species: deletions, point mutations or frame shift mutations suggesting redundancy in function, versus suppression of effector activity. In the latter case it seems that the pathogen cannot simply get rid of the effector without losing viability and, as a result, the matching NLR confers resistance that is more durable (Vleeshouwers *et al*., 2011, Kasuga & Gijzen, 2013). The current strategy is to stack multiple NLRs in one cultivar and then preferably NLRs that recognize RXLR effectors with diverse activities. (Haverkort *et al.*, 2008, Haesaert *et al.*, 2015, Jones *et al.*, 2014). So far, only a few host targets of RXLR effectors are known, but it is already clear that these effectors have the capacity to manipulate the host cell machinery at all levels and at different sites (Whisson *et al.*, 2016). It remains to be seen whether plant resistance conferred by NLRs can fully control late blight.

Most agrochemicals that are commercially available to combat *Phytophthora* diseases are derived from screenings in which random compounds are tested for inhibitory activity, for example on spore germination or lesion growth. Often the exact cellular or molecular target of the compound is not known. For a more rational design of agrochemicals it is essential to find very specific and unique targets in the pathogen that, when disrupted, harm the viability or virulence of the pathogen without having a detrimental effect on any other organism, including plants, humans, insects, and other microbes. One example of a potential drug target in oomycetes that has been identified in recent years, is a specific structure that is part of the actin cytoskeleton. The actin cytoskeleton is a highly dynamic network of actin polymers (F-actin). It is conserved in all organisms and involved in many cellular processes including cell motility and vesicle transport (van der Honing *et al.*, 2007, Schmidt & Hall, 1998). Despite this overall conservation, oomycetes have a novel actin structure next to actin cables. This so-called plaque is visible as a dot-like structure with a long life time and has been found exclusively in oomycetes (Meijer *et al.*, 2014a). In addition to plaques other novel actin configurations have been observed in *P. infestans* (Kots *et al.*, 2016) and this altogether offers potential for finding new targets for control. Other oomycete specific molecules that are attractive as target are the so-called GPCR bigrams. The GPCR-PIPKs (**Box 2**) are an example but there are a few other classes that match the same criteria (van den Hoogen *et al*., 2018). With the knowledge that many of the pharmaceutical drugs have GPCRs as target it is a conceivable that also these oomycete-specific GPCR bigrams are ideal candidates for further functional studies pursuing the aim to identify novel, oomycete-specific drug targets based on rational design.

Phytophthora infestans virulence factors

Studies on pathogenic oomycetes have identified several RXLR effectors as virulence factors using comparative and functional genomics (Jiang & Tyler, 2012, Sanju *et al.*, 2013, Torto-Alalibo *et al.*, 2005, Bos *et al.*, 2003, Adhikari *et al.*, 2013, McGowan & Fitzpatrick, 2017). Although very powerful, RXLR effectors are just part of the weaponry that *Phytophthora*

utilizes to damage the plant. Besides cell wall-degrading enzymes or proteases there are also numerous proteins with unknown (enzymatic) activity. Some of those are secreted in the host apoplast during infection while others such as the RXLR effectors are delivered into the host cell. The latter also includes the Crinklers, a large family of proteins which, similar to RXLR effectors, have a motif for host cell targeting at the N-terminus combined with vary diverse C-terminal domains, some of which have kinase or hydrolase activity (Haas *et al.*, 2009, Schornack *et al.*, 2009, van Damme *et al.*, 2012, Dong & Wang, 2016).

Enzymes as virulence factors

Enzymes are ubiquitous molecules, mainly proteins, that perform or accelerate chemical reactions and are involved in cellular metabolism (Cooper, 2000). There are many different enzymes and they are often grouped based on their activity (e.g. oxyreductases, hydrolases, ligases, etc), or their substrate (e.g. proteases, lipases, etc). Besides their functions in regular metabolic processes several microbial enzymes have been characterized that have a role in plant-microbe interactions. Below, we focus on microbial enzymes that may function as pathogenicity or virulence factor. Because of the subtle difference between pathogenicity and virulence and the fact that we cannot predict beforehand whether an enzyme is a pathogenicity factor or a virulence factor, we have chosen to use from now on one term: virulence factor, with the notice that the meaning can be broader than strictly 'having a role in virulence of the pathogen'.

Cell wall-degrading enzymes have been extensively studied as potential virulence factors in fungi and oomycetes and for their roles in establishing disease. The cell wall is one of the most important structural components of plant cells. Besides structural rigidity, it is important as defence barrier, located at the site where the first attempt for pathogen penetration is taking place. Cell wall-degrading enzymes provide the first ground for pathogen penetration and entrance to plant cells by the modification or breakdown of major cell wall components such as carbohydrates like cellulose and pectin (Bellincampi *et al.*, 2014). Major enzyme families are the glycoside hydrolases (GHs), the polysaccharide lyases and the carbohydrate esterases, which are grouped as Carbohydrate-Active enzymes (CaZymes) (Lombard *et al.*, 2014). Comparative genome and transcriptome analyses have indicated the large diversity of these enzymes, reflecting the adaptation of plant pathogens and the complexity of the interaction with their hosts (Zhao *et al.*, 2014, King *et al.*, 2011). In fungal pathogens like *Fusarium virguliforme*, *Verticillium dahliae* and *Botrytis cinerea*, cell wall-degrading enzymes have been found to contribute to virulence (Gui *et al.*, 2017, Chang *et al.*, 2016, ten Have *et al.*, 2002, Kubicek *et al.*, 2014).

In oomycetes and in *Phytophthora* species in particular, several genome mining studies revealed the large repertoires of cell-wall degrading enzymes while other studies highlighted their importance in virulence. These enzymes have been characterized in several *Phytophthora* species including *P. infestans* and *Phytophthora parasitica* (Ospina-

Giraldo *et al*., 2010, Blackman *et al.*, 2014). Transcriptomics analysis of genes encoding cell-wall degrading enzymes in *P. parasitica* showed induced expression during infection, while proteomics analysis of *P. infestans* showed an abundance of these enzymes in germinating cysts and appressoria (Blackman *et al.*, 2015, Resjö *et al.*, 2017). Olivera *et al.* (2016), showed that glycoside hydrolases in family GH20 are shared among all animal pathogenic oomycetes and suggested that they function as virulence factors. However, those particular enzymes were absent from plant pathogenic oomycetes. Functional analysis of the *P. sojae* glycoside hydrolase PsXEG1 showed that alteration of its expression results in reduced virulence. In addition, PsXEG1 is recognized by soybean as a PAMP thereby inducing PTI (Ma *et al*., 2015). Several RXLR effectors suppress the plant defense responses triggered by PsXEG1 recognition. Moreover, *P. sojae* has developed a decoy mechanism where an inactive paralog of PsXEG1, XLP1 binds with higher affinity to the enzyme inhibitor, by protecting the functional enzyme (Ma *et al.*, 2015, Su *et al.*, 2018). Recently, the PRR that recognizes XEG1 has been identified in *N. benthamiana*. It associates with XEG1 in the apoplast and forms a complex with the LRR receptor-like kinases BAK1 and SOBIR1 to induce defense responses (Wang *et al.*, 2018).

Phospholipases are an important group of enzymes that are capable of modifying phospholipids, the main structural components of cell membranes (Waite, 1996). They are also known to be involved in phospholipid signaling which in plants mediates growth, development, hormone regulation and responses to abiotic stress (Meijer & Munnik, 2003). Four classes of phospholipases are recognized based on their catalytic activity: i.e. phospholipases A, B, C and D. They hydrolyze phospholipids and convert them into lipid and non-lipid moieties. **Phospholipase D** (PLD) cleaves between the phosphate group and the head group, releasing phosphatidic acid (PA) and a free polar group. PA is a second messenger and has many different cellular functions such as membrane binding and modulation of enzymatic activities. It also has structural effects on cell membranes by modulation of ion channels and promotion of membrane curving (Kooijman *et al.*, 2005). PA can also directly or indirectly activate proteins (Testerink & Munnik, 2005). PLDs have a catalytic site with two highly conserved HxKxxxxD motifs (HKD1 and HKD2 respectively) (Testerink & Munnik, 2011, Selvy *et al.*, 2011). Analysis of the genomes of *P. sojae*, *P. ramorum* and *P. infestans* resulted in the identification of 18 PLDs, divided over five main classes (Meijer & Govers, 2006). Among the very conserved oomycete PLDs there are two subfamilies consisting of PLDs that have no perfect HKD motifs and vary in length, called PLD-likes and sPLD-likes (Fig. 1). In *P. infestans*, PLD activity is involved in zoospore encystment, likely through the formation of PA (Latijnhouwers *et al.*, 2002). Furthermore, phospholipases are thought to be involved in membrane modification or disruption processes that occur during host invasion. For pathogens, the plasma membrane is the second major barrier, after the cell wall, in host penetration. Evidence for secretion of PLDs by *P. infestans* came with the detection of PLD activity in extracellular medium. This implies that PLDs may play a role in pathogenicity (Ghannoum, 2000).

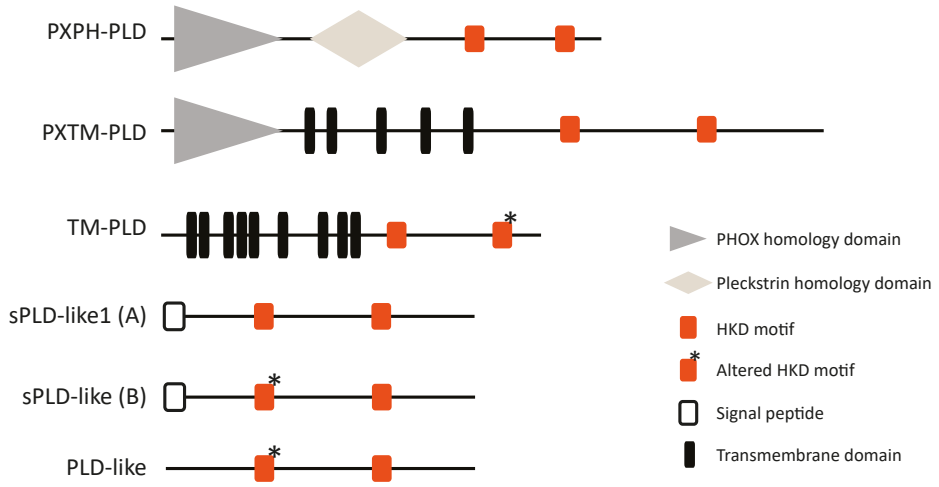


Figure 1. Schematic representation of the domain compositions of phospholipase D's in *P. infestans*.

Proteases, also referred to as proteinases, peptidases or proteolytic enzymes, are a major group of proteins that cleave other proteins. The *MEROPS* peptidase database classifies proteases based on their catalytic type and their evolutionary relationship in eight groups, namely aspartic proteases, metalloproteases, cysteine proteases, glutamic proteases, asparagine proteases, threonine proteases, proteases with mixed catalytic types and proteases not assignable to any group (Sanman & Matthew, 2014). Several aspartic, cysteine and metalloproteases have been shown to be involved in the fitness and virulence of mammalian and plant pathogens. Below, we address two groups of proteases: metalloproteases (MPs) and aspartic proteases (APs).

Metalloproteases (MPs) are characterized as such due to the metal ion, mostly zinc, that is contained in their active domain and that acts as a catalyst during hydrolysis. This group includes thermolysins, astacins and serralysins from the metzincins (or metzincin family) that were found to be involved in virulence of several mammalian and plant pathogens. A well-studied group in mammalian systems are the so-called sheddases, secreted transmembrane MPs known as matrix metalloproteases (MMPs) and An Disindegrin and Metalloprotease (ADAMs) that activate or inhibit membrane-spanning receptors by releasing the extracellular domain, a process referred to as ectodomain shedding (Hayashida *et al.*, 2010). In studies focusing on the analysis of the *P. infestans and Pythium ultimum* transcriptome during infection, the expression patterns of MP genes have been profiled, showing higher expression levels in early potato tuber infection stages (Ah-Fong *et al.*, 2017). In another study, two MPs were detected in the extracellular proteome of *P. infestans* (Meijer *et al.*, 2014b). However, no extensive studies have been done on MPs in oomycetes.

Aspartic or aspartyl **proteases** (APs) are enzymes that usually have two conserved aspartic acid residues in their catalytic domain. BACE1 (β-secretase) is an aspartic protease which is extensively studied due to its involvement in Alzheimer's disease (Dislich & Lichtenthaler, 2012, Fernández-Bachiller *et al.*, 2013). Another well studied AP is Plasmepsin V (PMV), an aspartic protease from the malaria pathogen *Plasmodium falciparum* that was found to be involved in the cleavage of PEXEL effectors prior to their transport into the host cell (Boddey *et al.*, 2010). The *Plasmodium* PEXEL effectors have a conserved RXLX$^{E/Q/D}$ motif, that similar to the RXLR motif in *Phytophthora* RXLR effectors, has a role in host cell targeting. In a study by Kay *et al*. (2011), AP genes from three *Phytophthora* species were identified and their properties based on their amino acid sequences were studied. Out of the twelve identified APs in *Phytophthora*, all but one, were predicted with a signal peptide, two were found to be similar to BACE1 AP and three to *Plasmodium* PMV. Another one has a G-protein-coupled receptor (GPCR) domain at the C-terminus resulting in a protein with a domain architecture that is unique to oomycetes (van den Hoogen *et al*., 2018).

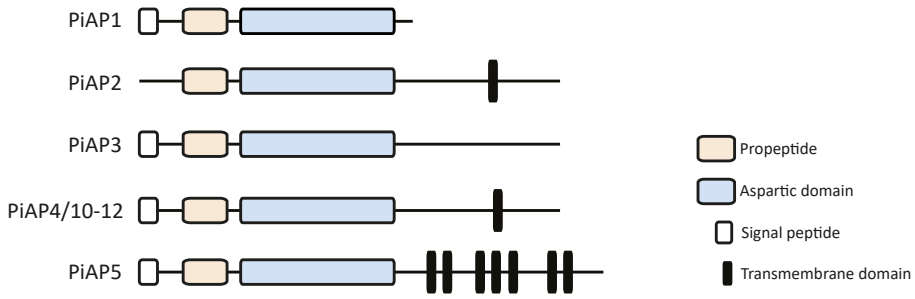


Figure 2. Schematic representation of the domain compositions of aspartic proteases in *Phytophthora infestans*.

Enzyme inhibitors as virulence factors

Besides all the various enzymes functioning as virulence factors, pathogens also employ enzyme inhibitors to counteract enzymes produced by the host as part of their defense machinery. Therefore, during evolution, it was beneficial for pathogens to develop molecules that act as enzyme inhibitors in order to protect themselves. Indeed, several pathogen-derived molecules are targeting plant enzymes, or block enzymatic activity in plant cells, thereby increasing the chances of pathogen survival into the host. There are several examples of *Phytophthora* inhibitors that have been found to block plant enzymes. In *P. sojae*, the apoplastic effector GIP1 inhibits the enzyme activity of soybean endo-β-1,3-glucanases (Bishop *et al.*, 2005). *P. infestans* has several glucanase inhibitors potentially blocking the activity of tomato endo-1,3-glucanases (Zuluaga *et al.*, 2015). Two *P. infestans* Kazal-like serine protease inhibitors, EPI1 and EPI10, were found to interact with a subtilisin-like serine protease and the apoplastic pathogenesis-related protease P69B from tomato (Tian *et al.*, 2007, Tian *et al.*, 2004). Furthermore, *P. infestans* cystatin-like cysteine protease inhibitors EPIC1 and

EPIC2B and their interaction with the cysteine papain-like apoplastic protease C14, cysteine proteases PIP1, and the defense-related protease Rcr3 in tomato, have been extensively studied (Tian *et al*., 2007, Kaschani *et al*., 2010, Song *et al*., 2009). Mutation by CRISPR/Cas9 of the cystatin-like cysteine protease inhibitor gene EPIC8 in *P. palmivora* resulted in increased papain sensitivity and reduced virulence (Gumtow et al., 2017). Moreover, it has been found that *Phytophthora* species have developed protease inhibitors specialized to the respective proteases of their host plant, by specific amino acid polymorphisms. The inhibitors seem to have stronger activity against the proteases from their own host, compared to similar proteases from non-hosts or less favourable hosts. This specialization is key to the adaptation of the different *Phytophthora* species to specific hosts (Dong *et al*., 2014).

Scope of this thesis

The research described in this thesis focuses on different groups of enzymes produced by the late blight pathogen *P. infestans* and explores their potential role during the interaction with host plants and as virulence factors.

Metalloproteases have been shown to play important roles in human diseases and in mammalian pathogens but so far, were not studied in oomycetes. **Chapter 2** presents the mining of the *P. infestans* genome for MP genes and the classification of *P. infestans* MPs in clans and families. Analysis of the domain architecture and expression profiling of *P. infestans* MPs lead to the identification of a few MPs with special features. This chapter provides a detailed overview of the MP repertoire in *P. infestans* and a valuable tool for selecting potential candidates for further functional analysis.

In the malaria pathogen *Plasmodium falciparum*, the aspartic protease PMV is found to be involved in the cleavage of PEXEL effectors prior to their translocation into the host cell. From the twelve aspartic proteases (APs) identified in *P. infestans,* three are similar to PMV and one has a domain architecture that is unique for oomycetes. These four were selected for further analysis. **Chapter 3** describes the functional analysis of the PMV homologs PiAP10, PiAP11 and PiAP12. To this end transgenic *P. infestans* lines in which the encoding genes are silenced or overexpressed were generated and their phenotypes with respect to virulence were analysed by performing infection assays. In addition, based on the similarities between *P. infestans* RXLR effectors and *Pl. falciparum* PEXEL effectors, the possibility of PiAPs having a similar function as PMV on *P. infestans* effectors was explored by biochemical assays using recombinant purified APs and RXLR effectors. **Chapter 4** focuses on the functional analysis of PiAP5, the aspartic protease that has a unique domain architecture with a GPCR domain in the C-terminal part of the protein. The effect of *PiAP5* silencing on growth, morphology and virulence of the pathogen was investigated and by biochemical analysis, the potential function of PiAP5 as protease was tested.

Phospholipase D's hydrolyse phospholipids and are widespread among all organisms. They are key enzymes for PA production and involved in many cellular processes, like membrane signaling and trafficking. In **Chapter 5** we focus on the functional analysis of a subset of PLDs that are potentially involved in virulence. We describe the effects of transient expression of three small PLD-like genes in *Nicotiana benthamiana* and use the transient expression as a tool to analyse the contribution of the small PLD-likes to virulence.

In the studies described in chapters 3, 4 and 5 we use leaves from potato, tomato or *Nicotiana benthamiana* for either testing the virulence of transgenic *P. infestans* lines in infection assays, or for transient expression of *P. infestans* genes to monitor their contribution to virulence. In fact, nearly all published studies focussing on *Phytophthora*-host interactions make use of whole plants or detached plant tissues such as leaves, stems or roots. In many cases infections on intact plants or differentiated plant tissues are suitable but there are also some disadvantages in particular when it comes to microscopy or synchronized infection. In order to circumvent these disadvantages, we developed a versatile infection system that exploits the tomato cell suspension line MsK8. In **Chapter 6** we show how this system can be used to study the *Phytophthora*-host interactions. Different experimental approaches are described, such as microscopy, monitoring of defense responses and gene expression analysis. Combined with the advantages of fast progressing and synchronized infections, we propose that this infection system can be further used for scaling-up experiments and for –omics studies.

Chapter 7 discusses our findings in the broader perspective of virulence factors and describes the contribution of pathogen-derived enzymes in the interaction with its host and the establishment of disease. Furthermore, the mechanisms underlying their function as virulence factors are addressed, and we propose directions for further research.

References

Adhikari, B.N., Hamilton, J.P., Zerillo, M.M., Tisserat, N., Lévesque, C.A. and Buell, C.R. (2013) Comparative genomics reveals insight into virulence strategies of plant pathogenic oomycetes. *PLoS ONE,* **8,** e75072.

Ah-Fong, A.M.V., Shrivastava, J. and Judelson, H.S. (2017) Lifestyle, gene gain and loss, and transcriptional remodeling cause divergence in the transcriptomes of *Phytophthora infestans* and *Pythium ultimum* during potato tuber colonization. *BMC Genomics,* **18,** 764.

Bakthavatsalam, D., Meijer, H.J.G., Noegel, A.A. and Govers, F. (2006) Novel phosphatidylinositol phosphate kinases with a G-protein coupled receptor signature are shared by *Dictyostelium* and *Phytophthora*. *Trends microbiol,* **14,** 378-382.

Barakate, A. and Stephens, J. (2016) An overview of CRISPR-based tools and their improvements: new opportunities in understanding plant–pathogen interactions for better crop protection. *Front. Plant Sci,* **7,** 765.

Bellincampi, D., Cervone, F. and Lionetti, V. (2014) Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Front. Plant Sci,* **5,** 228.

Bishop, J.G., Ripoll, D.R., Bashir, S., Damasceno, C.M.B., Seeds, J.D. and Rose, J.K.C. (2005) Selection on glycine β-1,3-endoglucanase genes differentially inhibited by a *Phytophthora* glucanase inhibitor protein. *Genetics,* **169,** 1009-1019.

Blackman, L., Cullerne, D. and Hardham, A. (2014) Bioinformatic characterisation of genes encoding cell wall degrading enzymes in the *Phytophthora parasitica* genome. *BMC Genomics,* **15,** 785.

Blackman, L.M., Cullerne, D.P., Torreña, P., Taylor, J. and Hardham, A.R. (2015) RNA-Seq analysis of the expression of genes encoding cell wall degrading enzymes during infection of lupin (*Lupinus angustifolius*) by *Phytophthora parasitica*. *PLoS ONE,* **10,** e0136899.

Boddey, J.A., Hodder, A.N., Gunther, S., Gilson, P.R., Patsiouras, H., Kapp, E.A., Pearce, J.A., de Koning-Ward, T.F., Simpson, R.J., Crabb, B.S. and Cowman, A.F. (2010) An aspartyl protease directs malaria effector proteins to the host cell. *Nature,* **463,** 627-631.

Bortesi, L. and Fischer, R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol Adv,* **33,** 41-52.

Bos, J.I.B., Armstrong, M., Whisson, S.C., Torto, T.A., Ochwo, M., Birch, P.R.J. and Kamoun, S. (2003) Intraspecific comparative genomics to identify avirulence genes from *Phytophthora*. *New Phytol,* **159,** 63-72.

Butler, N.M., Atkins, P.A., Voytas, D.F. and Douches, D.S. (2015) Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system. *PLoS ONE,* **10,** e0144591.

Chang, H.-X., Yendrek, C.R., Caetano-Anolles, G. and Hartman, G.L. (2016) Genomic characterization of plant cell wall degrading enzymes and in silico analysis of xylanses and polygalacturonases of *Fusarium virguliforme*. *BMC Microbiol,* **16,** 1-12.

Cook, D.E., Mesarich, C.H. and Thomma, B.P.H.J. (2015) Understanding plant immunity as a surveillance system to detect invasion. *Annu Rev Phytopathol,* **53,** 541-563.

Cooper, G.M. (2000) The central role of enzymes as biological catalysts. In: *The cell: A molecular approach. 2nd edition.* Sunderland (MA): Sinauer Associates.

Dangl, J.L., Horvath, D.M. and Staskawicz, B.J. (2013) Pivoting the plant immune system from dissection to deployment. *Science,* **341**.

Dislich, B. and Lichtenthaler, S.F. (2012) The membrane-bound aspartyl protease BACE1: Molecular and functional properties in Alzheimer's disease and beyond. *Front Physiol,* **3,** 8.

Domazakis, E. (2018) Unravelling the elicitin perception pathway against *Phytophthora* in potato. (PhD dissertation), Wageningen University & Research.

Dong, S., Stam, R., Cano, L.M., Song, J., Sklenar, J., Yoshida, K., Bozkurt, T.O., Oliva, R., Liu, Z., Tian, M., Win, J., Banfield, M.J., Jones, A.M.E., van der Hoorn, R.A.L. and Kamoun, S. (2014) Effector specialization in a lineage of the Irish potato famine pathogen. *Science,* **343,** 552-555.

Dong, S. and Wang, Y. (2016) Nudix effectors: a common weapon in the arsenal of plant pathogens. *PLoS Pathog,* **12,** e1005704.

Du, J., Verzaux, E., Chaparro-Garcia, A., Bijsterbosch, G., Keizer, L.C.P., Zhou, J., Liebrand, T.W.H., Xie, C., Govers, F., Robatzek, S., van der Vossen, E.A.G., Jacobsen, E., Visser, R.G.F., Kamoun, S. and Vleeshouwers, V.G.A.A. (2015) Elicitin

recognition confers enhanced resistance to *Phytophthora infestans* in potato. *Nat Plants,* **1,** 1-5.

Fang, Y. and Tyler, B.M. (2015) Efficient dsruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Mol Plant Pathol,* **17,** 127-139.

Fernández-Bachiller, M.I., Horatscheck, A., Lisurek, M. and Rademann, J. (2013) Alzheimer's disease: Identification and development of β-Secretase (BACE-1) binding fragments and inhibitors by dynamic Ligation screening (DLS). *Chem Med Chem,* **8,** 1041-1056.

Fry, W. (2008) *Phytophthora infestans*: The plant (and R gene) destroyer. *Mol. Plant Pathol,* **9,** 385-402.

Ghannoum, M.A. (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev,* **13,** 122-143.

Govers, F. and Gijzen, M. (2006) *Phytophthora* genomics: The plant destroyers' genome decoded. *Mol Plant Microbe Interact,* **19,** 1295-1301.

Gui, Y.-J., Chen, J.-Y., Zhang, D.-D., Li, N.-Y., Li, T.-G., Zhang, W.-Q., Wang, X.-Y., Short, D.P.G., Li, L., Guo, W., Kong, Z.-Q., Bao, Y.-M., Subbarao, K.V. and Dai, X.-F. (2017) *Verticillium dahliae* manipulates plant immunity by glycoside hydrolase 12 proteins in conjuction with carbohydrate-binding module 1. *Environ Microbiol,* **19,** 1914-1932.

Gumtow, R., Wu, D., Uchida, J. and Tian, M. (2017) A *Phytophthora palmivora* extracellular cystatin-like protease inhibitor targets papain to contribute to virulence on papaya. *Mol Plant Microbe Interact*.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T. *et al*. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature,* **461,** 393 - 398.

Haesaert, G., Vossen, J.H., Custers, R., De Loose, M., Haverkort, A., Heremans, B., Hutten, R., Kessel, G., Landschoot, S., Van Droogenbroeck, B., Visser, R.G.F. and Gheysen, G. (2015) Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions. *Crop Prot,* **77,** 163-175.

Haverkort, A.J., Boonekamp, P.M., Hutten, R., Jacobsen, E., Lotz, L.A.P., Kessel, G.J.T., Visser, R.G.F. and Van der Vossen, E.A.G. (2008) Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. *Potato Res,* **51,** 47-57.

Hayashida, K., Bartlett, A.H., Chen, Y. and Park, P.W. (2010) Molecular and cellular mechanisms of ectodomain shedding. *Anat Rec,* **293,** 925-937.

Hua, C., Meijer, H.J.G., de Keijzer, J., Zhao, W., Wang, Y. and Govers, F. (2013) GK4, a G-protein-coupled receptor with a phosphatidylinositol phosphate kinase domain in *Phytophthora infestans*, is involved in sporangia development and virulence. *Mol Microbiol,* **88,** 352-370.

Jiang, R.H.Y., Tripathy, S., Govers, F. and Tyler, B.M. (2008) RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc Natl Acad Sci,* **105,** 4874-4879.

Jiang, R.H.Y. and Tyler, B.M. (2012) Mechanisms and evolution of virulence in oomycetes. In: *Annu Rev Phytopathol,* pp. 295-318.

Jiang, R.H.Y., Tyler, B.M., Whisson, S.C., Hardham, A.R. and Govers, F. (2006) Ancient origin of elicitin gene clusters in *Phytophthora* genomes. *Mol Biol Evol,* **23,** 338 - 351.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B. and Weeks, D.P. (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Res,* **41,** e188.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature,* **444,** 323-329.

Jones, J.D.G., Witek, K., Verweij, W., Jupe, F., Cooke, D., Dorling, S., Tomlinson, L., Smoker, M., Perkins, S. and Foster, S. (2014) Elevating crop disease resistance with cloned genes. *Philos Trans R Soc Lond B Biol Sci,* **369,** 20130087.

Judelson, H.S. and Blanco, F.A. (2005) The spores of *Phytophthora*: weapons of the plant destroyer. *Nat Rev Microbiol,* **3,** 47-58.

Kamoun, S., Furzer, O., Jones, J.D., Judelson, H.S., Ali, G.S., Dalio, R.J., Roy, S.G., Schena, L., Zambounis, A., Panabieres, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B.M., Grünwald, N.J., Mukhtar, M.S.,

Tomé, D.F.A., Tör, M., Van Den Ackerveken, G., McDowell, J., Daayf, F., Fry, W.E., Lindqvist-Kreuze, H., Meijer, H.J.G., Petre, B., Ristaino, J., Yoshida, K., Birch, P.R.J. and Govers, F. (2015) The top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol,* **16,** 413-434.

Kamoun, S., Hraber, P., Sobral, B., Nuss, D. and Govers, F. (1999) Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. *Fungal Genet Biol,* **28,** 94-106.

Kamoun, S., van West, P., de Jong, A.J., de Groot, K.E., Vleeshouwers, V.G.A.A. and Govers, F. (1997) A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol Plant Microbe Interact,* **10,** 13-20.

Kaschani, F., Shabab, M., Bozkurt, T., Shindo, T., Schornack, S., Gu, C., Ilyas, M., Win, J., Kamoun, S. and van der Hoorn, R.A.L. (2010) An effector-targeted protease contributes to defense against *Phytophthora infestans* and is under diversifying selection in natural hosts. *Plant Physiol,* **154,** 1794-1804.

Kasuga, T. and Gijzen, M. (2013) Epigenetics and the evolution of virulence. *Trends microbiol,* **21,** 575-582.

Kay, J., Meijer, H.J.G., ten Have, A. and van Kan, J.A.L. (2011) The aspartic proteinase family of three *Phytophthora* species. *BMC Genomics,* **12,** 1-15.

King, B.C., Waxman, K.D., Nenni, N.V., Walker, L.P., Bergstrom, G.C. and Gibson, D.M. (2011) Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnol Biofuels,* **4,** 4-4.

Kooijman, E.E., Chupin, V., Fuller, N.L., Kozlov, M.M., de Kruijff, B., Burger, K.N.J. and Rand, P.R. (2005) Spontaneous curvature of phosphatidic acid and lysophosphatidic acid. *Biochemistry,* **44,** 2097-2102.

Kots, K., Meijer, H.J.G., Bouwmeester, K., Govers, F. and Ketelaar, T. (2016) Filamentous actin accumulates during plant cell penetration and cell wall plug formation in *Phytophthora infestans*. *Cellular and Molecular Life Sciences,* **74,** 909–920.

Kroon, L.P., Brouwer, H., Cock, A.W. and Govers, F. (2012) The genus *Phytophthora* anno 2012. *Phytopathology,* **102**.

Kroon, L.P.N.M., Brouwer, H., de Cock, A.W.A.M. and Govers, F. (2011) The Genus *Phytophthora* Anno 2012. *Phytopathology,* **102,** 348-364.

Kubicek, C.P., Starr, T.L. and Glass, N.L. (2014) Plant cell wall–degrading enzymes and their secretion in plant-pathogenic fungi. *Annu Rev Phytopathol,* **52,** 427-451.

Large, E.C. (1940) *The advance of the fungi*. Oxford: Alden Press.

Latijnhouwers, M., Munnik, T. and Govers, F. (2002) Phospholipase D in *Phytophthora infestans* and its role in zoospore encystment. *Mol Plant Microbe Interact,* **15,** 939-946.

Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M. and Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res,* **42,** D491.

Ma, Z., Song, T., Zhu, L., Ye, W., Wang, Y., Shao, Y., Dong, S., Zhang, Z., Dou, D., Zheng, X., Tyler, B.M. and Wang, Y. (2015) A *Phytophthora sojae* glycoside hydrolase 12 protein is a major virulence factor during soybean infection and is recognized as a PAMP. *Plant Cell,* **27,** 2057–2072.

McCarthy, C.G.P. and Fitzpatrick, D.A. (2017) Phylogenomic reconstruction of the oomycete phylogeny derived from 37 genomes. *mSphere,* **2,** e00095-00017.

McGowan, J. and Fitzpatrick, D.A. (2017) Genomic, network, and phylogenetic analysis of the oomycete effector arsenal. *mSphere,* **2,** 2:e00408-00417.

Meijer and Munnik T. (2003) Phospholipid-based signaling in plants. *Annu Rev Plant Biol,* **54,** 265-306.

Meijer, H.J.G. and Govers, F. (2006) Genomewide analysis of phospholipid signaling genes in *Phytophthora* spp.: Novelties and a missing link. *Mol Plant Microbe Interact,* **19,** 1337-1347.

Meijer, H.J.G., Hassen, H.H. and Govers, F. (2011) *Phytophthora infestans* has a plethora of phospholipase D enzymes including a subclass that has extracellular activity. *PLoS ONE,* **6,** e17767.

Meijer, H.J.G., Hua, C., Kots, K., Ketelaar, T. and Govers, F. (2014a) Actin dynamics in *Phytophthora infestans*; rapidly reorganizing cables and immobile, long-lived plaques. *Cell microbiol,* **16,** 948-961.

Meijer, H.J.G., Mancuso, F.M., Espadas, G., Seidl, M.F., Chiva, C., Govers, F. and Sabido, E. (2014b) Profiling the secretome and extracellular proteome of the potato late blight pathogen *Phytophthora infestans*. *Mol Cell Proteomics,* **13,** 2101-2113.

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., Wan, J., Gu, H. and Qu, L.-J. (2013) Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res,* **23,** 1233-1236.

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D.G. and Kamoun, S. (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol,* **31,** 691-693.

Olivera, I.E., Fins, K.C., Rodriguez, S.A., Abiff, S.K., Tartar, J.L. and Tartar, A. (2016) Glycoside hydrolases family 20 (GH20) represent putative virulence factors that are shared by animal pathogenic oomycetes, but are absent in phytopathogens. *BMC Microbiol,* **16,** 1-11.

Ospina-Giraldo, M.D., Griffith, J.G., Laird, E.W. and Mingora, C. (2010) The CAZyome of *Phytophthora* spp.: a comprehensive analysis of the gene complement coding for carbohydrate-active enzymes in species of the genus *Phytophthora*. *BMC Genomics,* **11,** 525.

Resjö, S., Brus, M., Ali, A., Meijer, H.J.G., Sandin, M., Govers, F., Levander, F., Grenville-Briggs, L. and Andreasson, E. (2017) Proteomic analysis of *Phytophthora infestans* reveals the importance of cell wall proteins in pathogenicity. *Mol Cell Proteomics,* **16,** 1958-1971.

Sanju, S., Thakur, A., Siddappa, S., Sreevathsa, R., Srivastava, N., Shukla, P. and Singh, B.P. (2013) Pathogen virulence of *Phytophthora infestans*: From gene to functional genomics. *Physiol Mol Biol Plants,* **19,** 165-177.

Sanman, L.E. and Matthew, B. (2014) Activity-based profiling of proteases. *Annu Rev Biochem,* **83,** 249-273.

Schmidt, A. and Hall, M.N. (1998) Signaling to the actin cytoskeleton. *Annu Rev Cell Dev Biol,* **14,** 305-338.

Schornack, S., Huitema, E., Cano, L.M., Bozkurt, T.O., Oliva, R., Van Damme, M., Schwizer, S., Raffaele, S., Chaparro-Garcia, A., Farrer, R., Segretin, M.E., Bos, J., Haas, B.J., Zody, M.C., Nusbaum, C., Win, J., Thines, M. and Kamoun, S. (2009) Ten things to know about oomycete effectors. *Mol Plant Pathol,* **10,** 795-803.

Seidl, M.F., Van den Ackerveken, G., Govers, F. and Snel, B. (2011) A domain-centric analysis of oomycete plant pathogen genomes reveals unique protein organization. *Plant Physiol,* **155,** 628-644.

Selvy, P.E., Lavieri, R.R., Lindsley, C.W. and Brown, H.A. (2011) Phospholipase D - enzymology, functionality, and chemical modulation. *Chem Rev,* **111,** 6064-6119.

Song, J., Win, J., Tian, M.Y., Schornack, S., Kaschani, F., Ilyas, M., van der Hoorn, R.A.L. and Kamoun, S. (2009) Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. *Proc Natl Acad Sci,* **106**.

Su, J., Spears, B.J., Kim, S.H. and Gassmann, W. (2018) Constant vigilance: plant functions guarded by resistance proteins. *Plant J,* **93,** 637–650

ten Have, A., Tenberge, K.B., Benen, J.A.E., Tudzynski, P., Visser, J. and van Kan, J.A.L. (2002) The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens. In: *Agricultural Applications.* (Kempken, F., ed.). Berlin, Heidelberg: Springer pp. 341-358.

Testerink, C. and Munnik, T. (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. *Trends Plant Sci,* **10,** 368-375.

Testerink, C. and Munnik, T. (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J Exp Bot,* **62,** 2349-2361.

Tian, M., Huitema, E., da Cunha, L., Torto-Alalibo, T. and Kamoun, S. (2004) A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. *J Biol Chem,* **279,** 26370-26377.

Tian, M., Win, J., Song, J., Van Der Hoorn, R., Van Der Knaap, E. and Kamoun, S. (2007) A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol,* **143,** 364-377.

Tian, P., Wang, J., Shen, X., Rey, J.F., Yuan, Q. and Yan, Y. (2017) Fundamental CRISPR-Cas9 tools and current applications in microbial systems. *Synthetic and Systems Biotechnology,* **2,** 219-225.

Torto-Alalibo, T., Tian, M., Gajendran, K., Waugh, M.E., West, P. and Kamoun, S. (2005) Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveal putative virulence factors. *BMC Microbiol,* **5,** 46.

van Damme, M., Bozkurt, T.O., Cakir, C., Schornack, S., Sklenar, J., Jones, A.M.E. and Kamoun, S. (2012) The Irish potato famine pathogen *Phytophthora infestans* translocates the CRN8 kinase into host plant cells. *PLoS Pathog,* **8,** e1002875.

van den Hoogen, D.J. and Govers, G. (2018) Attempts to implement CRISPR/Cas9 for genome editing in the oomycete *Phytophthora infestans*. *bioRxiv*, doi:https://doi.org/10.1101/274829.

van den Hoogen, D.J., Meijer, H.J.G., Seidl, M.F. and Govers, F. (2018) The ancient link between G-protein-coupled receptors and C-terminal phospholipid kinase domains. *mBio,* **9,** e02119-02117.

van der Honing, H.S., Emons, A.M.C. and Ketelaar, T. (2007) Actin based processes that could determine the cytoplasmic architecture of plant cells. *BBA-Mol Cell Res,* **1773,** 604-614.

van West, P., Kamoun, S., van 't Klooster, J.W. and Govers, F. (1999) Internuclear gene silencing in *Phytophthora infestans*. *Mol Cell,* **3,** 339-348.

Vleeshouwers, V.G.A.A., Raffaele, S., Vossen, J.H., Champouret, N., Oliva, R., Segretin, M.E., Rietman, H., Cano, L.M., Lokossou, A., Kessel, G., Pel, M.A. and Kamoun, S. (2011) Understanding and exploiting late blight resistance in the age of effectors. *Annu Rev Phytopathol,* **49,** 507-531.

Waite, M. (1996) Chapter 8 - Phospholipases. In: *New Comprehensive Biochemistry.* (Dennis, E. V. and Jean, E. V., eds.). Elsevier, pp. 211-236.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Qiu, J.-L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnol,* **32,** 947-951.

Wang, Y., Xu, Y., Sun, Y., Wang, H., Qi, J., Wan, B., Ye, W., Lin, Y., Shao, Y., Dong, S., Tyler, B.M. and Wang, Y. (2018) Leucine-rich repeat receptor-like gene screen reveals that *Nicotiana* RXEG1 regulates glycoside hydrolase 12 MAMP detection. *Nat Commun,* **9,** 594.

Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L. and Birch, P.R.J. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature,* **450,** 115 - 118.

Whisson, S.C., Boevink, P.C., Wang, S. and Birch, P.R.J. (2016) The cell biology of late blight disease. *Curr Opin Microbiol,* **34,** 127-135.

Zhao, Z.T., Liu, H.Q., Wang, C.F. and Xu, J.R. (2014) Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics,* **15**.

Zuluaga, A.P., Vega-Arreguín, J.C., Fei, Z., Matas, A.J., Patev, S., Fry, W.E. and Rose, J.K.C. (2015) Analysis of the tomato leaf transcriptome during successive hemibiotrophic stages of a compatible interaction with the oomycete pathogen *Phytophthora infestans*. *Mol Plant Pathol,* **47,** 42-54.

Chapter 2

Phytophthora infestans metalloproteases: an inventory of protein modifying enzymes

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This chapter has been submitted for publication

2

Abstract

Pathogens deploy a wide range of pathogenicity factors that modify host tissue or manipulate host defenses, among which are proteases. Metalloproteases (MPs) have been indicated as important determinants of virulence in a variety of animal and plant pathogens. In this study we investigated the repertoire of MPs in the oomycete *Phytophthora infestans*, a notorious plant pathogen that causes late blight on potato and tomato. The genome of *P. infestans* was scrutinized for MPs resulting in 99 putative MP-encoding genes. Based on homology and phylogenetic analyses these were classified in 9 clans and 20 families as defined in the *MEROPS* peptidase database. Of the 99 proteins, 29 contain a signal peptide or signal anchor suggesting that these are secreted. Comparison of the *P. infestans* MP genes with their homologs in other oomycetes and other species within the Stramenopile lineage showed an expansion of these genes in *Phytophthora* species. Domain architecture and accessory domain composition analyses revealed a few MPs with a novel domain architecture exclusively found in *Phytophthora* species. Expression profiling of the MP genes using whole transcriptome sequencing data of various *in vitro* growth stages showed that nearly all MP genes are expressed and that they can be grouped in eight clusters with distinct expression profiles. We report the full genome-based inventory of *P. infestans* MPs. To our knowledge, this is the first systematic inventory of MPs in an oomycete. Based on these results MP genes can be selected as candidates for future functional studies in *P. infestans* and other oomycetes.

Keywords: Secreted metalloproteases, oomycete, *MEROPS,* peptidase, protein domain architecture, plant pathogen, Stramenopile

Introduction

Proteases play important roles in all organisms, not only within the organism itself but also in interactions with other organisms. They can function in the synthesis and degradation of proteins and are indispensable in numerous molecular and cellular processes (Barrett & Rawlings, 2007). Pathogenic microbes often deploy proteases as pathogenicity factors, for example for degrading or modifying host tissue, or disrupting the basic cellular machinery in the host in order to create suitable conditions for successful colonisation (Bellincampi *et al.*, 2014, Miyoshi & Shinoda, 2000, Monod *et al.*, 2002).

Proteases, also referred to as peptidases or proteolytic enzymes, are divided in seven major groups according to their catalytic types: namely aspartic-, cysteine-, glutamic-, metallo-, asparagine lyase, serine and threonine proteases. In addition, there are proteases of mixed or unknown catalytic type. *MEROPS* is a peptidase database that currently contains around 4000 entries. It groups proteases into families based on significant amino acid sequence similarities. At a higher hierarchical level, homologous protein families are grouped into clans (Rawlings & Morton, 2008, Rawlings *et al.*, 2016). As such, the *MEROPS* classification, is an excellent tool for categorizing proteases resulting from genome annotations and for predicting potential functions of invalidated proteases.

Metalloproteases (MPs) comprise a group of proteases that function by virtue of a divalent metal cation positioned at their catalytic site, in most cases zinc (zinc-dependent MPs). Zinc-binding by MPs is mainly accommodated by the two histidine residues in the motif HEXXH, which is a motif conserved in over 50% of the MPs (Rawlings & Barrett, 1995). In animals, MPs were reported to play a role in receptor modification, thereby leading to loss of function of the receptor or alteration of downstream signaling mediated by such a receptor (Sanderson *et al.*, 2006). These so-called sheddases include several members of the A Disintegrin And Metalloproteases (ADAMs) and Matrix metalloproteases (MMPs) families. They shed the extracellular part of membrane proteins, a process referred to as ectodomain shedding. This results in a truncated receptor and an extracellular protein, both functioning independently of each other, likely leading to a gain of function (Hayashida *et al.*, 2010, Higashiyama *et al.*, 2011). In a recent study, MP inhibition assays in rodent cell lines indicated that ADAMs are responsible for cleavage and processing of the N-terminal part of the GPCR receptor GPR37L1, leading to its inactivation (Coleman *et al.*, 2016).

MPs have been suggested to play an important role in virulence in several pathogenic microorganisms (Miyoshi & Shinoda, 2000, Li & Zhang, 2014). For example, leishmanolysin GP63, a zinc-binding MP from several protozoan *Leishmania* species, was shown to have proteolytic activity on protein-tyrosine phosphatases of macrophages during infection and to enhance migration of the pathogen through the extracellular matrix (McGwire *et al.*,

2003, Yao *et al.*, 2003, Gomez *et al.*, 2009). Deletion of leishmanolysins in *Leishmania major* resulted in reduced lesion formation in mice, identifying GP63 as a virulence factor (Joshi *et al.*, 2002). In the mammalian pathogen *Vibrio cholerae*, the extracellular Zn-dependent metalloprotease hemagglutinin, also reported as vibriolysin, is involved in the modification of toxins, degradation of mucus barriers and disruption of host intestinal junctions (Benitez & Silva, 2016). MPs were also reported to play a role in the interaction of bacteria with nematodes. Metalloprotease ColB from a nematicidal strain of *Bacillus thuringiensis* was found to be involved in the colonization of nematodes (Peng *et al.*, 2016).

Also in plant pathogens, MPs play a role in interactions with their hosts. It was shown for example that the rice blast fungus *Magnaporthe oryzae* has a MP-like protein that directly interacts with the rice resistance protein Pi-ta and is recognized as the avirulence factor AVR-Pita (Jia *et al.*, 2016, Jia *et al.*, 2000). However, as yet there is no biochemical evidence that AVR-Pita is a functionally active MP (Orbach *et al.*, 2000). In the phytopathogenic bacterium *Erwinia amylovora* (renamed *Pectobacterium*) the lack of PtrA, a secreted extracellular zinc-binding MP, resulted in reduced host-plant colonization. In line with this, inhibition of PtrA activity with EDTA led to a similar reduced host-plant colonization phenotype. PtrA is the most abundant secreted protein in *E. amylovora* and possesses proteolytic activity against the general substrate gelatin (Zhang *et al.*, 1999).

Our study focuses on the oomycete *Phytophthora infestans*, the causal agent of potato and tomato late blight (Schoina & Govers, 2015). This filamentous plant pathogen has a large economic impact on potato production worldwide. Farmers heavily invest in chemical spraying to protect the canopy against late blight (Haverkort *et al.*, 2008). Oomycetes belong to the Stramenopiles, an eukaryotic lineage that also includes diatoms and brown algae. Besides the more than hundred plant pathogenic species in the *Phytophthora* genus, there are many other oomycete pathogens (Kroon *et al.*, 2011, Kamoun *et al.*, 2015). Major groups are the downy mildews, obligate biotrophs that entirely depend on their plant hosts to survive, *Pythium* spp., that are mainly plant pathogens although some parasitize on mammals or other microbes, and *Saprolegnia* spp., several of which are well known fish pathogens (Kamoun *et al.*, 2015, Seidl *et al.*, 2012, Runge *et al.*, 2011, Blair *et al.*, 2008, Jiang *et al.*, 2013).

P. infestans is a hemibiotrophic pathogen that depends on living host tissue during the first stages of infection. Therefore, avoidance of recognition by the host is important for successful penetration of host cells and subsequent colonization. Extensive research on *Phytophthora*-plant interactions has provided insights into the molecular mechanisms employed by the pathogen to escape recognition and successfully infect its host. Genome sequencing revealed that *P. infestans* has over 500 genes encoding cytoplasmic effectors and around 200 encoding apoplastic effectors (Haas *et al.*, 2009). Several of these effectors are known to function in virulence by supressing host defense responses

(Whisson *et al.*, 2016). Next to these effector genes, *P. infestans* has many other putative pathogenicity genes including genes encoding hydrolases, proteases and protease inhibitors (Seidl *et al*., 2012, Haas *et al*., 2009). Examples are the aspartic proteases (APs), a family with twelve members including one, PiAP5, in which the protease domain is fused to a C-terminal G-protein-coupled receptor domain (Kay *et al.*, 2011). Such a peculiar domain combination is a typical example of a *Phytophthora*- or oomycete-specific bigram that is not found outside the oomycete lineage. A comprehensive study of the protein domain organisation in 67 eukaryotes revealed that especially *Phytophthora* spp. have a significantly higher number of distinct domain combinations (bigrams) compared to species with a comparable number of domain types (Seidl *et al.*, 2011). Several of the proteins with overrepresented domain combinations were predicted to be secreted. Indeed, for *P. infestans*, 259 genes encoding proteins with overrepresented domains were found to be differentially regulated during infection, the majority of those having a signal peptide (Seidl *et al*., 2011). Moreover, proteomic analyses of the *P. infestans* secretome revealed that besides effectors, several proteases were detected in the extracellular medium, including cysteine, aspartic and metalloproteases, confirming that these enzymes are secreted by the pathogen and could potentially play a role in the host-pathogen interplay (Meijer *et al.*, 2014).

While MPs have been shown to have various roles in cellular processes and in pathogenicity, they have hardly been studied in oomycetes. The aim of this study was to identify the full repertoire of MPs present in *P. infestans,* and to analyze their characteristics. The MPs resulting from a bioinformatics search were divided in clans and families and their distribution within the oomycete and Stramenopile lineage was studied. Further analyses focussed on the domain architecture of MPs aimed at finding unique oomycete- or *Phytophthora*-specific MPs, and included expression profiling to reveal which MP genes are expressed in pre-infection life stages. Together these results provide a basis for selecting candidates for future studies on the role of MPs as pathogenicity factors in *P. infestans* and related oomycetes.

Results

Identification of metalloprotease genes in *P. infestans*

To identify genes in *P. infestans* encoding putative metalloproteases (MPs) we followed the procedure depicted in Fig. 1. As a first step a word search was performed for genes annotated as 'metalloprotease' in the *P. infestans* T30-4 reference genome, resulting in 42 genes. Nucleotide sequences and deduced protein sequences were used to perform BLAST searches against the *P. infestans* reference genome and 24 additional genes were identified. In parallel, blastp search was performed using as query protein sequences from 100 different MPs, derived from the *MEROPS* database (Finn *et al.*, 2014, Rawlings *et al*.,

2016). Based on the two searches, a total of 103 MP genes were identified (Table S1A). By carefully examining the predicted gene models in the genome browser and inspecting the alignments with ESTs and transcript sequences from RNA-Seq data, 53 predicted gene models were found to be correct, while the remaining 50 required manual correction. In most cases this concerned adaptations in the length of the open reading frame (ORF) due to an erroneous prediction of start and/or stop codon(s), the introduction or removal of introns, or in few cases it concerned splitting or merging of gene models (Table S1B). In five gene models, premature stop codons were found that were not rescued by correcting RNA-Seq reads, and therefore these five were considered to be pseudogenes. The sequences of all the corrected genes were re-blasted against the *P. infestans* genome sequence, which resulted in the detection of one additional MP gene (Fig. 1, Table S1A). Based on the available RNA-Seq reads from *P. infestans* life stages the gene models were validated. This revealed that 14 of the MP genes show alternative splicing, often resulting in more splice variants (Table S1C).

We also analyzed the positioning of the MP genes on the genome and this revealed that the genes are randomly distributed over the genome. There are no indications for strong clustering of different MP genes. Five pairs of highly similar MP genes were found, with sequence similarity at the protein level ranging from 67 to 91% suggesting gene duplications (Table S1D). Moreover, an analysis of the intergenic distances did not reveal a bias for MP genes towards gene-dense or gene-sparse regions (Table S1E, Fig. S1). Only a few MPs were located in the gene-sparse regions, and surrounded by repeats. In conclusion, the genome mining and subsequent correction of several wrongly predicted gene models resulted in the identification of a total of 104 *P. infestans* MP genes including five pseudogenes. In a few cases, alternative splicing was found as well as indications of gene duplication.

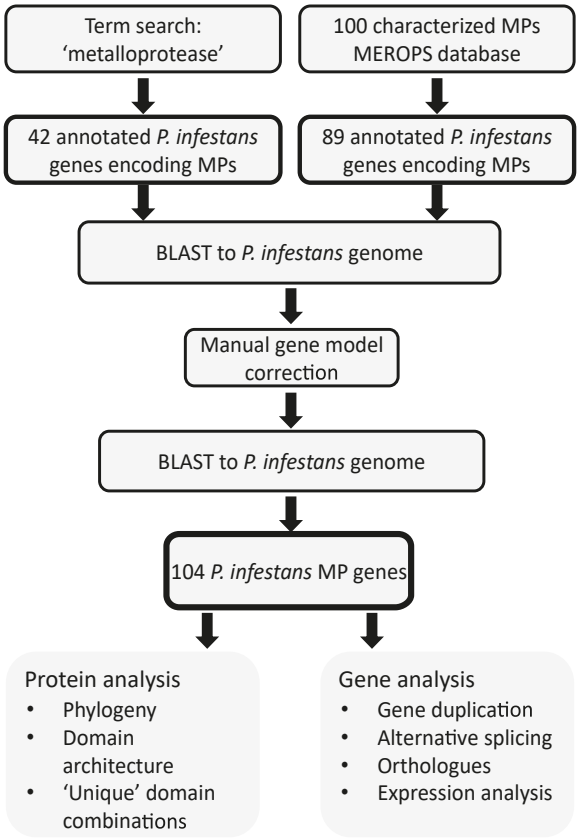


Figure 1. Pipeline for the identification of putative MP genes in *P. infestans*. Protein sequences derived from *P. infestans* gene models annotated as MPs were retrieved via a term search from The Broad Institute database and sequences from characterised MPs from 100 different families were derived from the *MEROPS* database. These sequences were used as query for performing blastp against the *P. infestans* genome. Gene models were manually corrected based on ESTs from *P. infestans* strain T30-4 and transcriptomics data from *P. infestans* strain T20-2 and on their similarity with *P. sojae* and *P. ramorum* homologous genes. The corrected gene models were blasted against the *P. infestans* T30-4 reference genome (blastn). The derived MPs were further analysed as indicated.

P. infestans MPs are classified in 20 metalloprotease families

The *MEROPS* database uses a structure-based classification of proteases and groups metalloproteases in 71 families based on their amino acid similarity in the peptidase unit. Families with similarities in structure or common sequence motifs in the catalytic domains are considered to be 'homologous' and are further grouped in 16 different clans (Rawlings *et al*., 2016). According to the numbers included in the *MEROPS* database, MPs constitute 24% of all *P. infestans* proteases, making them one of the largest protease groups, together with cysteine proteases (Fig. S2A). In order to classify the *P. infestans* MP genes, BLAST searches were performed against the *MEROPS* and PFAM databases

using amino acid sequences of the *P. infestans* MPs as query. Based on the identified MP domains, the 104 *P. infestans* MPs (including the ones derived from pseudogenes) were divided over 20 families. For further grouping, 10 families were assigned to clan MA and three to clan MH. The clans are coded according to the *MEROPS* database with M referring to metalloprotease while the second capital letter is arbitrary (Rawlings *et al*., 2016). For six other clans (MC, ME, MF, MG, MJ and MP *P. infestans* comprises only one family. In line with the classification in the *MEROPS* database family M79 is not assigned to a specific clan (unassigned, U) (Fig. 2). From the identified MPs, the majority belongs to clan MA (41), while clan MH contains 15 MPs and clan MG contains 10. The 38 remaining MPs are divided over five clans (MC, ME, MF, MJ and MP) or not assigned (U). It should be noted that family M22, which comprises O-sialoglycoproteases, used to be included in the *MEROPS* database in clan MA but was recently removed from that clan due to lack of experimental evidence supporting peptidase function (Aravind & Koonin, 1999). Nevertheless, for the two identified *P. infestans* M22 members we maintained the original classification. In summary, based on their MP domain, the 104 *P. infestans* MPs are divided over 19 families assigned to eight clans, and an additional family (M79) that is not assigned to any clan.

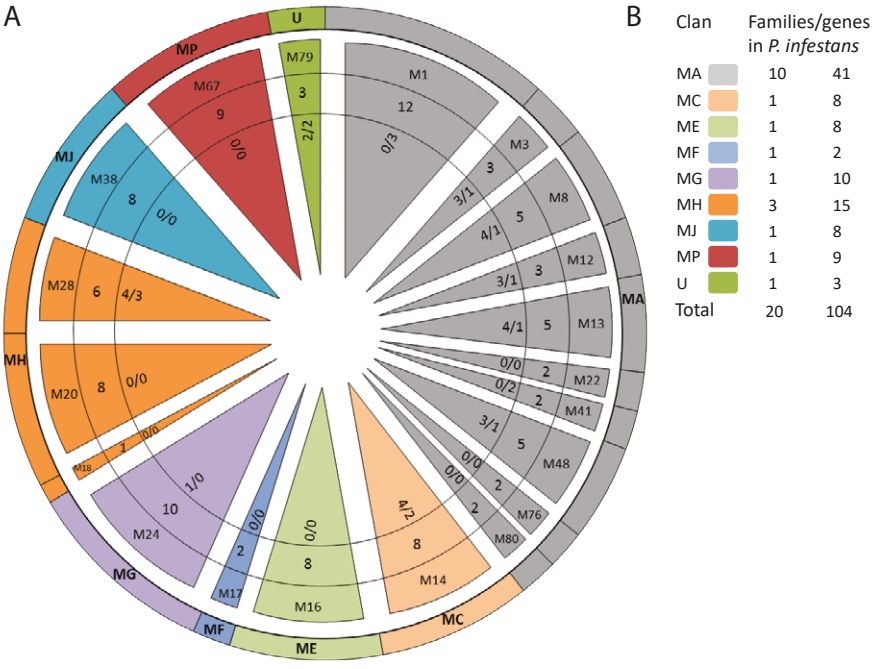
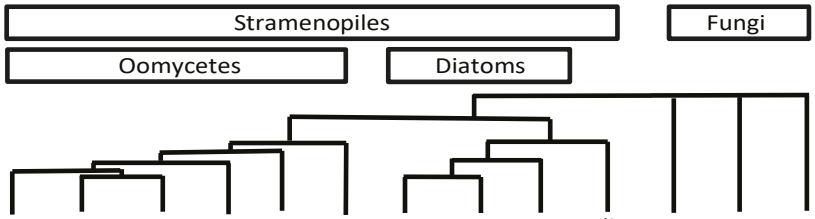


Figure 2. *MEROPS* classification of *P. infestans* MPs. A) Letters and numbers in the outer to inner concentric rings refer to clans, families and number of MPs per family, respectively. Clans are coded according to the *MEROPS* database. Numbers in the inner pie charts show the number of MP proteins per family with a signal peptide or transmembrane domain, respectively. B) Numbers of families and genes in *P. infestans* per clan.

Homologs of *P. infestans* MPs in other Stramenopiles

In order to identify homologs of *P. infestans* MPs in other oomycetes and to gain more insight on how these enzymes have evolved within the Stramenopiles, a BLAST search was conducted. Genomes of five oomycetes, four diatoms and one brown algae were screened and genomes of three fungal species were included as outgroups (Fig. 3; Table S2). Results indicate that most *P. infestans* MPs are conserved among Stramenopiles. From the total number of MP genes in specific families, it can be seen that some families are expanded in oomycetes when compared to diatoms and the more distant fungal species. This is for example the case for M14 and M20. Two MP families, i.e. M12 and M80, are restricted to oomycetes while M79 is only present in one of the seven non-oomycete species included in this search. Among the three *Phytophthora* spp. there are more or less similar numbers of MPs in each family with the exception of M8 and M28. The latter has far more members in *P. ramorum* than in *P. infestans*, 12 versus 6, while the reverse is true for M8, 1 versus 5.

Counting the number of proteases present in the *MEROPS* database for each species, revealed quite some variation in total numbers of proteases (Fig. S2B). Of the six oomycetes included, *Pythium ultimum* has the highest number of MPs in *MEROPS* (i.e. 94) and *P. ramorum* and *Hyaloperonospora arabidopsidis* the lowest (i.e. 48). Since the numbers of MPs in the *MEROPS* database are, in all cases, smaller than the numbers obtained by the BLAST searches performed in this study we think that our analysis gives a more realistic picture. Our approach though, has the limitation that with a search for homologs we miss the MPs that are not present in *P. infestans.* For example, one MP classified in family M54 was found in *P. ramorum* and *P.* sojae, but is lacking in *P. infestans* (Table S3). Nevertheless, we can conclude that nearly all *P. infestans* MPs have homologs in other *Phytophthora* spp. and other oomycetes, and that only a few of the MPs seem to be oomycete specific.



Clan	Family	*Phytophthora infestans*	*Phytophthora ramorum*	*Phytophthora sojae*	*Hyaloperonospora arabidopsidis*	*Pythium ultimum*	*Saprolegnia parasitica*	*Thalassiosira pseudonana*	*Phaeodactylum tricornutum*	*Aureococcus anophagefferens*	*Ectocarpus siliculosus*	*Fusarium graminearum*	*Saccharomyces cerevisiae*	*Ustilago maydis*
MA	M1	12	12	9	8	10	6	4	3	3	11	3	3	3
	M3	3	3	3	1	3	8	4	3	2	2	5	2	1
	M8	5	1	4	-	1	3	3	2	-	-	-	-	-
	M12	3	3	3	2	3	6	-	-	-	-	-	-	-
	M13	5	3	3	6	3	8	1	1	-	-	-	-	1
	M22	2	2	2	1	1	2	1	2	2	2	2	2	2
	M41	2	2	2	3	1	2	3	4	4	3	2	3	1
	M48	5	5	5	2	5	6	4	3	-	2	2	2	2
	M76	2	3	2	2	2	2	1	1	1	2	1	-	1
	M80	2	1	1	1	1	1	-	-	-	-	-	-	-
MC	M14	8	7	7	3	8	14	1	1	4	3	1	1	-
ME	M16	8	7	7	6	5	10	7	7	4	6	5	6	4
MF	M17	2	1	1	2	2	4	1	2	4	2	-	-	1
MG	M24	10	13	11	8	6	11	10	7	12	7	9	6	9
MH	M18	1	1	1	1	-	1	2	2	2	1	2	2	1
	M20	8	6	7	4	6	5	2	2	-	2	3	1	1
	M28	6	12	7	2	6	3	1	1	-	2	4	2	2
MJ	M38	8	7	10	5	7	6	2	3	6	4	12	2	5
MP	M67	9	8	8	10	5	8	6	6	8	7	7	3	7
U	M79	3	2	3	2	2	3	-	-	-	-	1	-	-
	Sum	**104**	**99**	**96**	**69**	**77**	**109**	**53**	**50**	**52**	**56**	**59**	**35**	**41**

Figure 3. Number of homologous *P. infestans* MP genes in other species. MPs with more than 60% similarity to *P. infestans* MPs are considered homologs. Three fungal species including yeast were used as outgroups. The tree is drawn based on NCBI taxonomy.

P. infestans MPs have diverse domain architectures

To gain more insight in the domain architecture of *P. infestans* MPs, we performed a domain analysis of all 99 functional MPs. Twenty nine MPs were predicted to have a signal peptide or signal anchor and 17 contained one or more transmembrane domains (Figs. 2, S1). In 30 cases, alternative domain architectures were predicted for one protein (Table S1A), which in several cases results in a different type of protein. One example is shown in Fig. 4 for PITG_12517 which has either two peptidase in tandem or a range of transmembrane domains. The domain analysis revealed that the *P. infestans* MPs are highly diverse with

respect to their peptidase domains and show a high diversity in domain architecture, even among MPs of the same family (Fig. 4, Table S1). Forty seven MPs have just the peptidase domain, and no other protein domain, like for example MPs from family M16. In some cases there is a second similar peptidase domain. The other 52 MPs have additional domains. In 19 of these the additional domain is another peptidase domain while the remaining ones have a variety of accessory domains. Family M1 is the largest family with eleven members (plus one pseudogene) and with the most diverse domain combinations among all MP families identified in *P. infestans.* In contrast, M48 is the family whose members all have the same domain composition (Fig. 4). Phylogenetic analyses of M1 and M48 based on MP domains show that MPs with the same domain architecture cluster together, and are thus more similar to each other than to the other members in the same MP family (Fig. 4).

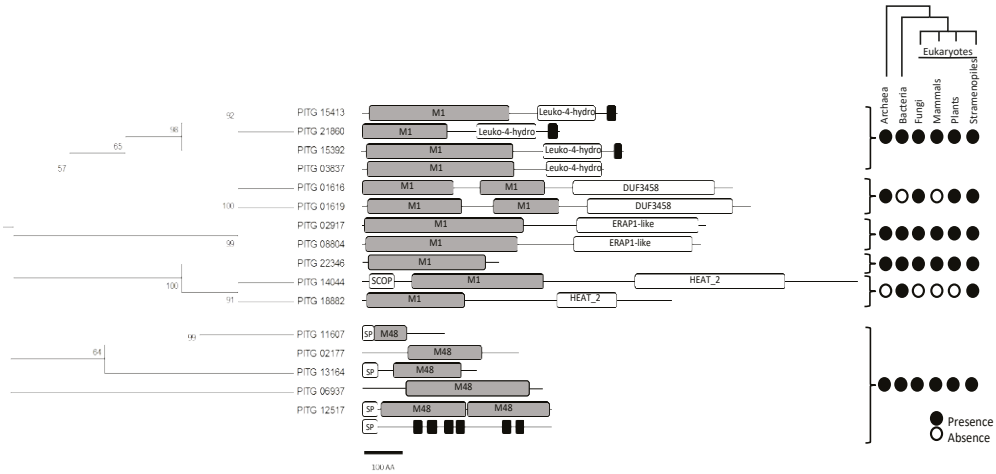


Figure 4. Domain composition of MPs two diverse *P. infestans* MPs in M1 and M48 families, both belonging to clan MA. In the schematic representation MP domains are depicted in grey, transmembrane domains in black and all other protein domains as open boxes. On the left, the phylogenetic relationship between MPs in the families is depicted. On the right, the occurrence of the MP architecture in different phylogenetic lineages is depicted.

P. infestans MPs with a unique domain architecture

In previous studies, it was shown that *Phytophthora* spp. have proteins with unique domain combinations which are only found in oomycetes or are even restricted to the *Phytophthora* genus (Meijer & Govers, 2006, Seidl *et al*., 2011). We scanned the NCBI database for the occurrence of proteins with similar architecture and determined to which taxonomic group they belong. Six main groups were distinguished, four in the eukaryotes, i.e. fungi, plants, mammals and Stramenopiles, and two prokaryotic lineages, archaea and bacteria. While for most *P. infestans* MPs, counterparts with a similar domain

architecture were found in several organisms and in most taxonomic groups, there were some MPs with a domain architecture not found outside the oomycetes. Among the 99 *P. infestans* MPs, there were seven that seem to have a domain architecture that is less common in other organisms. Two of these are members of family M1. Most members of this family have a domain architecture that occurs in MPs present in both eukaryotes and prokaryotes but the domain architecture in two M1 MPs, i.e. PITG_14044 and PITG_18882, was found only in MPs present in bacteria and other Stramenopiles (Fig. 4). Two MPs belonging to family M13 (i.e. PITG_08425 and PITG_07643) and one member of the family M80 (PITG_13744) have a domain architecture that is restricted to MPs found in plants and in other Stramenopiles (Fig. 5). The other member in family M80 (PITG_15925) has a single peptidase domain named WML and no additional domains. Within the Stramenopiles, MPs with a single WML domain are restricted to *Phytophthora* spp. and within the other taxonomic lineages to bacteria, fungi and plants. The domain architecture of three MPs, two of which belonging to family M20 (PITG_00577 and PITG_06978) and one to family M67 (PITG_16722), was unique for Stramenopiles. Actually PITG_00577 has two MP domains from different families; an amidohydrolase domain (M38) and a M20 peptidase domain. Its neighboring gene PITG_00578 encodes a MP carrying an amydohydrolase domain and was classified in family M38. MPs with a similar architecture were not found in any other lineage. Moreover, within the Stramenopiles the two M20 members are found only in *Phytophthora* spp. suggesting that these are truly *Phytophthora*-specific MPs (Fig. 5, Table S1F). In conclusion, about one third of the *P. infestans* MPs has a variety of accessory domains. The domain architecture of seven *P. infestans* MPs is not universal among eukaryotes and three MPs have a novel domain architecture that is unique for *Phytophthora* spp.

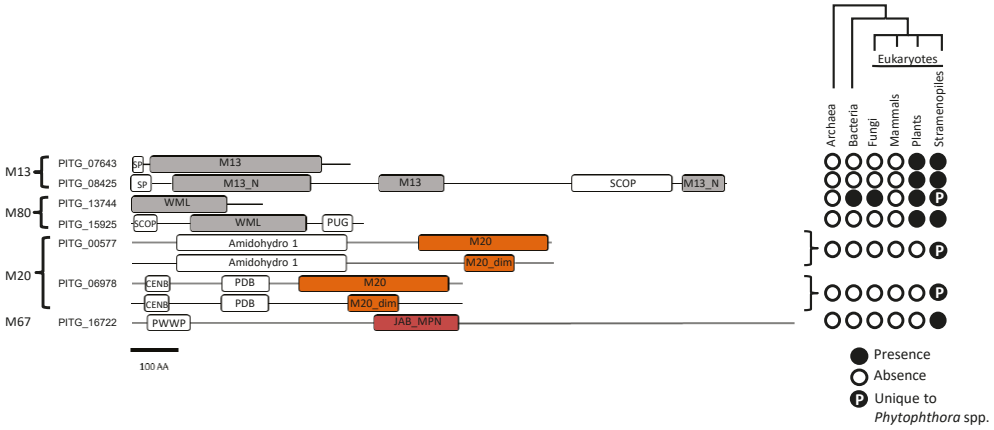


Figure 5. *P. infestans* MPs with a unique domain architecture and their occurrence in other species. MP domains are depicted in grey (MA), orange (MH) or red (MP) based on their clan. All other protein domains are depicted as open boxes. On the right, the occurrence of MPs with a similar domain composition and architecture in different phylogenetic lineages is depicted. A 'P' indicates that the domain architecture is unique for *Phytophthora* spp.

Clustering of MP genes based on expression profiles

We generated RNA-Seq from four life stages of *P. infestans* strain T20-2. After quality control and data normalization we analyzed the expression profiles of the MP genes during the four life stages, i.e. zoospores, germinating cysts, mycelium and sporangia. All MP genes are expressed in the four life stages, but at different levels. Genes were empirically clustered based on their expression patterns leading to eight distinct clusters (Fig. 6). Almost half

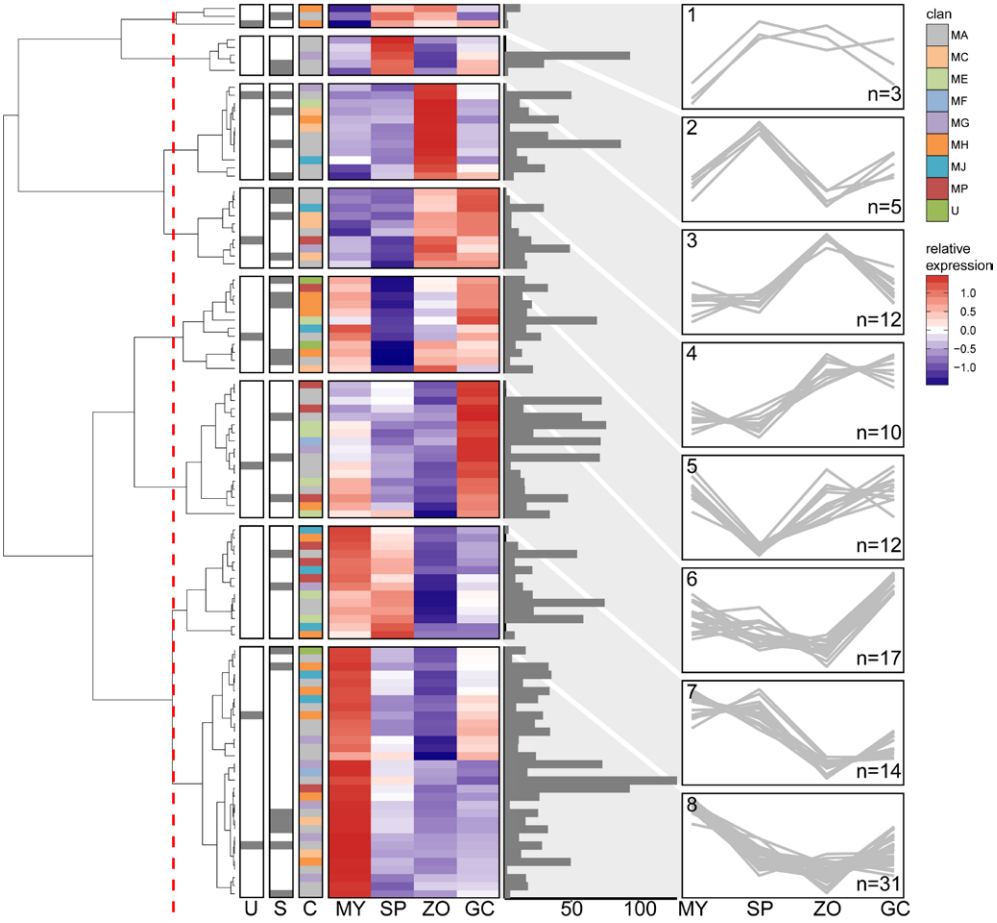


Figure 6. Clustering of MP genes based on expression patterns during the *P. infestans* life cycle. The dendrogram on the left displays the hierarchical clustering of the expression patterns. The red-dashed vertical line indicates the cut off for the clustering. The grey bars in the columns U and S mark the MPs with a unique domain architecture or with a predicted signal peptide, respectively. The colour-coded bars in column C indicate the clan in *MEROPS* comprising the MP. The heatmap shows the relative expression of each gene based on the stage-wise, z-score transformed expression values in mycelium (MY), sporangia (SP), zoospores (ZP) and germinating cysts (GC). The adjacent bar plot shows the mean TPM value for each gene. The line plots on the right display the stage-wise expression profiles of the eight clusters, resulting from the cut off and the number (n) of genes in each cluster.

of the MP genes show the highest transcript levels in mycelium (clusters 7 and 8 with 45 genes in total). MP genes in cluster 2 show the highest expression in sporangia and germinating cysts, as opposed to the expression pattern of the MPs in cluster 3 with the highest expression in zoospores. In cluster 4, transcript levels in mycelium and sporangia are low compared to the level in the other two stages with the highest expression in zoospores. Cluster 5 contains MPs that show upregulation in zoospores and germinating cysts when compared to sporangia but this cluster differs from cluster 4 because of the relatively high transcript levels in mycelium. In cluster 6, the germinating cysts stage is the stage with the highest transcript levels, while in cluster 1, transcript levels are high in sporangia and zoospores. As shown in Fig. 6, the cluster analysis did not reveal any apparent correlation between expression patterns and specific features of the MPs. Genes encoding MPs with a signal peptide or with a unique domain composition are randomly distributed over the eight clusters and each cluster contains MPs from different clans. Taken together, these results suggest that MPs are active throughout the *P. infestans* life cycle but that each growth stage exploits a different pool of MPs.

Discussion

Despite several studies in mammalian systems that highlight the importance of MPs in pathogenicity, knowledge on the role of MPs in the virulence of plant pathogens is very limited. The aim of this study was on the one hand, the identification of MP genes in the late blight pathogen *P. infestans*, and on the other hand, the analysis of the features of the encoded MPs. By a thorough mining of the *P. infestans* genome and other public databases, and bioinformatic and transcriptome analyses of the candidate genes, we identified the metalloprotease repertoire of this devastating pathogen. A total of 99 *P. infestans* MPs were identified, divided over 9 clans and 20 families. Some MPs were found to have a domain architecture unique for Stramenopiles or even for *Phytophthora* species. To our knowledge, this is the first systematic study of MPs in a *Phytophthora* species and in oomycetes in general.

To identify candidate MPs with a potential role in virulence we assessed if *P. infestans* has MPs belonging to the same families as MPs that have been associated with pathogenicity in other organisms. These include MPs distributed over the following ten families: human sheddases belonging to families M10 (MMPs) and M12B (ADAMs) that are implicated in Alzheimer's disease and breast cancer (Miller *et al.*, 2017, Hartmann *et al.*, 2013, Tallant *et al.*, 2010, Edwards *et al.*, 2008); nardilysins from family M16 that have implications in human brain diseases (Bernstein *et al.*, 2013); the leishmanolysin GP63 in family M8 that acts as a virulence factor in *Leishmania* parasites (Gomez *et al*., 2009, Hallé *et al.*, 2009, Joshi *et al.*, 2002, Kaye & Scott, 2011); serralysins in family M14 implicated in

virulence of *Serratia* bacteria infecting mammalians (Semenova & Rudenskaya, 2009); the *V. cholerea* vibriolysin in family M4 (Benitez & Silva, 2016); the *B. thuringiensis* ColB in family M9B (Peng *et al*., 2016); the neprylysin PtrA in family M13 produced by the bacterial plant pathogen *Erwinia amylovora* (Zhang *et al*., 1999); astacins in family M12A that are produced by animal-parasitic nematodes and modify the extracellular matrix of the host by hydrolyzing collagen (Stepek *et al*., 2015, Semenova & Rudenskaya, 2009); the *M. oryzae* avirulence protein AVR-Pita in family M35 (Jia *et al*., 2000); and fungalysins in family M36 that have proteolytic activity against collagen and chitin and act as virulence factors in the fungal pathogens *Colletotichum graminicola*, *Fusarium verticillioides* and *Aspergillus fumigatus* (Sanz-Martín *et al*., 2016, Fernandez *et al*., 2013, Naumann *et al*., 2011). *P. infestans* has MPs in five out of these ten, namely the families M8, M13 and M12A in clan A, family M14 in clan C and family M16 in clan E. Two of these families, the astacins (in M12A) and the serralysins (in M14), are classified as metzincins, a group that also comprises MMPs (M10) and ADAMs (M12B) and includes MPs that share the zinc-binding HEXXH motif in their catalytic domain (Semenova & Rudenskaya, 2009). Remarkably all members of the *P. infestans* M8 and M12 family have a predicted signal peptide or anchor (four and three, respectively; pseudogene PITG_13513 in M8 is excluded) while in family M13 this accounts for four out of five members and in family M14 for three out of seven (excluding the pseudogene PITG_13564) (Fig. 2). So, the majority, namely 14 out of the 25 MPs having a signal peptide/anchor belong to just four families, members of which are implicated in pathogenicity in other organisms. Despite the low similarity of the *P. infestans* M8, M12, M13 and M14 MPs to the ones described as pathogenicity factors in other organisms, the domain architectures are similar. Therefore, it is tempting to speculate that those MPs could play a role in the interaction with the host.

Hallmarks of effector genes or pathogenicity genes in oomycetes are on the one hand, their expression profiles with a higher expression in pre-infection and early infection stages, and on the other hand, their genomic context; effector genes are often located in gene sparse regions surrounded by repeats or transposable elements, and subject to duplication events resulting in expanded gene families (Haas *et al*., 2009). Several of the *P. infestans* MP genes are flanked by relatively long intergenic regions often composed of repeats (Table S1E, Fig. S1). However, there is no bias towards a specific MP clan or MP family and so far, we have no indications that these MP genes, nor MP genes in gene dense regions, are subject to dynamic alterations or expansions. Apart from five cases of possible gene duplication, no tendency to expansions was observed, which was corroborated by comparisons of the MP gene repertoire among *Phytophthora* spp. This in contrast to, for example, RXLR effector genes or NPP genes that are highly expanded in the various *Phytophthora* species, often in a species-specific manner (Haas *et al*., 2009, Jiang *et al*., 2008, Tyler *et al*., 2006). An example of a variable MP gene is *Avr-Pita* in the

fungus *M. oryzae*. This avirulence gene is located in a dynamic genomic region that is subject to a high mutation rate and this has resulted in field isolates that gained virulence on rice cultivars carrying the resistance gene *Pi-ta* (Jia *et al*., 2016). *P. infestans* uses a similar strategy of adaptation to avoid recognition by resistant cultivars. *Phytophthora* resistance is governed by intracellular Nucleotide-binding Leucine Rich repeat (NLR) proteins that are each activated by a specific effector. These *Phytophthora* effectors are highly diverse but they all share the host cell targeting motif RXLR. Since none of the MPs identified in this study has such an RXLR motif it is highly unlikely that MPs function as avirulence factor in a similar manner as Avr-Pita or RXLR effectors. Based on the expression profiles the genes in clusters 4 and 6 are the ones showing a relatively higher expression in a pre-infection stage, i.e. germinating cysts, a pattern that is supportive for a putative role in pathogenicity. We could not detect any bias in the clustering with respect to MP clans or families, or other characteristics. For example, a few MPs in cluster 4 and 6 do have a signal peptide, another hallmark for a pathogenicity factor, but since also the other six clusters comprise MPs with a signal peptide the distribution seems to be random. What is noteworthy though, is the occurrence of two M12 members and two M14 members in cluster 4, the only ones out of the 10 MPs in cluster 4 with a signal peptide. As mentioned above, we speculate that MPs in these families are likely to play a role in the interaction with the host. In fact, one of the M12 astacins (i.e. PITG_09851) was detected in a proteome analysis aimed at identifying extracellular proteins secreted by *P. infestans* in culture medium (Meijer *et al*., 2014). The increased expression of this MP12 gene in pre-infection stages suggests that astacin is present in the plant apoplast during infection.

The main characteristic of a MP is the peptidase domain. Most *P. infestans* MPs have one or two peptidase domains with or without a signal peptide/anchor at the N-terminus. However, about one third of the *P. infestans* MPs has accessory domains that are highly diverse. This may point to a second biochemical activity apart from or next to the protease activity, an association with other proteins or protein complexes, a specific subcellular location or maybe a module that is required for the tertiary structure of the MP. Based on the high diversity in accessory domains and domain architecture it can be assumed that the *P. infestans* MPs have very diverse functions. Another phenomenon that may result in more diversity in MPs is alternative splicing. For 14 MP genes splice variants were found, but in only two cases this influenced the domain architecture of the encoded MPs (Table S1). Alternative splicing in oomycetes has been reported previously, often with splice variants associated with specific life stages (Betz *et al.*, 2016, Burkhardt *et al.*, 2014, Judelson, 2012, Sêton Bocco & Csűrös, 2016).

In most families, the diversity in domain architecture is rather limited (as shown for family M48 in Fig. 4), but in family M1 for example, the diversity is high. Such diversity among MP family members and in between MP families and clans is well documented (Mansfeld & Andrew, 2007, Rawlings & Barrett, 1995) and in most cases the domain organization of the *P. infestans* MPs was similar to the domain organization found in MPs from other organisms. With the notion that *Phytophthora* spp. and oomycetes in general, possess a relatively high proportion of proteins with a unique domain architecture (Seidl *et al*., 2011), we were keen to identify MPs that are unique for *Phytophthora* spp. as this could hint to a specialization or gain of function of the MPs. Among the few MPs with a domain architecture that is less common in other taxonomic lineages there were two *Phytophthora* unique MPs, both in family M20, and one Stramenopile unique MP in family M67 (Fig. 5). There are no indications though, that these MPs are genuine pathogenicity factors. All three lack a signal peptide and only the latter, the M67 member, shows an increased expression in pre-infection stages. Nevertheless, their unique domain architecture justifies further studies on these MPs if only to learn how far this uniqueness reaches. Is their protease activity unique or is the substrate a *Phytophthora* or Stramenopile specific compound?

In conclusion, the analyses presented in this study provide new insights into the MP repertoire in *P. infestans*. In addition, analyses of MP domain architecture and expression profiles allows us to pinpoint several candidate MPs for further study. While analyzing the gene models we encountered the pitfalls of a poorly assembled and annotated genome. More than half of the predicted MP models in the reference T30-4 genome appeared to be incorrectly annotated, an issue that has been noted previously in other gene inventory studies (Kay *et al*., 2011). Despite the manual annotation and the reiterated searches there is still a chance that we missed some MP genes and this could explain why we found MPs in other oomycetes and Stramenopiles, that were not detected in *P. infestans*. Alternatively, the lack of those genes could also be due to gene losses, with the acquirement of novel *Phytophthora*-unique MPs through gene gains as counterbalance. Seidl *et al*. (2012) showed that massive gene gains, duplications and losses have shaped the evolutionary history of oomycetes and in particular *Phytophthora,* and likely this has been a drive for speciation in this genus (Seidl *et al*., 2012). We anticipate that for the basic cellular machinery the functions of missing MPs are compensated by MPs belonging to the same or other families with similar enzymatic activities. For pathogenicity or host specificity, pathogens may have acquired additional MPs. Systematic analyses of the MP repertoire in other *Phytophthora* spp. are needed to see to what extent MPs have changed during speciation and whether or not they play a role in host specificity.

Conclusions

Metalloproteases are involved in several proteolytic processes and as such, in maintaining the cellular machinery in a balanced state, while some have been described to play a role in interactions between organisms as pathogenicity factors of pathogens. In this study, a manual genome search was performed in parallel to screening of the available peptidase databases, all with the aim to identify the full repertoire of MPs in *P. infestans*. A thorough bioinformatics analysis of the gene and protein properties, including domain architecture analysis and expression profiling, allows us the categorise and cluster the MP genes based on their properties. This study is the first inventory of MPs in an oomycete and provides an extensive description of their characteristics. Based on these analyses, interesting properties of *P. infestans* MPs were revealed, including their large diversity, the potential secretion of a subset of MPs, cases of alternative splicing and gene duplication, an expansion of the number of MPs in *Phytophthora* spp. as compared to other Stramenopiles and MPs with unique domain architecture. Furthermore, expression profiling and clustering revealed several MPs with increased expression during host infection. Together, these data provide a foundation for further studies on the biochemistry and function of these enzymes in *Phytophthora* and in oomycetes in general.

Materials & Methods

Data sources and MPs sequence identification

Genomic and protein sequences were used from various oomycetes, Stramenopiles and fungi, retrieved from public genome repositories (Table S2). Genes annotated to encode metalloproteases were retrieved using an initial word search with the term 'metalloprotease' that was performed in the *P. infestans* database at the time at The Broad institute website, currently hosted at NCBI (bioproject 17665). Obtained sequences were aligned to the *P. infestans* transcript sequences (Haas *et al*., 2009) using blastn (https://blast.ncbi.nlm.nih.gov/) at default settings. Resulting hits with significant alignment scores (> 80% identity), were retrieved and aligned to the *P. infestans* T30-4 genome sequence (Haas *et al*., 2009) to identify any unannotated genes. To optimize the sensitivity of the alignments, gene models in the *P. infestans* genome annotation were manually corrected based on RNA-Seq coverage. Additionally, representative MP protein sequences from all families were retrieved from the *MEROPS* database (release 9.12) (Rawlings *et al*., 2014) and were aligned against *P. infestans* genome sequence using tblastx, default settings. Family classification of MPs was based on the *MEROPS* classification of metalloproteases (Fig. 1). Identified gene models were further characterized and, when necessary, the gene models were corrected based on RNA-Seq data from *P. infestans* strain T20-2. In case of insufficient read coverage,

gene models were corrected based on homologous gene sequences of *P. sojae* and *P. ramorum* retrieved from JGI database (Table S2). The 5' and 3' intergenic distances were determined in base pairs (bp) between every consecutive pair of genes annotated in the reference genome *P. infestans* T30-4, using the method as described by (Haas *et al*., 2009).

Identification of MP homologs in Stramenopiles

To identify MP homologs, the *P. infestans* MP sequences were blasted against the proteome of six oomycetes, three diatoms and a brown algae species belonging to Stramenopiles and three fungi as outgroup species (Table S2). An E-value of 10^{-5} and a 60% query coverage cut-off were maintained.

MP protein domain prediction

Protein domain composition was determined via PFAM searches (v.27.0) (Finn *et al*., 2014), and using the web tools SMART (v.7.0) (Letunic *et al.*, 2015) and InterProScan 5 (Jones *et al.*, 2014). Transmembrane domains were predicted by TMHMM (v.2.0) (Krogh *et al.*, 2001), HMMTOP (v.2.0) (Tusnady & Simon, 2001) and SOSUI (v.1.11) (Hirokawa *et al.*, 1998). Signal peptides were predicted by SignalP (v.3.0) (Bendtsen *et al.*, 2004), using Neural Networks and Hidden Markov Models. Prediction of signal peptides was performed on the first 70 amino acids at the N-terminus of each protein sequence, using a threshold of 0.7. Prediction of signal anchors was accepted with the same threshold.

To identify MPs with unique domain architecture, blastp searches were performed against the NCBI non-redundant database. Protein sequences with an alignment score above 50% identity were selected for in-depth domain composition analysis.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignments of MP domains were constructed using ClustalOmega (v.2.1) with the default settings (Sievers *et al.*, 2011). Maximum likelihood phylogenetic trees were created using MEGA6 (Tamura *et al.*, 2013) and the robustness of the trees was assessed using 1000 bootstrap replicates.

RNA sequencing

P. infestans strain T20-2 was grown on rye sucrose medium at 18°C and 10-days old mycelium was collected for RNA extraction. Sporangia were isolated from 10-days old mycelium growing on rye agar medium flooded with 10 ml water. To isolate zoospores, 10-days old mycelium was flooded with ice-cold water for 3 hours. Zoospores were

collected after filtration with a 50 µm mesh. Germinating cysts were obtained from collected zoospores that were left to germinate at room temperature for 3 hours. RNA was extracted using NucleoSpin RNA II extraction kit (Macherey-Nagel) according to manufacturer's instructions. RNA sequencing was performed using an Illumina HiSeq™ 2000, producing 90 bp paired-end reads. FastQC v11.5 was used to check the quality of the reads, and fastx_trimmer from the fastx-toolkit v0.0.14 was used to trim off. the first 14 nucleotides. Reads were aligned to the *P. infestans* reference genome using Tophat v.2.1.0 (Haas *et al*., 2009, Langmead *et al.*, 2009). All settings were left default, except for the minimal intron size, which was set to 100 bp.

Gene expression profiling

The relative expression per transcript was quantified using Kallisto (v0.42.4) (Bray *et al.*, 2016). Relative transcript expression was expressed in Transcripts Per Million (TPM) units. The TPM values for each transcript were z-score transformed. The normalized TPM values were clustered hierarchically using correlation distances and average linkage. The dendrogram was cut at a height of 1.75 to divide the transcripts into eight appropriate clusters, determined by the elbow method.

Authors' contributions

CS, KB and FG designed the research. CS, SR, MFS and HJGM performed the analyses. CS and KB integrated the data. CS, KB and FG wrote the manuscript. All authors read and approved the manuscript.

Aknowledgements

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References

Aravind, L. and Koonin, E.V. (1999) Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches. *J Mol Biol,* **287,** 1023-1040.

Barrett, A.J. and Rawlings, N.D. (2007) 'Species' of peptidases. In: *Biol Chem* pp. 1151.

Bellincampi, D., Cervone, F. and Lionetti, V. (2014) Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Front Plant Sci,* **5,** 228.

Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol,* **340,** 783–795.

Benitez, J.A. and Silva, A.J. (2016) *Vibrio cholerae* hemagglutinin(HA)/protease: An extracellular metalloprotease with multiple pathogenic activities. *Toxicon,* **115,** 55-62.

Bernstein, H.G., Stricker, R., Dobrowolny, H., Steiner, J., Bogerts, B., Trübner, K. and Reiser, G. (2013) Nardilysin in human brain diseases: both friend and foe. *Amino Acids,* **45,** 269-278.

Betz, R., Walter, S. and Requena, N.C. (2016) Alternative splicing – an elegant way to diversify the function of repeat-containing effector proteins? *New Phytol,* **212,** 306-309.

Blair, J.E., Coffey, M.D., Park, S.-Y., Geiser, D.M. and Kang, S. (2008) A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genet Biol,* **45,** 266-277.

Bray, N.L., Pimentel, H., Melsted, P. and Pachter, L. (2016) Near-optimal probabilistic RNA-seq quantification. *Nat biotechnol* **34,** 525-527.

Burkhardt, A., Buchanan, A., Cumbie, J.S., Savory, E.A., Chang, J. and Day, B. (2014) Alternative splicing in the obligate biotrophic oomycete pathogen, *Pseudoperonospora cubensis*. *Mol Plant Microbe Interact,* **22,** 298-309.

Coleman, J.L.J., Ngo, T., Schmidt, J., Mrad, N., Liew, C.K., Jones, N.M., Graham, R.M. and Smith, N.J. (2016) Metalloprotease cleavage of the N terminus of the orphan G protein–coupled receptor GPR37L1 reduces its constitutive activity. *Sci Signal,* **9,** ra36.

Edwards, D.R., Handsley, M.M. and Pennington, C.J. (2008) The ADAM metalloproteinases. *Mol Aspects Med,* **29,** 258-289.

Fernandez, D., Russi, S., Vendrell, J., Monod, M. and Pallares, I. (2013) A functional and structural study of the major metalloprotease secreted by the pathogenic fungus *Aspergillus fumigatus*. *Acta Crystallogr D,* **69,** 1946-1957.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y. and Eddy, S.R. (2014) Pfam: the protein families database. *Nucleic Acids Res,* **42,** 222-230.

Gomez, M.A., Contreras, I., Halle, M., Tremblay, M.L., McMaster, R.W. and Olivier, M. (2009) *Leishmania* GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. *Sci Signal,* **2,** 1-12.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T. *et al*. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature,* **461,** 393 - 398.

Hallé, M., Gomez, M.A., Stuible, M., Shimizu, H., McMaster, W.R., Olivier, M. and Tremblay, M.L. (2009) The *Leishmania* surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. *J Biol Chem,* **284,** 6893-6908.

Hartmann, M., Herrlich, A. and Herrlich, P. (2013) Who decides when to cleave an ectodomain? *Trends Biochem Sci,* **38,** 111-120.

Haverkort, A.J., Boonekamp, P.M., Hutten, R., Jacobsen, E., Lotz, L.A.P., Kessel, G.J.T., Visser, R.G.F. and Van der Vossen, E.A.G. (2008) Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. *Potato Res,* **51,** 47-57.

Hayashida, K., Bartlett, A.H., Chen, Y. and Park, P.W. (2010) Molecular and cellular mechanisms of ectodomain shedding. *Anat Rec,* **293,** 925-937.

Higashiyama, S., Nanba, D., Nakayama, H., Inoue, H. and Fukuda, S. (2011) Ectodomain shedding and remnant peptide signalling of EGFRs and their ligands. *J Biochem,* **150,** 15-22.

Hirokawa, T., Boon-Chieng, S. and Mitaku, S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics,* **14,** 378-379.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J,* **19,** 4004-4014.

Jia, Y., Zhou, E., Lee, S. and Bianco, T. (2016)

Coevolutionary dynamics of rice blast resistance gene *Pi-ta* and *Magnaporthe oryzae* avirulence gene *AVR-Pita 1*. *Phytopathology,* **106,** 676-683.

Jiang, R.H.Y., de Bruijn, I., Haas, B.J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Bulone, V., Díaz-Moreno, S.M., Dumas, B., Fan, L., Gaulin, E., Govers, F., Grenville-Briggs, L.J., Horner, N.R., Levin, J.Z., Mammella, M., Meijer, H.J.G., Morris, P., Nusbaum, C., Oome, S., Phillips, A.J., van Rooyen, D., Rzeszutek, E., Saraiva, M., Secombes, C.J., Seidl, M.F., Snel, B., Stassen, J.H.M., Sykes, S., Tripathy, S., van den Berg, H., Vega-Arreguin, J.C., Wawra, S., Young, S.K., Zeng, Q., Dieguez-Uribeondo, J., Russ, C., Tyler, B.M. and van West, P. (2013) Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. *PLoS Genet,* **9,** e1003272.

Jiang, R.H.Y., Tripathy, S., Govers, F. and Tyler, B.M. (2008) RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc Natl Acad Sci,* **105,** 4874-4879.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.-Y., Lopez, R. and Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics,* **30,** 1236-1240.

Joshi, P.B., Kelly, B.L., Kamhawi, S., Sacks, D.L. and McMaster, W.R. (2002) Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. *Mol Biochem Parasitol,* **120,** 33-40.

Judelson, H.S. (2012) Dynamics and innovations within oomycete genomes: insights into biology, pathology, and evolution. *Eukaryot Cell,* **11,** 1304–1312.

Kamoun, S., Furzer, O., Jones, J.D., Judelson, H.S., Ali, G.S., Dalio, R.J., Roy, S.G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B.M., Grünwald, N.J., Mukhtar, M.S., Tomé, D.F.A., Tör, M., Van Den Ackerveken, G., McDowell, J., Daayf, F., Fry, W.E., Lindqvist-Kreuze, H., Meijer, H.J.G., Petre, B., Ristaino, J., Yoshida, K., Birch, P.R.J. and Govers, F. (2015) The top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol,* **16,** 413-434.

Kay, J., Meijer, H.J.G., ten Have, A. and van Kan, J.A.L. (2011) The aspartic proteinase family of three *Phytophthora* species. *BMC Genomics,* **12**.

Kaye, P. and Scott, P. (2011) Leishmaniasis: Complexity at the host-pathogen interface. *Nat Rev Microbiol,* **9,** 604-615.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol,* **305,** 1-14.

Kroon, L.P.N.M., Brouwer, H., de Cock, A.W.A.M. and Govers, F. (2011) The Genus *Phytophthora* Anno 2012. *Phytopathology,* **102,** 348-364.

Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol,* **10,** R25.

Letunic, I., Doerks, T. and Bork, P. (2015) SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res,* **43,** 257-260.

Li, J. and Zhang, K.-Q. (2014) Independent expansion of zincin metalloproteinases in *Onygenales* fungi may be associated with their pathogenicity. *PLoS ONE,* **9,** e90225.

Mansfeld, J.C.J.P. and Andrew, P.M.E. (2007) *Metalloproteases*.

McGwire, B.S., Chang, K.-P. and Engman, D.M. (2003) Migration through the extracellular matrix by the parasitic protozoan *Leishmania* is enhanced by surface metalloprotease gp63. *Infect Immun,* **71,** 1008-1010.

Meijer, H.J.G. and Govers, F. (2006) Genomewide analysis of phospholipid signaling genes in *Phytophthora* spp.: Novelties and a missing link. *Mol Plant Microbe Interact,* **19,** 1337-1347.

Meijer, H.J.G., Mancuso, F.M., Espadas, G., Seidl, M.F., Chiva, C., Govers, F. and Sabido, E. (2014) Profiling the secretome and extracellular proteome of the potato late blight pathogen *Phytophthora infestans*. *Mol Cell Proteomics,* **13,** 2101-2113.

Miller, M.A., Sullivan, R.J. and Lauffenburger, D.A. (2017) Molecular pathways: receptor ectodomain shedding in treatment, resistance, and monitoring of cancer. *Clin Cancer Res,* **23,** 623-629.

Miyoshi, S.I. and Shinoda, S. (2000) Microbial metalloproteases and pathogenesis. *Microbes Infect,* **2,** 91-98.

Monod, M., Capoccia, S., Léchenne, B., Zaugg, C., Holdom, M. and Jousson, O. (2002) Secreted proteases from pathogenic fungi. *International*

Journal of Medical Microbiology, **292,** 405-419.

Naumann, T.A., Wicklow, D.T. and Price, N.P.J. (2011) Identification of a chitinase-modifying protein from *Fusarium verticillioides*: truncation of a host resistance protein by a fungalysin metalloprotease. *J Biol Chem,* **286,** 35358-35366.

Orbach, M.J., Farrall, L., Sweigard, J.A., Chumley, F.G. and Valent, B. (2000) A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. *Plant Cell,* **12,** 2019-2032.

Peng, D., Lin, J., Huang, Q., Zheng, W., Liu, G., Zheng, J., Zhu, L. and Sun, M. (2016) A novel metalloproteinase virulence factor is involved in *Bacillus thuringiensis* pathogenesis in nematodes and insects. *Environ Microbiol,* **18,** 846-862.

Rawlings, N.D. and Barrett, A.J. (1995) Evolutionary families of metallopeptidases. In: *Methods in Enzymology*. (Barrett Alan, J., ed.). Academic Press, pp. 183-228.

Rawlings, N.D., Barrett, A.J. and Finn, R. (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res,* **44,** D343-D350.

Rawlings, N.D. and Morton, F.R. (2008) The MEROPS batch BLAST: a tool to detect peptidases and their non-peptidase homologues in a genome. *Biochimie,* **90,** 243-259.

Rawlings, N.D., Waller, M., Barrett, A.J. and Bateman, A. (2014) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res,* **42,** D503-509.

Runge, F., Telle, S., Ploch, S., Savory, E., Day, B., Sharma, R. and Thines, M. (2011) The inclusion of downy mildews in a multi-locus-dataset and its reanalysis reveals a high degree of paraphyly in *Phytophthora*. *IMA Fungus,* **2,** 163-171.

Sanderson, M.P., Dempsey, P.J. and Dunbar, A.J. (2006) Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. *Growth Factors,* **24,** 121-136.

Sanz-Martín, J.M., Pacheco-Arjona, J.R., Bello-Rico, V., Vargas, W.A., Monod, M., Díaz-Mínguez, J.M., Thon, M.R. and Sukno, S.A. (2016) A highly conserved metalloprotease effector enhances virulence in the maize anthracnose fungus *Colletotrichum graminicola*. *Mol Plant Pathol,* **17,** 1048-1062.

Schoina, C. and Govers, F. (2015) The oomycete *Phytophthora infestans*, the Irish potato famine pathogen. In: *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture.* (Lugtenberg, B., ed.). Springer International Publishing, pp. 371-378.

Seidl, M.F., Van den Ackerveken, G., Govers, F. and Snel, B. (2011) A domain-centric analysis of oomycete plant pathogen genomes reveals unique protein organization. *Plant Physiol,* **155,** 628-644.

Seidl, M.F., Van den Ackerveken, G., Govers, F. and Snel, B. (2012) Reconstruction of oomycete genome evolution identifies differences in evolutionary trajectories leading to present-day large gene families. *Genome Biol Evol,* **4,** 199-211.

Semenova, S.A. and Rudenskaya, G.N. (2009) The astacin family of metalloproteinases. **3,** 17-32.

Sêton Bocco, S. and Csűrös, M. (2016) Splice sites seldom slide: Intron evolution in oomycetes. *Genome Biol Evol,* **8,** 2340-2350.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D. and Higgins, D.G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol,* **7,** 539.

Stepek, G., McCormack, G., Winter, A.D. and Page, A.P. (2015) A highly conserved, inhibitable astacin metalloprotease from T*eladorsagia circumcincta* is required for cuticle formation and nematode development. *Int J Parasitol,* **45,** 345-355.

Tallant, C., Marrero, A. and Gomis-Rüth, F.X. (2010) Matrix metalloproteinases: Fold and function of their catalytic domains. *BBA-Mol Cell Res,* **1803,** 20-28.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution,* **30,** 2725-2729.

Tusnady, G.E. and Simon, I. (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics,* **17,** 849-850.

Tyler, B.M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R.H.Y., Aerts, A., Arredondo, F.D., Baxter, L., Bensasson, D. and Beynon, J.L. (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science,* **313,** 1261 - 1266.

Whisson, S.C., Boevink, P.C., Wang, S. and Birch, P.R.J. (2016) The cell biology of late blight disease. *Curr Opin Microbiol,* **34,** 127-135.

Yao, C., Donelson, J.E. and Wilson, M.E. (2003) The major surface protease (MSP or GP63) of *Leishmania* sp. Biosynthesis, regulation of expression, and function. *Mol Biochem Parasitol,* **132,** 1-16.

Zhang, Y., Bak, D.D., Heid, H. and Geider, K. (1999) Molecular characterization of a protease secreted by *Erwinia amylovora*. *J Mol Biol,* **289,** 1239-1251.

Supplemental data

Table S1. Characteristics of the identified *Phytophthora infestans* MP genes.

Table S1A. Identified P. infestans MPs. Domain architecture analysis, presence of domain architecture in other species and protein sequences.

Clan	Family	Gene model	Domain composition**	SP[a]	TM[b]	Fungi	Stramenopiles	Mammals	Plants
MA	M1	PITG_21284[#]	M1 (14-231)	-/-	-/1/-	+	+	+	+
		PITG_22346[*]	M1 (12-206)	-/-	-/-/-	+	+	+	+
		PITG_18882[*]	M1 (12-262) - SCOP:d1gw5a[1,3] (501-652)	-/-	-/1/-		+	+	+
		PITG_14044	SCOP:d1hs6a2[1] (28-86) - M1 (131-475) - SCOP:d1gw5a[1,3] (714-1102)	-/-	-/-/-		+	+	+
		PITG_01616[*]	M1 (1-243) - M1[1,2] (312-483) - DUF3458 (558-932)	-/-	-/-/-		+		+
			M1 - MA_2[1,2] - DUF3458	-/-	-/-/-		+		+
		PITG_01619	M1 (16-257) - M1[1,2] (339-510) - DUF3458 (585-959)	-/-	-/-/-		+		+
			M1 - MA_2[1,2] - DUF3458	-/-	-/-/-		+		+
		PITG_02917	M1 (7-413) - ERAP1_C (553-863)	-/-	-/-/-	+	+	+	+
			MA_2[1] - ERAP1_C	-/-	-/-/-	+	+	+	+
		PITG_08804[*]	M1 (10-414) - ERAP1_C (563-876)	-/-	-/-/-	+	+	+	+
			MA_2[1] - ERAP1_C	-/-	-/-/-	+	+	+	+
		PITG_03837	M1 (14-402) - Leuk-A4-hydro (486-634)	-/-	-/-/-	+	+	+	+
			MA_2[1] - Leuk-A4-hydro	-/-	-/-/-	+	+	+	+
		PITG_15392	M1 (14-389) - Leuk-A4-hydro (466-615) - TM (648-667)	-/-	1/1/1	+	+	+	+
			MA_2[1] - Leuk-A4-hydro - TM	-/-	1/1/1	+	+	+	+
		PITG_15413[*]	M1 (17-391) - Leuk-A4-hydro (467-619) - TM (652-674)	-/-	1/1/-	+	+	+	+
			MA_2[1] - Leuk-A4-hydro - TM	-/-	1/1/-	+	+	+	+
		PITG_21860[*]	M1 (1-219) - Leuk-A4-hydro (295-447) - TM (479-501)	-/-	1/1/-	+	+	+	+
			MA_2[1] - Leuk-A4-hydro - TM	-/-	1/1/-	+	+	+	+
	M3	PITG_02711	SP (1-32) - M3 (264-725)	-/+	-/2/-	+	+	+	+
		PITG_08599	SP (1-28) - M3 (247-700)	+/+	-/-/-	+	+	+	+
		PITG_08197	POLXc[1] (47-136) - M3 (253-704)	-/+	1/-/1	+	+	+	+

MA	M8	PITG_09118*	SP (1-22) - M8 (146-289) - M8 (299-612)	+/+	-/-/-		+	+	+
		PITG_09119*	SP (1-26) - M8 (169-301) - M8 (307-618)	+/+	-/-/-		+	+	+
		PITG_13513#	M8 (1-142)	-/-	-/-/-		+	+	+
		PITG_14228*	SP (1-21) - M8 (160-269) - M8 (294-602)	+/+	-/-/2		+	+	+
		PITG_08774*	SA (1-51) - M8 (62-610) - EGF[1] (637-673) - EGF[1] (676-714) - EGF_like (718-749) - EGF_like (893-925) - EGF_like[1] (948-1000)	sa	-/1/-		+		+
			SA (1-51) - EGF1 (602-634) - EGF[1] (637-673) - EGF[1] (676-714) - EGF_like (718-749) - EGF_like (893-925) - EGF_like[1] (948-1000)	sa	-/1/-		+		+
	M12	PITG_09824	SP (1-17) - Astacin (94-240)	+/+	-/-/-	+	+		
		PITG_09851*	SP (1-21) - Astacin (138-297)	+/+	-/-/-	+	+		
		PITG_13752	SP (1-25) - Astacin (590-740) - TM (861-883)	+/+	1/-/-	+	+		
	M13	PITG_07643*	SP (1-22) - M13_N (39-402)	+/+	-/-/-		+		+
		PITG_09870	SP (1-20) - M13_N (38-415) - M13 (471-676)	+/+	-/-/-	+	+	+	+
		PITG_10317	SP (56-78) - M13_N (115-491) - M13 (544-772)	sa	-/1/-	+	+	+	+
		PITG_18701*	SA (1-22) - M13_N (39-402) - M13 (457-607)	+/+	-/-/-	+	+	+	+
		PITG_08425	TM (1-41) - M13_N (89-459) - M13 (510-651) - SCOP:d1fgja[1] (905-1120) - M13 (1134-1222)	-/-	1/1/1		+		
	M22	PITG_05594*	M22 (37-315) / Gcp-like domain	-/-	-/-/-	+	+	+	+
		PITG_06894	M22 (41-343) / Gcp-like domain	-/-	-/-/-	+	+	+	+
	M41	PITG_01087	AAA (253-389) - M41 (447-646) - TM (177-199)	-/-	-/-/1	+	+	+	+
		PITG_10147	FtsH_ext (158-315) - AAA (415-555) - M41 (615-817) - TM (327-348)	-/-	-/2/1	+	+	+	+
	M48	PITG_02177	M48 (122-317)	-/-	-/1/-	+	+	+	+
		PITG_06937	M48 (75-279)	-/-	-/3/-	+	+	+	+
		PITG_11607	SP (1-30) - M48 (1-119)	+/-	-/1/-	+	+	+	+
		PITG_12517	SA (1-39) - M48 (48-268) - M48 (271-481)	sa	7/7/5	+	+	+	+
			SA (1-39) - M48 (48-268) - 6TM (133-155, 170-192, 213-235, 240-262, 360-382, 392-414)	sa	7/7/5	+	+	+	+
		PITG_13164	SA (1-39) - M48 (80-253)	sa	-/-/-	+	+	+	+
	M76	PITG_08420*	M76 (9-186)	-/-	-/-/-	+	+	+	+
		PITG_18055	M76 (23-193)	-/-	-/-/-	+	+	+	+
	M80	PITG_13744*	WLM (4-208)	-/-	-/-/-	+	+	+	+
		PITG_15925*	SCOP:d1euvb[1] (6-53) - WLM (124-360) - PUG (395-456)	-/-	-/-/-		+		+

Table S1A. *Continued*

Clan	Family	Gene model	Domain composition**	SP[a]	TM[b]	Fungi	Stramenopiles	Mammals	Plants
MC	M14	PITG_00756	SP (1-21) - M14 (182-463)	+/+	-/1/1	+	+	+	+
		PITG_06195*	M14 (308-466)	-/-	-/-/-	+	+	+	+
		PITG_06850*	SP (1-16) - M14 (80-303)	+/+	-/1/-	+	+	+	+
		PITG_09251*	M14 (229-519) - TM (758-780)	-/-	1/1/1	+	+	+	+
		PITG_11126*	SP (1-23) - M14 (81-224) - TM (740-768)	+/+	1/2/2	+	+	+	+
		PITG_13564#	SP (1-20) - M14 (78-277)	+/+	1/1/1	+	+	+	+
		PITG_23044*	M14 (272-539)	-/-	-/2/-	+	+	+	+
		PITG_05548*	Armadillo-like helical[3] (6-429) - M14 (1063-1317)	-/-	-/2/-	+	+	+	
ME	M16	PITG_00203	M16 (46-193) - M16_C (198-381)	-/-	-/-/-	+	+	+	+
		PITG_04726*	M16 (60-207) - M16_C (211-370)	-/-	-/-/-	+	+	+	+
		PITG_20378*	M16 (99-247) - M16_C (252-446)	-/-	-/1/-	+	+	+	+
		PITG_08991	M16 (30-168) - M16_C (193-375) - M16_M (379-672) - M16_C (675-867)	-/-	-/-/-	+	+	+	+
		PITG_17056*	M16 (33-171) - M16_C (196-376) - M16_M (382-668) - M16_C (672-856)	-/-	-/-/-	+	+	+	+
		PITG_01569*	M16 (84-217) - M16_C[1,2] (243-431) - M16_M (436-722) - M16_C (726-911)	-/-	-/1/-	+	+	+	+
		PITG_16759*	M16 (78-218)- M16_C[1,2] (238-415) - M16_M (438-737) - M16_C[3] (820-1006)	-/-	-/-/-	+	+	+	+
		PITG_13437	M16 (97-208) - M16_C (245-445) - M16_assoc (518-773) - M16_C[3] (847-1022)	-/-	-/-/-	+	+	+	+
MF	M17	PITG_04838	M17_ N (56-162) - M17 (201-510)	-/-	-/-/-	+	+	+	+
		PITG_11569	M17 (209-523)	-/-	-/1/-	+	+	+	+
MG	M24	PITG_09504*	M24 (130-441)	-/-	-/1/-	+	+	+	+
		PITG_12211*	PFAM:zf-C6H2 (18-65) - M24 (140-368)	-/-	-/-/-	+	+	+	+
		PITG_12220	PFAM:zf-C6H2 (6-53) - M24 (125-353)	-/-	-/-/-	+	+	+	+
		PITG_13658*	M24 (57-284)	-/-	-/-/-	+	+	+	+
		PITG_13014	Winged helix-turn-helix DNA binding[3] (257-327) - M24 (36-313)	-/-	-/-/-	+	+	+	+
		PITG_01206	AMP_N (51-182) - M24 (219-458)	-/-	-/-/-	+	+	+	+
		PITG_07955*	SP (1-20) - AMP_N (84-224) - M24 (263-530)	+/+	-/2/-	+	+	+	+
		PITG_04875	Creatinase_N (33-173) - Creatinase_N[1] (200-333) - M24 (337-557) - M24_C (568-630)	-/-	-/-/-				

MG	M24		Creatinase_N (33-173) - Creatinase_N_2 (176-335) - M24 (337-557) - M24_C (568-630)	-/-	-/-/-			
		PITG_10266*	SCOP:d1xgsa2[1] (151-351) - Creatinase_N[3] (13-134) - M24[3] (153-353)	-/-	-/1/-	+	+	+	+
		PITG_08392	FACT-Spt16 Nlob (31-193) - Peptidase M24 (206-439) - SPT16 domain (572-727) - Rtt106 (848-938)	-/-	-/1/-	+	+	+	+
MH	M18	PITG_02114	M18 (20-449)	-/-	-/-/-	+	+	+	+
			M42[1] (239-443)	-/-	-/-/-	+	+	+	+
	M20	PITG_05866#	M20 (76-190)	-/-	-/-/-	+	+	+	+
		PITG_00028*	M20 (74-414)	-/-	-/-/-	+	+	+	+
			M20_dimer (187-317)	-/-	-/-/-	+	+	+	+
			M28 (59-266)	-/-	-/-/-	+	+	+	+
		PITG_00029	M20 (71-411)	-/-	-/-/-	+	+	+	+
			M20_dimer (184-315)	-/-	-/-/-	+	+	+	+
			M28 (55-241)	-/-	-/-/-	+	+	+	+
		PITG_02852*	M20 (73-404)	-/-	-/-/-	+	+	+	+
			M20_dimer (178-308)	-/-	-/-/-	+	+	+	+
			M28[1] (57-253)	-/-	-/-/-	+	+	+	+
		PITG_05858#	M20 (92-402)	-/-	-/-/-	+	+	+	+
			M20_dimer (200-329)	-/-	-/-/-	+	+	+	+
		PITG_05861*	M20 (90-431)	-/-	-/-/-	+	+	+	+
			M20_dimer (199-322)	-/-	-/-/-	+	+	+	+
		PITG_00577*	Amidohydro 1 (95-482) - M20 (592-859)	-/-	-/-/-		+		
			Amidohydro 1 (95-482) - M20_dimer (685-788)	-/-	-/-/-		+		
		PITG_06978*	CENP-B_N (27-74) - PDB[1] (187-282) - M20 (346-653)	-/-	-/-/-		+		
			CENP-B_N (27-74) - PDB[1] (187-282) - M20_dimer (451-552)	-/-	-/-/-		+		
	M28	PITG_10813*	SA (43-59) - M28 (159-354) - 8TM (393-412, 425-447, 457-479, 495-517, 521-539, 552-577, 592-614, 621-640)	sa	9/9/8	+	+	+	+
		PITG_00273	TM (68-90) - PA (221-310) - M28 (436-590) - TFR_dimer (749-863)	-/-	1/3/1	+	+	+	+
		PITG_00289	TM (47-66) - PA (206-344) - M28 (390-612) - TFR_dimer (658-776)	-/-	1/1/1	+	+	+	+
		PITG_12818*	SA (1-16) - PA (235-325) - M28 (420-640) - TFR_dimer (691-814)	sa	-/1/1	+	+	+	+
		PITG_12824*	SA (1-50) - PA (182-281) - M28 (366-586) - TFR_dimer (641-755)	sa	-/1/1	+	+	+	+
		PITG_14835	SP (1-19) - Nicastrin (241-490) - TM (685-707)	+/+	1/1/2	+	+	+	+
			SP (1-19) - M28[1] (231-474) - TM (685-707)	+/+	1/1/2	+	+	+	+

Table S1A. *Continued*

Clan	Family	Gene model	Domain composition**	SP[a]	TM[b]	Fungi	Stramenopiles	Mammals	Plants
MJ	M38	PITG_00578	Amidohydro_4	-/-	-/-/-	+	+	+	+
			Amidohydro_1 (272-430)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1] - Amidohydro_3[1] (113-431)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1] - Amidohydro_3[1]	-/-	-/-/-	+	+	+	+
		PITG_03605*	SP (1-20) - Amidohydro_1 (62-413)	-/-	-/-/-	+	+	+	+
			SP - Amidohydro_4[1]	-/-	-/-/-	+	+	+	+
			SP (1-20) - Amidohydro_3[1] (177-405)	-/-	-/-/-	+	+	+	+
			SP - Amidohydro_5[1] - Amidohydro_4[1]	-/-	-/-/-	+	+	+	+
			SP - Amidohydro_5[1] - Amidohydro_3[1]	-/-	-/-/-	+	+	+	+
		PITG_05263	Amidohydro_1 (62-447)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1]	-/-	-/-/-	+	+	+	+
		PITG_06129*	Amidohydro_3 (48-519)	-/-	-/-/-	+	+	+	+
			Amidohydro_1[1] (296-518)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1]	-/-	-/-/-	+	+	+	+
		PITG_07820	Amidohydro_1 (66-438)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1]	-/-	-/-/-	+	+	+	+
			Amidohydro_3[1] (109-439)	-/-	-/-/-	+	+	+	+
		PITG_08142	Amidohydro_1 (75-438)	-/-	-/-/-	+	+	+	+
			Amidohydro_4[1]	-/-	-/-/-	+	+	+	+
			Amidohydro_3[1] (123-436)	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1] - Amidohydro_4[1]	-/-	-/-/-	+	+	+	+
			Amidohydro_5[1] - Amidohydro_3[1]	-/-	-/-/-	+	+	+	+
		PITG_15694	Amidohydro_1 (16-320)	-/-	-/-/-	+	+	+	+
			Amidohydro_2[1] (73-209)	-/-	-/-/-	+	+	+	+
		PITG_00636	Urease γ[1,2] (1-99) - Urease β[1,2] (134-231) - Urease α (271-391) - Amidohydro_1 (397-730)	-/-	-/1/-	+	+		+
			Urease γ[1,2] - Urease β[1,2] - Amidohydro_1	-/-	-/1/-	+	+		+
			Urease γ[1,2] - Urease β[1,2] - Amidohydro_5[1]	-/-	-/1/-	+	+		+

MP	M67	PITG_03561	JAB_MPN (41-205)	-/-	-/-/-	+	+		+
		PITG_20689	JAB_MPN (54-185)	-/-	-/1/-	+	+		+
		PITG_03459	JAB_MPN (32-173) - MitMem_reg (194-307)	-/-	-/-/-	+	+	+	+
		PITG_08676	JAB_MPN (32-167) - MitMem_reg (175-308)	-/-	-/-/-	+	+	+	+
		PITG_09289	JAB_MPN (13-144) - MitMem_reg (166-277)	-/-	-/-/-	+	+	+	+
		PITG_11063	JAB_MPN (13-147) - MitMem_reg (170-281)	-/-	-/1/-	+	+	+	+
		PITG_16722*	PWWP (36-122) - JAB_MPN (503-638)	-/-	-/-/-		+		
		PITG_05600*	USP8 dimer (32-139) - JAB_MPN (276-407)	-/-	-/2/-	+	+	+	+
		PITG_10969*	PRO8NT (73-224) - PROCN (415-821) - RRM_4 (1006-1097) - U5_2-snRNA_bdg (1230-1363) - U6_2-snRNA _bdg (1463-1621) - PRP8 domain IV (1781-2010) - JAB-MNP (2120-2254) - PROCT[2,3] (2233-2353)	-/-	-/3/1	+	+	+	+
U	M79	PITG_03965	3TM (115-137, 149-171, 209-231) - Abi domain (255-406)- TM (434-456)	-/-	9/9/7		+		
			3TM (115-137, 149-171, 209-231) - 6TM (252-271, 291-313, 320-342, 362-384, 397-419, 434-456)	-/-	9/9/7		+		
		PITG_04001	SP (1-18)- 2TM (43-65, 85-107) - Abi domain (3-246) - 2TM (206-228, 260-282[1])	+/+	5/5/3		+		
		PITG_17962*	SP (1-24) - 2TM (39-56, 68-90) - Abi domain (114-219) - 3TM (153-170, 190-212, 232-251[1])	+/+	5/5/6		+		

*: Gene models corrected and verified based on RNAseq data

#: pseudogene

**: Protein domains present in MPs predicted by 1: SMART, 2: PFAM, 3: InterproScan5, when no indication, gene models were predicted by all three softwares/databases

[a] : Signal peptide prediction by SignalP 3.0 based on neural networks (NN)/ Hidden Markov motifs (HMM) by ; +: predicted, -: not predicted, sa: signal anchor

[b] : Transmembrane domain prediction based on TMHMM / HMMtop / SOSUI. , -: not predicted, 1-9: number of predicted TMs

Respective domain positions are idicated within brackets

Table S1B. Corrected gene models. Corrections that have been made in the MP gene sequences

Clan	Family	Gene model	Wrong start	Wrong stop	Intron removed (nr if >1)	Intron introduction (Nr if >1)	Split model	Merge models	Wrong splice donor	Wrong splice acceptor	Insertion (bp)
MA	M1	PITG_21284 #									
		PITG_22346*		X	X						
		PITG_18882*			4		into 3				
		PITG_01616*			X						X
		PITG_08804*	X								
		PITG_15413*			X						
		PITG_21860*			X						
	M8	PITG_09118*	X	X	X						
		PITG_09119*	X	X	X						
		PITG_13513#									
		PITG_14228*	X	X	X						
		PITG_08774*	X			X					
		PITG_09851*			X						
	M13	PITG_07643*		X	2						
		PITG_18701*	dispute								
	M22	PITG_05594*					X				
	M76	PITG_08420*		X					X		
	M80	PITG_13744*		X							
		PITG_15925*			X						
MC	M14	PITG_06195*			3						
		PITG_06850*	X		2						
		PITG_09251*	dispute								
		PITG_11126*	X								
		PITG_13564#									
		PITG_23044*						merge with PITG_23043			
		PITG_05548*			X	2		X			

Clan	Family	Gene model	Wrong start	Wrong stop	Intron removed (nr if >1)	Intron introduction (Nr if >1)	Split model	Merge models	Wrong splice donor	Wrong splice acceptor	Insertion (bp)
ME	M16	PITG_04726*					X				
		PITG_20378*			X						
		PITG_17056*			X			X			
		PITG_01569*	X		X		X				
		PITG_16759*			X						
MG	M24	PITG_09504*			X						
		PITG_12211*	X								
		PITG_13658*				X					
		PITG_07955*	X		2						
		PITG_10266*			2						
	M20	PITG_05866#									
		PITG_00028*				X			X	X	
		PITG_02852*								X	
		PITG_05858#									
		PITG_05861*		X		X					
		PITG_00577*			X						
		PITG_06978*			3						
	M28	PITG_10813*			X						
		PITG_12818*	X								
		PITG_12824*	X		X						
	M38	PITG_03605*			2						X (frame shift)
		PITG_06129*			X						
	M67	PITG_16722*	X	X	X			X			
		PITG_05600*	X						X		
		PITG_10969*			X	X					
	M79	PITG_17962*			X						

Table S1C. MP genes with splice variants

Clan	Family	PITG	Splice variants
MA	M12	PITG_09851	2
	M22	PITG_05594	2
	M48	PITG_02177	2
	M76	PITG_08420	5*
MC	M14	PITG_05548	2
MG	M24	PITG_13658	3
	M24	PITG_04875	2
MH	M20	PITG_00029	2
	M20	PITG_02852	3
	M20	PITG_00577	4
	M28	PITG_14835	2
MJ	M38	PITG_07820	4
MP	M67	PITG_16722	2
	M67	PITG_10969	4*

*: Splice variants result in a different protein

Table S1D. MP genes that are indicative for gene duplication

Clan	Family	PITG	% Protein similarity	Orientation	Repeat sequences
MA	M1	PITG_01616	91	>	
		PITG_01619		>	upstream
MA	M8	PITG_09118	71.8	>	
		PITG_09119		>	
MH	M20	PITG_00028	67.4	>	
		PITG_00029		>	
MH	M20	PITG_05858#	71.7	>	up- and downstream
		PITG_05861		>	
MG	M24	PITG_02211	83.6	>	
		PITG_02220		>	

#: pseudogene

Table S1E. Features of several MP genes located in gene-sparse regions of *P. infestans* genome

Clan	Family	Gene	Genome organisation		Orientation		Protein features
			repeats	various			
MA	M1	PITG_02917	downstream		>		
	M1	PITG_18882	up- and downstream		<		
	M12	PITG_09851	downstream	splice variants	<	SP	
	M13	PITG_07643	up- and downstream		<	SP	unique architecture
	M13	PITG_18701	up- and downstream		<	SP	
	M41	PITG_01087	none		>		
	M48	PITG_02177	upstream	splice variants	>		
MC	M14	PITG_06195	up- and downstream		<		
	M14	PITG_13564[#]	up- and downstream		<	SP	
ME	M16	PITG_01569	up- and downstream	gene upstream	<		
	M16	PITG_04726	up- and downstream		<		
	M16	PITG_16759	upstream		<		
MG	M24	PITG_07955	up- and downstream		>	SP	
	M24	PITG_10266	up- and downstream		<		
MH	M20	PITG_05858[#]	up- and downstream	duplication	>		
	M28	PITG_12824	downstream		<	SA	
MJ	M38	PITG_07820	none	splice variants; gene upstream	<		
U	M79	PITG_03965	up- and downstream	gene downstream	>		

Table S1E. MPs that have a unique domain architecture

Clan	Family	PITG
MA	**M13**	PITG_07643
	M13	PITG_08425
	M80	PITG_13744
	M80	PITG_15925
MH	**M20**	PITG_00577
	M20	PITG_06978
MP	**M67**	PITG_16722

Table S2. Genomes used in this study.

Species	Version	Source	URL
Phytophthora infestans	V1.0	BROAD*	https://www.broadinstitute.org/scientific-community/data
Phytophthora ramorum	V1.1	JGI	http://genome.jgi.doe.gov/
Phytophthora sojae	V3.0	JGI	http://genome.jgi.doe.gov/
Hyaloperonospora arabidopsidis	V2.0	BROAD*	https://www.broadinstitute.org/scientific-community/data
Pythium ultimum	V4.0	BROAD*	https://www.broadinstitute.org/scientific-community/data
Saprolegnia parasitica	V2.0	BROAD*	https://www.broadinstitute.org/scientific-community/data
Thalassiosira pseudonana	V3.0	JGI	http://genome.jgi.doe.gov/
Phaeodactylum tricornutum	V2.0	JGI	http://genome.jgi.doe.gov/
Aureococcus anophagefferens	V1.0	JGI	http://genome.jgi.doe.gov
Ectocarpus siliculosus	V2.0	BEG	http://bioinformatics.psb.ugent.be
Fusarium graminearum	V1.0	BROAD*	https://www.broadinstitute.org/scientific-community/data
Saccharomyces serevisiae		BROAD*	https://www.broadinstitute.org/scientific-community/data
Ustilago maydis	V1.0	BROAD*	https://www.broadinstitute.org/scientific-community/data

*: currently hosted at NCBI (bioproject 17665)

Table S3: Number of MP genes in *Phytophthora* spp., Stramenopiles, diatoms and three fungal species, according to *MEROPS* database.

Clan	Families presence in *P. infestans*	Families absence in *P. infestans*	*Phytophthora infestans*	*Phytophthora sojae*	*Phytophthora ramorum*	*Hyaloperonospora arabidopsidis*	*Pythium ultimum*	*Saprolegnia parasitica*	*Thalassiosira pseudonana*	*Phaeodactylum tricornutum*	*Aureococcus anophagefferens*	*Ectocarpus siliculosus*	*Fusarium graminearum*	*Saccharomyces cerevisiae*	*Ustilago maydis*
MA	M1		7	6	7	5	8	5	4	3	3	5	4	5	2
	M3		3	3	3	4	3	8	5	4	4	3	4	2	1
		M4	-	-	-	-	-	-	-	-	-	-	3	-	-
		M6	-	-	-	-	-	-	2	4	-	-	1	-	-
	M8		4	5	1	-	1	3	9	2	-	4	-	-	-
		M10	-	-	-	-	-	-	-	-	1	1	4	-	2
		M11	-	-	-	-	-	-	-	5	-	-	-	-	-
	M12		3	3	3	2	3	6	1	-	1	-	4	-	2
	M13		5	3	3	10	8	10	1	1	1	-	1	-	1
		M32	-	-	-	-	-	-	1	1	1	1	-	-	-
		M35	-	-	-	-	-	-	-	-	-	-	1	-	-
		M36	-	-	-	-	-	-	-	-	-	-	1	-	-
	M41		2	2	2	3	2	2	4	5	7	5	2	3	1
		M43	-	-	-	-	-	-	2	4	-	1	2	-	3
	M48		2	1	1	1	3	2	4	2	3	3	2	2	2
		M49	-	-	-	-	-	-	-	-	-	-	2	1	-
		M54	-	1	1	-	-	1	-	-	-	-	1	-	-
		M72	-	-	-	-	-	-	-	-	-	5	-	-	-
	M76		2	2	3	2	2	2	2	2	1	1	1	1	1
	M80		1	1	-	-	-	-	1	2	-	2	3	1	1
MC	M14		8	11	5	3	12	14	4	2	7	5	9	1	1
ME	M16		11	10	8	6	12	10	12	9	11	14	7	16	6
MF	M17		2	2	1	2	2	1	2	3	6	3	-	-	1
MG	M24		8	8	-	-	7	-	8	6	9	8	10	6	5
MH	M18		1	1	1	-	1	1	2	2	2	1	2	2	1
	M20		7	4	3	3	7	10	2	2	6	5	17	4	1
	M28		4	8	-	-	7	-	-	1	1	2	17	12	5
MJ	M38		2	2	2	1	8	2	2	1	5	1	20	11	1
		M19	-	-	-	-	-	-	-	-	1	-	2	-	1
MM		M50	-	-	-	-	-	-	1	2	1	-	1	-	-
MO		M23	-	-	-	-	-	-	-	1	1	1	-	-	-
MP	M67		5	4	3	4	6	4	3	2	5	6	7	3	4
U	M79		2	2	1	2	2	2	2	3	3	5	1	1	1
		M82	-	-	-	-	-	1	-	-	-	-	-	-	-
	Sum		79	79	48	48	94	83	74	69	80	82	129	71	43

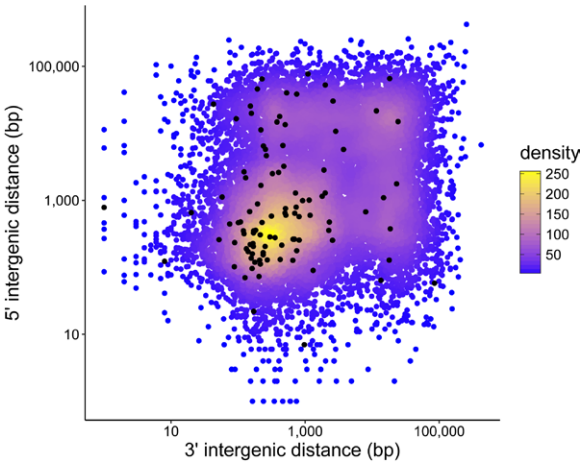

Figure S1. Intergenic distances at the 5′ and 3′ ends between each consecutive pair of *P. infestans* genes. MP genes are shown as black dots with one gene per dot. All other genes are shown by colored dots. The number of genes represented by each dot can be deduced from the heatmap on the right.

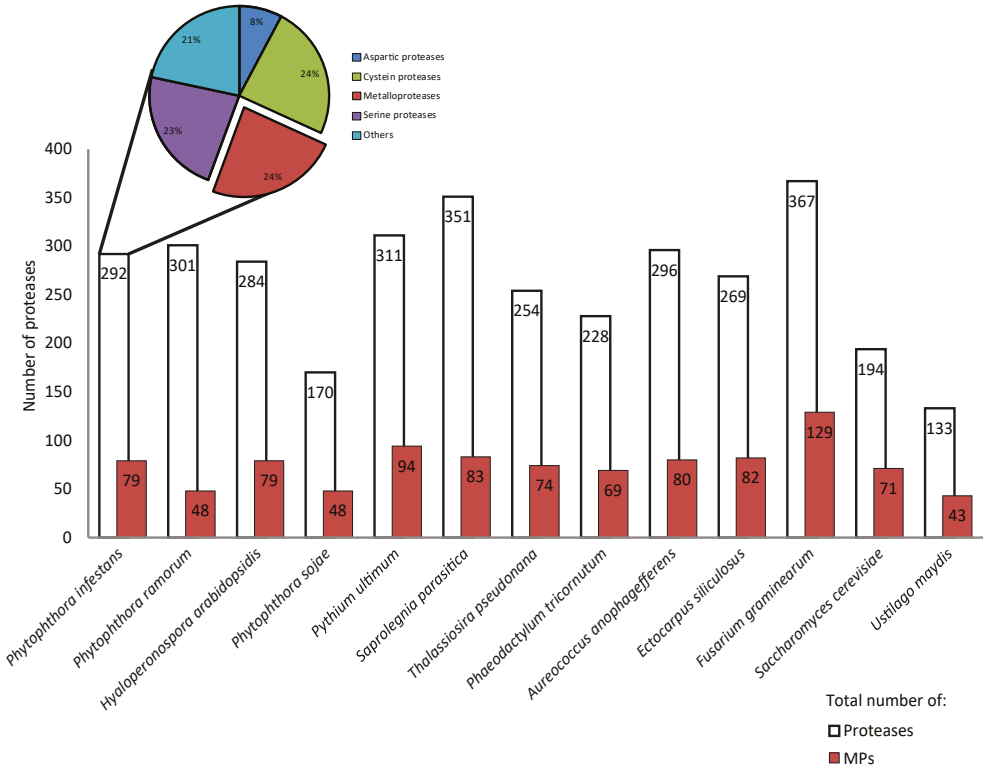

Figure S2. Total number of proteases and MPs indicated in the *MEROPS* database for the species mentioned in Figure 3. The pie chart shows the relative proportion (in percentages) of each class of proteases in *P. infestans*.

Chapter 3

Clade 5 aspartic proteases of *Phytophthora infestans* are virulence factors implied in RXLR effector cleavage

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3

Abstract

Late blight caused by the oomycete pathogen *Phytophthora infestans* is one of the most destructive diseases in potato cultivation. To successfully colonize its host, *P. infestans* secretes a suite of effector proteins that undermine plant immunity, many of which contain a conserved N-terminal RXLR motif that strongly resembles the host targeting motif in effectors of the malaria parasite *Plasmodium falciparum*. In this study, we focus on three *P. infestans* clade 5 aspartic protease (APs) that are homologous to Plasmepsin V (PMV), a *Pl. falciparum* AP responsible for cleaving effectors prior to translocation into red blood cells. Malaria parasites expressing mutated PMV are impaired in effector translocation and are less virulent. To determine whether clade 5 PiAPs play similar roles in virulence, we characterized *P. infestans* transformants with either reduced or enhanced *PiAP* expression levels.

P. infestans transformants with altered *PiAP10* or *PiAP12* expression were found to be impaired in mycelial growth and sporangia production, and were hampered in their virulence on potato leaves. Similar effects were not observed in PiAP11 transformants. Activity assays revealed that clade 5 PiAPs possess protease activity, including PiAP10 and PiAP12 that potentially cleave the RXLR effector PiAVR4, but not a version with a mutated RXLR motif. These findings imply that *P. infestans* APs function in the proteolytic cleavage of RXLR effectors.

Keywords: Late blight disease, enzymatic activity, pathogenicity, plant-pathogen interactions, proteolysis

Introduction

Plant pathogenic microorganisms - including bacteria, fungi and oomycetes - secrete effector proteins that suppress host immune responses to facilitate successful host colonization. The potato late blight pathogen *Phytophthora infestans* has an extended arsenal of effector proteins, of which the majority contains the conserved N-terminal RXLR (Arginine-x-Leucine-Arginine) peptide motif. These so-called RXLR effectors are considered to be translocated into host cells via specialized infection structures, such as haustoria. Multiple studies show that the RXLR motif is essential for the delivery of *P. infestans* effectors into host cells, and that mutation of the RXLR motif hampers effector translocation (Bozkurt *et al*., 2012, Whisson *et al*., 2007, Schornack *et al*., 2009). It was only recently shown that RXLR effectors indeed shuttle into host cells by visualizing an mRFP-tagged RXLR effector that accumulates in the plant cell nucleus (Wang *et al.*, 2017). The exact mechanism that supports translocation of RXLR effectors has, however, not been fully resolved.

The *Phytophthora* RXLR motif strongly resembles the host translocation motif RXLX$^{E/Q/D}$ of PEXEL effectors secreted by the malaria pathogen *Plasmodium falciparum*. Studies by Boddey *et al.* (2010) and Russo *et al.* (2010) showed that the N-terminal RXLX$^{E/Q/D}$ motif of *Plasmodium* PEXEL effectors is cleaved after the L residue by the aspartic protease Plasmepsin V (PMV) prior to host translocation into red blood cells. PMV was found to reside in the endoplasmic reticulum (ER) of the pathogen (Russo *et al*., 2010). Inhibition of PMV activity using HIV inhibitors, the aspartic protease inhibitor pepstatin or a PEXEL-mimetic inhibitor severely impacted effector export and resulted in loss of virulence (Russo *et al*., 2010, Sleebs *et al.*, 2014). Translocation of cleaved PEXEL effectors into host cells is mediated by the *Plasmodium* translocon of exported proteins (PTEX) complex, which comprises as core components, the exported protein EXP2, the AAA^{+}-ATPase HSP101 and PTEX150, a novel *Plasmodium* protein with unknown function (Elsworth *et al.*, 2014).

The strong resemblance of host translocation motifs (RXLX$^{E/Q/D}$ versus RXLR) in effectors of *Phytophthora* and *Pl. falciparum* suggested that both organisms make use of similar export mechanisms to deliver effectors into host cells (Bhattacharjee *et al.*, 2006, Bouwmeester *et al.*, 2011). The current predominant model emphasizes on cleavage of the RXLR motif to initiate effector translocation into host cells (Bouwmeester *et al*., 2011, Wang *et al*., 2017, Petre & Kamoun, 2014). *Phytophthora* genomes, however, seem not to have genes encoding homologs of PTEX components and as such, the mechanism by which RXLR effectors are translocated remains elusive (Bouwmeester *et al*., 2011). Another model suggests that the RXLR motif binds to phosphatidylinositol 3-phosphate (PI(3)P) in host membranes, thereby activating effector translocation by pathogen-independent lipid raft-mediated endocytosis (Kale *et al.*, 2010).

Kay *et al.*, (2011) identified 12 aspartic proteases (APs) in *P. infestans*, including three clade 5 PiAPs - i.e. PiAP10, PiAP11 and PiAP12 - that are homologous to *Pl. falciparum* PMV. APs are endopeptidases usually containing two active aspartate residues in their catalytic site that bind a water molecule to initiate cleavage of protein substrates. Well-known eukaryotic APs are pepsins, cathepsins and renins (Revuelta *et al.*, 2014). Several plant APs have been shown to play keys roles in pathogen defense and abiotic stress response (Xia *et al.*, 2004, Simões & Faro, 2004). In fungi, APs are involved in various physiological processes, including fitness and growth. Inhibition of APs in diverse plant pathogenic fungi, such as *Alternaria* and *Fusarium* species, was shown to result in growth inhibition (Dash *et al.*, 2001). Examples of APs involved in pathogen-host interactions are HIV-1, which functions in protein cleavage of structural components to generate infectious HIV virions, and the secretory APs SAP1-10 of the yeast *Candida albicans* that are involved in hyphal formation and cleavage of host proteins (Craig *et al.*, 1998, Monod & Zepelin, 2002, Naglik *et al.*, 2003, Silva *et al.*, 2014). ASP5, an AP from the zoonotic parasite *Toxoplasma gondii*, was found to be located in the Golgi apparatus and plays a key role in the cleavage of GRA effectors at a PEXEL-like motif. Deletion of ASP5 resulted in reduced parasite fitness and virulence (Hammoudi *et al.*, 2015, Cassone *et al.*, 2016).

This study focuses on *P. infestans* clade 5 PiAPs that are homologues to *Plasmodium* PMV. Here, we determined the involvement of PiAPs in virulence by functionally characterizing *P. infestans* transformants with altered *PiAP5* expression levels. In order to test whether PiAPs have a similar role in effector modification as PMV, we assessed the potential activity of PiAPs in effector cleavage using the *P. infestans* RXLR effector AVR4 as substrate.

Results

PiAP expression is enhanced in pre-infection stages and peaks during host colonization

To gain insight into the biological functions of clade 5 *PiAPs* (Fig. 1A), we first assessed the expression of *PiAP10*, *PiAP11*, and *PiAP12* in four different *P. infestans* life stages, i.e. growing mycelia, sporangia, zoospores and germinating cysts. Quantitative RT-PCR using gene-specific primers revealed that clade 5 *PiAPs* are expressed in all developmental stages, with highest transcript levels in germinating cysts (Fig. 1B). Expression of *PiAP10* and *PiAP11* was also found to be induced in zoospores, whereas *PiAP12* transcription is elevated in sporangia. Next, we determined *PiAP* expression during potato leaf infection, i.e. at time points 0 to 72 hours post inoculation. The three *PiAPs* were found to be expressed during all time points, and showed remarkably similar profiles including a sharp increase in expression at 36 hpi (Fig. 1C). This distinct peak in expression could be related to full establishment of disease or to haustoria development (Zuluaga *et al.*, 2016).

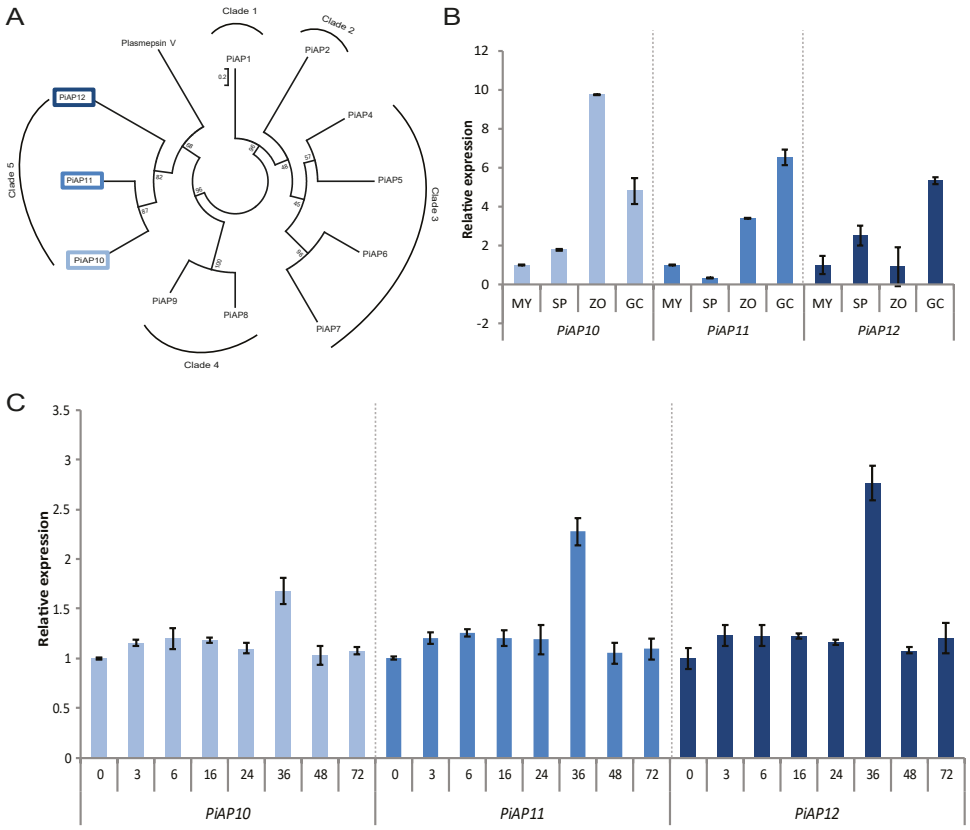

Figure 1. Expression of clade 5 *PiAPs* in different *P. infestans* life stages and during potato infection. (A): Phylogenetic tree of *P. infestans* APs based on full-length amino acid sequences. Clade 5 PiAPs clustering with *Pl. falciparum* PMV are outlined in blue. Numbers at nodes indicate bootstrap values representing the level of clade support inferred by Maximum Likelihood. (B): Expression levels of *PiAP10*, *-11* and *-12* in different life stages, i.e. mycelium (MY), sporangia (SP), zoospores (ZO), and germinating cysts (GC). Expression levels were calculated relative to transcript values in mycelium using *PiActA* as endogenous control. (C): Expression of *PiAP10*, *-11* and *-12* during potato leaf infection. Leaf samples for RNA isolation were taken at different time points (hours) post-inoculation. Transcript values were calculated relative to the expression level at time point 0. Bars represent averages ± SD of two biological replicates.

Altered *PiAP* expression in *P. infestans* affects colony growth

To further investigate the roles of clade 5 PiAPs, we transformed *P. infestans* isolate T35-3 with constructs encoding full length PiAPs harboring mCherry tags. Transformants selected for their resistance to geneticin were verified by PCR, and subsequently analyzed by qRT-PCR to determine *PiAP* expression levels. Transformants with a >25% higher level in *PiAP* expression were considered to be overexpressing lines (PiAP-OX), whereas those with a reduction in gene expression of at least 25% were selected as silenced lines (PiAP-Sil). In total, we obtained 16 PiAP-OX lines; i.e. eight lines for PiAP10, four lines for PiAP11

and four lines for PiAP12. Transformation also resulted in seven PiAP-Sil lines; three for PiAP10, two for PiAP11 and two silenced lines for PiAP12 (Fig. 2). Additional expression analysis revealed that transformation of the target gene did not affect expression of the other two *PiAPs*. Two *P. infestans* transformants that only express *mCherry* were selected as empty vector controls (mCh22 and mCh25).

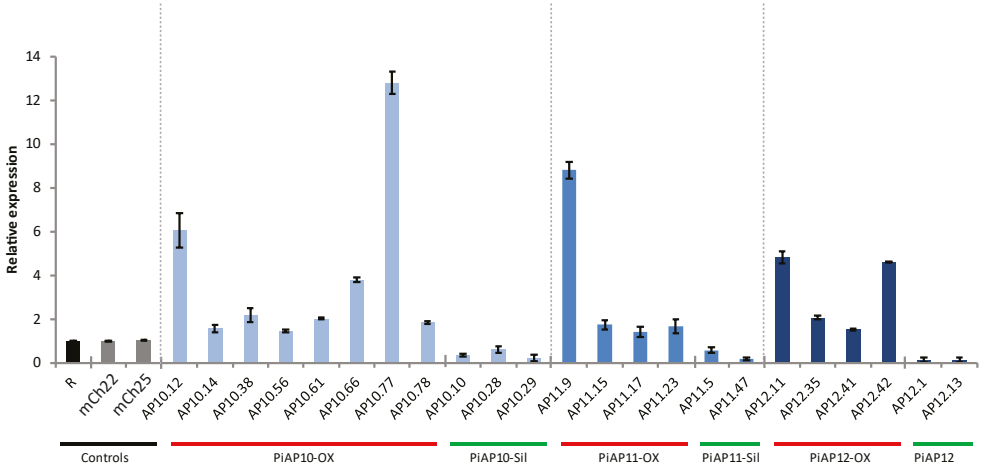

Figure 2. *PiAP*-overexpressing and -silenced transformants. *PiAP10*, *-11*, and *-12* expression in *P. infestans* transformants relative to transcript levels in the recipient strain T35-3 (R). *PiActA* was used as endogenous control. Transformants with increased or decreased *PiAP* expression were selected by qRT-PCR, and are grouped as overexpressing (-OX) and silenced lines (-Sil). Bars represent averages ± SD of two biological replicates.

Phenotypic characterization showed that transformants enhanced or silenced in *PiAP10* and *PiAP12* expression are affected in colony morphology. All PiAP10- and PiAP12-Sil lines showed reduction in mycelial growth and sporangia production (Fig. 3), whereas such a clear effect on development was not found among PiAP10- and PiAP12-OX lines. In contrast, both PiAP11-Sil and -OX lines seem to be slightly enhanced in growth and production of sporangia (Fig. 3). No correlation could be found between the degree of silencing or overexpression and the observed phenotypic effects. All transformants, regardless of their transgene expression level, were able to produce zoospores in amounts comparable to that of the control lines. Taken together, these results suggest that altered expression levels of *PiAP10* and *PiAP12* affects *P. infestans* growth and sporangia production.

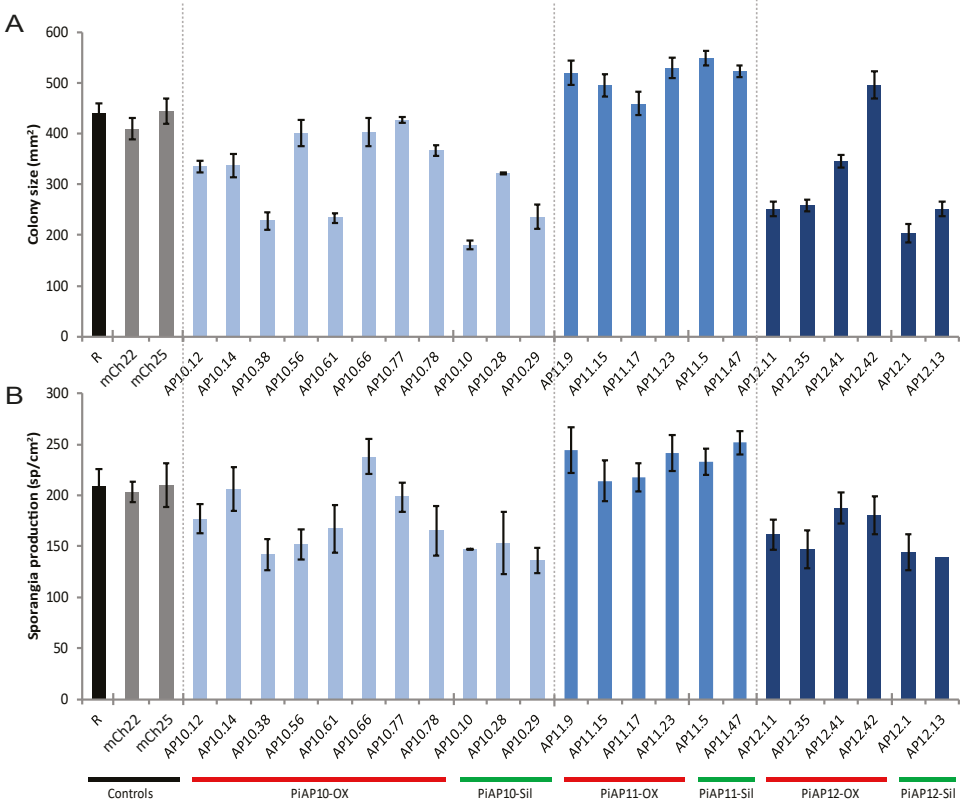

Figure 3. Altered *PiAP* expression affects *P. infestans* growth and sporulation. Colony size (A) and sporangia production (B) of *P. infestans* -OX and -Sil lines at 9 dpi. Lines transformed with the empty vector pMCherryN (mCh22 and mCh25) were included as controls. Bars represent averages ± SD of two biological repeats.

P. infestans transformants with altered *PiAP10* or *PiAP12* expression are hampered in virulence

In a next step, we assayed whether alteration of *PiAP* expression influences *P. infestans* virulence. Potato leaves were inoculated with similar amounts of zoospores, and infection efficiency and lesion formation were measured after 5 days. Control lines mCh22 and mCh25 were found to behave in a similar way as the recipient isolate T35-3. In contrast, potato leaves inoculated with *P. infestans* lines that overexpress either *PiAP10* or *PiAP12*, resulted in significantly smaller lesions (Fig. 4A). It should be noted that these OX-lines also have lower infection efficiencies. This is largely due to the fact that inoculation with these lines frequently resulted in cell death (Fig. 4B). Transformants overexpressing *PiAP11* (PiAP11-OX) developed lesions comparable to the control, with no reduction in efficiency and no induction of cell death (Fig. 4). PiAP10- and PiAP12-Sil lines have a low infection efficiency and show significantly reduced lesion sizes on inoculated potato leaves, but

did not induce cell death (Fig. 4). Similar to the PiAP11-OX lines, transformants silenced for *PiAP11* did not show any reduction in infection rate or lesion size in comparison to the control lines (Fig. 4).

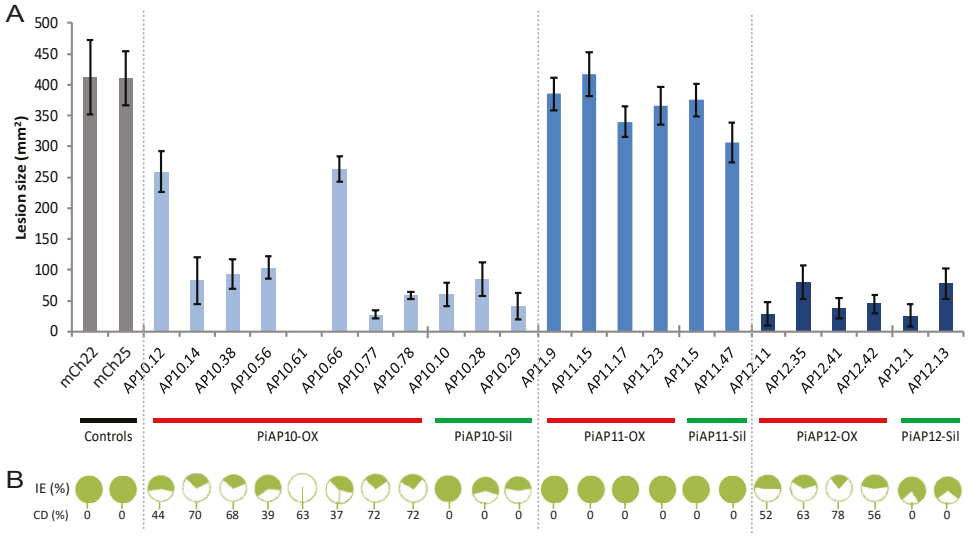

Figure 4. Infection assay of *P. infestans* PiAP transformants on detached potato leaves. (A): *P. infestans* lesion sizes on potato leaves at 5 dpi. Data are an average of three biological repeats ± SD (n=18). (B): Infection efficiency of PiAP transformants. Green sectors in pie charts depict percentages of successful infection. Numbers below white sectors represent percentages of cell death observed among unsuccessful infections at 5 dpi. Data are an average of three biological repeats (n=30).

Expression of *PiAP10* and *PiAP12* in *Nicotiana benthamiana* impairs colonization by *P. infestans*

To investigate if the observed cell death induced by PiAP10- and PiAP12-OX lines is pathogen-independent, we transiently expressed *PiAPs* in *N. benthamiana* leaves by agro-transformation. No cell death was observed by the naked eye in *PiAP*-expressing zones at 5 dpi. Empty vector and the cell death-inducing protein CRN2 (Torto *et al.*, 2003) were used as negative and positive controls, respectively. In addition, we monitored ion leakage as a measure of cell death. Agroinfiltrated leaves that transiently express *PiAP10* and *PiAP12* showed an increase in ion leakage compared to the negative control, but this was not comparable to the strong response initiated by the positive control (Fig. 5A). Transient expression of *PiAP11* had only a minor effect. Next, we performed infection assays on agroinfiltrated leaves with *P. infestans* zoospores. *In planta* expression of *PiAP10* and *PiAP12* significantly reduces leaf colonization, while leaves that express *PiAP11* are unaffected in lesion growth (Fig. 5B).

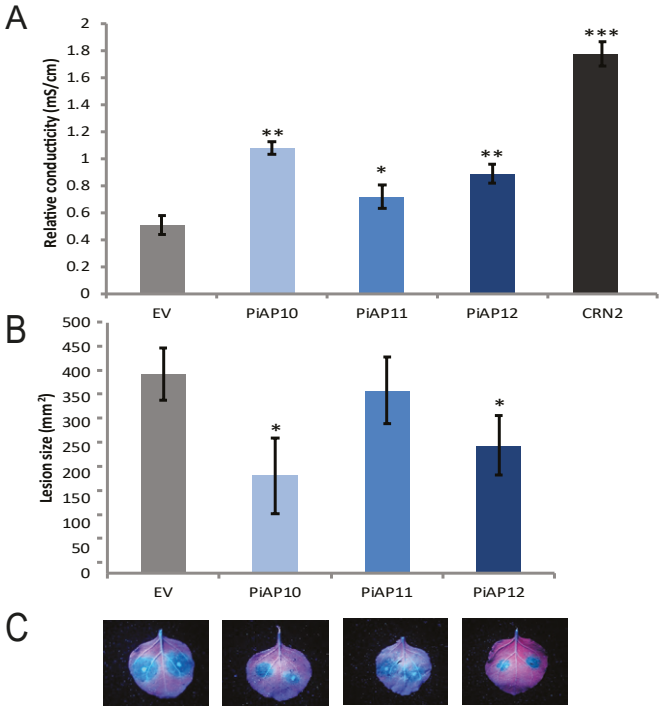

Figure 5. Expression of *PiAP10* and *PiAP12* in *N. benthamiana* results in smaller *P. infestans* lesions. (A): Ion leakage in *N. benthamiana* leaves transiently expressing *PiAPs*. Conductivity values were measured 5 days after agroinfiltration. Data are an average of three biological repeats (n=15), with error bars indicating SD. Asterisks indicate significant differences compared to the control (ANOVA, p<0,05). (B): Mean sizes of *P. infestans* lesions on *N. benthamiana* leaves transiently expressing *PiAPs* at 5 dpi. Bars represent averages ± SD of three biological repeats (n=30). Asterisks indicate significant differences compared to the control (ANOVA, p<0,05). (C): Images taken under UV light of representative *P. infestans* lesions on agroinfiltrated *N. benthamiana* leaves.

Clade 5 PiAPs have enzymatic activity

To determine whether clade 5 PiAPs have enzymatic activities we constructed FLAG/His-tagged gene fragments encoding the aspartic domains of the individual PiAPs (PiAP10; $AA_{66\text{-}413}$, PiAP11; $AA_{96\text{-}441}$, PiAP12; $AA_{91\text{-}430}$). Recombinantly produced PiAPs were analyzed by so-called in-gel zymography; assays based on enzymatic degradation of high-molecular-weight substrates integrated in polyacrylamide gels that can be visualized by electrophoresis (Choi *et al.*, 2009). In our assays we used gelatin, a widely applied general substrate to determine enzymatic activity. Gelatin is a mixture of proteins derived after enzymatic hydrolysis of collagen. As control we included trypsin, a serine protease that hydrolyses proteins by cleaving peptide bonds on the carboxyl side of lysine and arginine residues. Results show that trypsin was able to digest gelatin efficiently, as indicated by the strong discoloration of the gel (Fig. 6). Discoloration of the gel was also observed upon

incubation with both soluble and refolded PiAP10, -11, and -12, although this was not as strong as observed with trypsin (Fig 6). When we added the aspartic protease inhibitor pepstatin no discoloration was observed, suggesting that clade 5 PiAPs have aspartic protease activity.

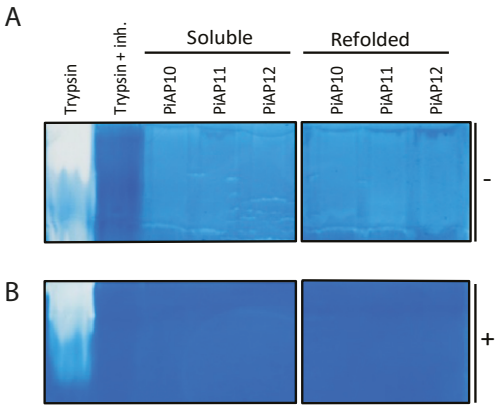

Figure 6. *P. infestans* APs have enzymatic activity against gelatine. In-gel zymography with soluble and refolded APs using 0.1% gelatin as substrate. Gels were stained with Coomassie Brilliant Blue to detect proteolytic activity as discoloration. Enzymatic assays were conducted in the absence (-) and presence (+) of the aspartic protease inhibitor pepstatin.

PiAP10 and PiAP12 cleave *P. infestans* RXLR effector AVR4

Based on the fact that the aspartic protease PMV of the malaria parasite *Pl. falciparum* cleaves PEXEL effectors after the L residue within the motif $RXLX^{E/Q/D}$, we performed activity assays to determine if PiAPs can cleave RXLR effectors in a similar fashion. For this purpose, we heterologously produced a tagged version of the *P. infestans* RXLR effector AVR4 ($AVR4^{RFLR}$). Two bands were detected by immunoblotting, one with an expected size of approximately 40 kDa and a lower band at 25 kDa that could be a degradation product. Incubation of $AVR4^{RFLR}$ with PiAP10 and PiAP12 resulted in the appearance of a third band, slightly smaller than $AVR4^{RFLR}$. The size of this band is around 35 kDa, which is the expected size of AVR4 when cleaved in, or near the RXLR motif. A similar sized band was not detected in assays using PiAP11 and PMV, suggesting some sort of enzyme specificity (Fig. 7B). These results suggest enzymatic cleavage of AVR4 at the N-terminus.

To determine the involvement of the RXLR motif we produced a version of AVR4 in which the RXLR motif is mutated into AAAA ($AVR4^{AAAA}$). Activity assays using $AVR4^{AAAA}$ as substrate did not result in a comparable sized extra band with any of the recombinant PiAPs (Fig. 7C). This indicates that either AVR4 is cleaved in the RXLR motif, or that the RXLR motif is important for the cleavage process.

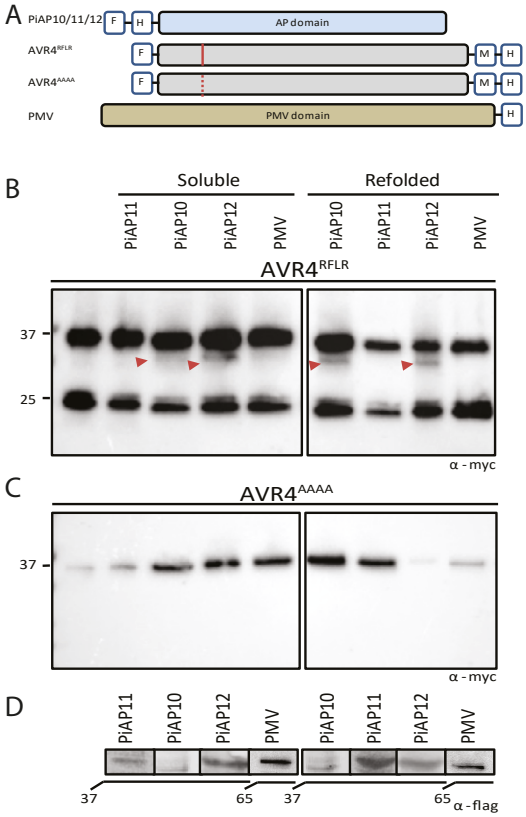

Figure 7. *P. infestans* effector AVR4 is cleaved by PiAP10 and PiAP12. (A): Cartoons of recombinantly produced PiAPs, PMV and versions of PiAVR4. Red lines indicate the position of the RXLR motif in AVR4. Squares outlined in blue indicate the used tags (M: Myc, F: FLAG, H: His). (B): AVR4^{RFLR} is cleaved by PiAP10 and PiAP12, but not by PiAP11 and PMV. Arrowheads mark the lower molecular weight PiAVR4 fragments obtained after cleavage. (C): PiAP10 and PiAP12 are unable to cleave the mutated version AVR^{AAAA}. (D): Immunoblots of soluble and refolded PiAPs detected by α-FLAG antibodies. Numbers indicate protein sizes in kDa.

Discussion

APs are an important class of enzymes involved in virulence of several mammalian parasites, and shown to play key roles in host cell manipulation to facilitate disease. Well-studied examples are PMV from *Pl. falciparum* that modifies PEXEL effectors to activate translocation into the host erythrocytes and ASP5 from *Toxoplasma gondii*, which modifies effectors at the PEXEL-like motif and plays a role in the pathogen fitness and virulence (Russo *et al.*, 2010, Boddey *et al.*, 2010, Hammoudi *et al.*, 2015). A large number of effectors secreted by plant pathogenic oomycetes harbour a N-terminal RXLR motif sequence highly similar in sequence to the PEXEL motif. This led to the tempting hypothesis that a

similar mechanism exists for RXLR effector translocation. Early studies showed that PEXEL and RXLR motifs are interchangeable, however, PMV was not able to cleave RXLR effectors (Bhattacharjee *et al*., 2006). PMV-mediated modification and subsequent transport of PEXEL effectors into the erythrocytes has been questioned. Another model suggests that the PEXEL motif binds PI(3)P at the ER. A recent study by (Boddey *et al*., 2016) could not find sufficient evidence to support this model and showed that the modification of the PEXEL effectors by PMV is the essential step to initiate effector translocation.

RXLR effectors are found to accumulate around haustoria, the site where they supposedly enter the host cells. Several studies have been focussing on the mechanism responsible for effector translocation into the host cell, and most of them indicate that the RXLR motif is essential (Schornack *et al*., 2009). However, several studies show that the presence of the pathogen is not necessary for the uptake of RXLR effectors in host cells. The *P. sojae* effector AVR1b was found to enter host cells via a mechanism independent of the pathogen (Dou *et al*., 2008, Whisson *et al*., 2007). However, experiments showing this autonomous uptake were not repeatable by others (Wawra *et al*., 2013). A different mechanism of translocation that is mediated by external PI(3)P binding of the RXLR-motif of effectors has been suggested (Kale *et al*., 2010). This model has also been under heavy debate, with more recent studies indicating that the RXLR motif is not sufficient for PI(3)P binding and that the uptake is pathogen-dependent (Petre & Kamoun, 2014, Petre *et al*., 2016).

In this study we focused on three clade 5 APs of *P. infestans* that show similarity to PMV of *Pl. falciparum*. We hypothesized that these APs might be involved in virulence of *P. infestans* by modifying RXLR effectors. As a first approach, the potential role of PiAPs on *P. infestans* virulence was examined by altering their expression. Overexpression of *PiAP*s did not have a severe effect on growth and sporulation. However, silencing of *PiAP10* and *PiAP12* resulted in reduced colony growth and sporulation suggesting that these APs have a role in pathogen fitness. Similar results have been reported in other studies, where inhibition of APs resulted in growth inhibition of several fungal species (Dash *et al*., 2001), while deletion of ASP5 in *T. gondii* led to reduced fitness of the pathogen (Hammoudi *et al*., 2015).

To study the role of the PiAPs in virulence, we performed disease assays with transformants in which *PiAP* expression was silenced. Lines that were silenced in *PiAP10*- or *PiAP12*- but not *PiAP11*-expression were found to be reduced in virulence, as indicated by the formation of significantly smaller lesions on potato leaves. Inoculation of potato leaves with transformants overexpressing *PiAP10* or *PiAP12* induced cell death and the lesions were smaller. This was not observed in *PiAP11*-overexpressing lines. Our agroinfiltration assays showed that transient expression of *PiAP10* and *PiAP12* in *N. benthamiana* leaves and subsequent inoculation with *P. infestans* results in smaller lesions compared to the empty vector controls. This could suggest that PiAPs are active inside host cells, potentially

stimulating plant defense and hindering host colonization. Protein structure prediction suggests that PiAP10 and PiAP12 are membrane-bound and may function as homodimers. PiAP11 is membrane bound as well, but it lacks the essential cysteine residues to form dimers (Kay *et al*., 2011). We hypothesize that this could be a potential explanation for the observed differences between PiAP10, PiAP11 and PiAP12.

To determine whether clade 5 PiAPs have a similar biochemical function as PMV, we performed *in vitro* experiments with heterologously produced AP domains of PiAP10, PiAP11 and PiAP12. Enzymatic activity assays using gelatine as a general substrate showed that the AP domains of all three clade 5 PiAPs harbour proteolytic activity and this was verified by inhibition of their activity with the general AP inhibitor pepstatin. Activity assays using recombinant PiAPs suggest that both PiAP10 and PiAP12 are capable of cleaving the *P. infestans* RXLR effector AVR4. Furthermore, we show that the RXLR motif in AVR4 is important, since mutation of the motif resulted in loss of enzymatic cleavage. In a recent study it was shown that the *P. infestans* RXLR effector AVR3 is cleaved in the N-terminus before secretion, however, none of the clade 5 PiAPs was found to modify the effector (Wawra *et al.*, 2017). This is in contrast to our findings; however, this could be due to the fact that the experimental procedures were different. In the study by Wawra *et al.* (2017), activity assays with *P. infestans* PiAPs were performed at 37°C and in acidic/basic conditions more similar to the PMV assays (Boddey *et al*., 2010). Furthermore, a different effector was used, under a different experimental set-up, and proteins under study were produced in a different way to ours. It is possible though, that not all RXLR effectors are modified by the same enzyme and that this is a more specialized process.

Taken together, our results indicate that PiAP10 and PiAP12 play a role in *P. infestans* virulence and that they are key components in the infection process, but the underlying mechanisms are not yet clear. Based on our findings, we propose a model where specific PiAPs act as key components in the modification of one or a particular group of RXLR effectors and therefore promote disease in a very precise and balanced procedure, and that alteration of this balance affects the fitness and the virulence of the pathogen. Reduced levels of PiAP interfere with effector secretion, leading to reduced virulence. Overproduction leads to uncontrolled activity and causes imbalanced effector export, culminating in cell death. Although it is very tempting to make this hypothesis, more experimental evidence is needed to further investigate how PiAPs are involved in *P. infestans* virulence and effector modification.

Materials & Methods

Phytophthora culture conditions and plant infection assays

Phytophthora infestans isolates were grown at 18°C on rye sucrose (RS) agar plates supplemented with appropriate antibiotics. *P. infestans* transformants were selected and grown on RS agar plates supplemented with 5 mg/l geneticin (G418). Zoospores were isolated by flooding two-week old mycelium with ice-cold water for 2-3 hours. Inoculum concentrations were adjusted to $1*10^5$ zoospores/ml. Five-week old potato plants (cv Désirée) were spray-inoculated with *P. infestans* zoospore suspensions until run-off. Inoculation assays on detached *Nicotiana benthamiana* and potato leaves were performed by placing 10 µl droplets of inoculum on abaxial leaf sides. Mock-inoculation was performed with water. Inoculated plants were incubated at 18°C and high humidity. Lesion sizes were determined at 5 dpi as described by Vleeshouwers *et al*., 2008.

Nucleic acid isolation and quantitative RT-PCR

P. infestans DNA was extracted using TRIzol reagent, and RNA was isolated with a Quick-RNA Mini-prep kit according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using oligo(dT) primers and M-MLV reverse transcriptase. Quantitative RT-PCR was performed in triplicate on an ABI7300 SDS real-time qPCR machine. Transcript levels of *PiAPs* were quantified using gene-specific primers and *PiActA* as endogenous control (Table S1). Data were analyzed based on the $2^{-\Delta\Delta C}{}_T$ method (Schmittgen & Livak, 2008).

Plasmid construction

Full-length sequences of *PiAP10*, *-11* and *-12* were PCR-amplified from genomic DNA of *P. infestans* isolate 88069 using proof-reading DNA polymerase and primers that generate restriction sites (Table S1). PCR fragments were introduced by directional cloning into plasmid pMCherryN, which carries a geneticin resistance cassette and a mCherry sequence under control of a *ham34* promoter (Ah-Fong and Judelson, 2011). Sequences encoding PiAP domains ($PiAP10_{66\text{-}413}$, $PiAP11_{96\text{-}441}$, $PiAP12_{91\text{-}430}$) were amplified from cDNA with primers containing a 6His sequence (Table S1). Sequences encoding C-terminal 6His/3Myc-tagged $PiAVR4^{RFLR}$ ($AA_{25\text{-}287}$) and the RXLR-mutant version $PiAvr4^{AAAA}$ (RFLR→AAAA) were obtained via gene synthesis. Gene fragments were introduced by restriction digestion to plasmid pFLAG-ATS. Plasmid PMV-3His encoding *Plasmodium falciparum* Plasmepsin V was provided by Boddey *et al*. (2010). All constructs were introduced to *E. coli* strain BL-21 for protein production.

Full length *PiAPs* sequences amplified from cDNA were introduced in plasmid pENTR/D-TOPO, and subsequently in binary plasmid pGWB5 via LR reaction (Invitrogen). Resulting constructs were transformed by electroporation into *A. tumefaciens* strain Agl1 for *Agrobacterium*–mediated transient transformation assays.

Transformation of *Phytophthora infestans*

Protoplasts of *P. infestans* T35-3 were obtained by treating 2-days-old mycelium for 1 hour with 5 mg/ml cellulase and 10 mg/ml β-glucanase. Prior to transformation, 25 µg plasmid DNA was mixed with 60 µl lipofectin and incubated for 15 min at RT. Protoplasts were transformed using the PEG transformation protocol as described by Ah-Fong *et al.* (2008). Transformed protoplasts were kept 24 hours in liquid RS medium containing 5 mg/l geneticin (G418) and subsequently plated on RS agar plates with geneticin. Colonies were cultured on fresh RS agar plates and covered with a thin layer of RS agar supplemented with 5 mg/l geneticin. Emerging colonies were isolated and grown for 2 weeks on selective medium. Mycelial growth and sporangia production were determined 12 days after inoculation of a mycelial plug (5 mm diameter) in the centre of a fresh RS agar plate.

Agroinfiltration assays

Agrobacterium tumefaciens Agl1 strains carrying binary vectors were grown overnight at 37°C in LB medium supplemented with appropriate antibiotics. Bacterial pellets were resuspended to an OD_{600} of 1.0 in induction medium (per l: 20 g sucrose, 5 g MS basal salt mixture, 1.95 g MES, 200mM acetosyringone, pH 5.6). Agroinfiltrations were performed on abaxial sides of *N. benthamiana* leaves using a needleless syringe. Plants were kept in a climate chamber at 21°C and a 70% relative humidity.

Electrolyte leakage assays

Six leaf disks (9 mm in diameter) were excised per agroinfiltrated area and incubated for 3 hours in 5 ml MQ under gentle agitation. After incubation, sample conductivity was measured using a digital conductivity meter equipped with LabX direct PH 2.1 software (Mettler Toledo). Total conductivity was measured after boiling leaf disks in solution for 15 minutes. Electrolyte leakage was calculated by dividing sample conductivity by the total conductivity. Experiments were repeated in triplicate each consisting three replicates per sample.

Protein production and purification

Recombinant *E. coli* strains were grown overnight as pre-cultures in 25 ml LB medium containing appropriate antibiotics at 37°C. Individual pre-cultures were used to inoculate in 1 l of LB medium, and grown at 37°C for 2 hours to an OD_{600} of 0.5. Protein production was induced by adding 0.35 mM IPTG and cultures were subsequently grown for 4 hours at 30°C. Cells were collected by centrifugation at 5000 rpm for 30 min, and lysed in 40 ml lysis buffer (per 100 ml: 7.4 g sodium phosphate, 10 ml glycerol, 300 ml lysozyme, 6.26 ng DNAse) for 1 hour at RT. Samples were snap-frozen in liquid nitrogen and thaw at RT. Supernatants (soluble protein fractions) were obtained by centrifugation at 14000 rpm for 45 min. Pellets were resuspended in 5 ml denaturing buffer (100 mM NaH_2PO_4, 100 mM

Tris-HCl, 6 M GdnHCl, pH 8.0), incubated for 40 min at RT, and subsequently centrifuged at 14000 rpm for 45 minutes (denatured protein fractions). Protein fractions were stored at -20°C.

Protein purification was performed with Ni-NTA agarose beads. Protein extracts were mixed with beads (200 µl beads per 1 ml extract) and incubated for 2 hours at 4°C. Beads were collected by centrifugation at 800 rpm for 5 min and rinsed twice with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Bound proteins were eluted by incubation for 15 minutes at 4°C with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Purified proteins from soluble fractions were dialysed (Spectra/Por 3 RC dialysis tubing, MWCO 3,5 kDa, Spectrum Labs, USA) against 20 volumes of dialysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0) overnight at 4°C.

Denatured protein fractions were washed and eluted in buffers additionally supplemented with 6 M GdnHCl. Purified denatured proteins were step-wise refolded using dialysis buffer with decreasing GdnHCl concentrations (6, 5, 4, 3, 2, 1, 0.5 and 0 M). Dialysis buffer was changed/refreshed every 12-15 hours. Purified proteins were stored in 20% glycerol at -80°C.

Protein detection

Proteins samples were boiled for 5 min in SDS-loading buffer and separated on 4-20% gradient Mini-PROTEAN TGX Stain-free Precast protein gels (Bio-Rad). After electrophoresis, proteins were transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad). Proteins were detected by incubating membranes with α-His or α-FLAG antibodies (diluted to 1:5000 in TBST) in 5% skimmed milk for 1 hour at RT. Western blot signals were visualized using SuperSignal West Dura substrate (Thermo Fisher Scientific) using a ChemiDoc XRS system. Protein blots were subsequently stained with Coomassie Brilliant Blue R250.

Zymography and *in vitro* cleavage assays

In-gel zymography assays were performed according to Hassani *et al*. (2014) with slight modifications. In brief, 12% polyacrylamide gels containing 1.5 mg/ml gelatine were loaded with purified soluble and refolded PiAP proteins at a concentration of 10 µg. Trypsin was used as a positive control for protein digestion. Trypsin protease activity was inhibited using 50 µM protease inhibitor mix (cOmplete ULTRA, Sigma-Aldrich). After electrophoresis, gels were incubated with 1% Triton X-100 for 1 hour at RT, and subsequently washed overnight in 50 mM Tris-Cl pH 7.4, 5 mM, $CaCl_2$ and 1 mM ZnCl, at RT under constant agitation. Gels were stained with Coomassie Brilliant Blue R250. *In vitro* activity assays were performed according to Boddey *et al*. (2010), with modifications. Purified PiAPs and PMV (2 µg) were mixed with soluble $PiAVR4^{RFLR}$ or $PiAVR4^{AAAA}$ (1 µg) in

100 µl activity buffer (25 mM Tris, 25 mM MES, pH 6.5), and incubated for 1 hour at RT under constant shaking. Hereafter, samples were boiled for 5 min in SDS-loading buffer prior to SDS-PAGE. Proteases and substrate proteins were detected with immunoblotting using α-FLAG and α-His antibodies, respectively.

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References

Bhattacharjee, S., Hiller, N.L., Liolios, K., Win, J., Kanneganti, T.-D., Young, C., Kamoun, S. and Haldar, K. (2006) The malarial host-targeting signal is conserved in the Irish potato famine pathogen. *PLoS Pathog,* **2,** e50.

Boddey, J.A., Hodder, A.N., Gunther, S., Gilson, P.R., Patsiouras, H., Kapp, E.A., Pearce, J.A., de Koning-Ward, T.F., Simpson, R.J., Crabb, B.S. and Cowman, A.F. (2010) An aspartyl protease directs malaria effector proteins to the host cell. *Nature,* **463,** 627-631.

Boddey, J.A., O'Neill, M.T., Lopaticki, S., Carvalho, T.G., Hodder, A.N., Nebl, T., Wawra, S., van West, P., Ebrahimzadeh, Z., Richard, D., Flemming, S., Spielmann, T., Przyborski, J., Babon, J.J. and Cowman, A.F. (2016) Export of malaria proteins requires co-translational processing of the PEXEL motif independent of phosphatidylinositol-3-phosphate binding. *Nat Commun,* **7,** 10470.

Bouwmeester, K., Meijer, H.J.G. and Govers, F. (2011) At the frontier; effectors crossing the *Phytophthora*-host interface. *Front Plant Sci,* **2**.

Bozkurt, T.O., Schornack, S., Banfield, M.J. and Kamoun, S. (2012) Oomycetes, effectors, and all that jazz. *Curr Opin Plant Biol,* **15,** 483-492.

Cassone, A., Vecchiarelli, A. and Hube, B. (2016) Aspartyl proteinases of eukaryotic microbial pathogens: from eating to heating. *PLoS Pathog,* **12,** e1005992.

Choi, N.-S., Kim, B.-H., Park, C.-S., Han, Y.J., Lee, H.W., Choi, J.H., Lee, S.-G. and Song, J.J. (2009) Multiple-layer substrate zymography for detection of several enzymes in a single sodium dodecyl sulfate gel. *Anal Biochem,* **386,** 121-122.

Craig, C., Race, E., Sheldon, J., Whittaker, L., Gilbert, S., Moffatt, A., Rose, J., Dissanayeke, S., Chirn, G.W., Duncan, I.B. and Cammack, N. (1998) HIV protease genotype and viral sensitivity to HIV protease inhibitors following saquinavir therapy. *AIDS (London, England),* **12,** 1611-1618.

Dash, C., Ahmad, A., Nath, D. and Rao, M. (2001) Novel bifunctional inhibitor of xylanase and aspartic protease: implications for inhibition of fungal growth. *Antimicrob Agents Chemother,* **45,** 2008-2017.

Dou, D., Kale, S.D., Wang, X., Jiang, R.H.Y., Bruce, N.A., Arredondo, F.D., Zhang, X. and Tyler, B.M. (2008) RXLR-mediated entry of *Phytophthora sojae* effector AVR1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell,* **20,** 1930-1947.

Elsworth, B., Matthews, K., Nie, C.Q., Kalanon, M., Charnaud, S.C., Sanders, P.R., Chisholm, S.A., Counihan, N.A., Shaw, P.J., Pino, P., Chan, J.-A., Azevedo, M.F., Rogerson, S.J., Beeson, J.G., Crabb, B.S., Gilson, P.R. and de Koning-Ward, T.F. (2014) PTEX is an essential nexus for protein export in malaria parasites. *Nature Research letter,* **511,** 587-591.

Hammoudi, P.-M., Jacot, D., Mueller, C., Di Cristina, M., Dogga, S.K., Marq, J.-B., Romano, J., Tosetti, N., Dubrot, J., Emre, Y., Lunghi, M., Coppens, I., Yamamoto, M., Sojka, D., Pino, P. and Soldati-Favre, D. (2015) Fundamental roles of the Golgi-associated *Toxoplasma* aspartyl protease, ASP5, at the host-parasite interface. *PLoS Pathog,* **11,** e1005211.

Kale, S.D., Gu, B., Capelluto, D.G.S., Dou, D., Feldman, E., Rumore, A., Arredondo, F.D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C.B., Shan, W. and Tyler, B.M. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell,* **142,** 284 - 295.

Kay, J., Meijer, H.J.G., ten Have, A. and van Kan, J.A.L. (2011) The aspartic proteinase family of three *Phytophthora* species. *BMC Genomics,* **12**.

Monod, M. and Zepelin, M.B.-v. (2002) Secreted aspartic proteases as virulence factors of *Candida* species. *Biol Chem,* **383,** 1087-1093.

Naglik, J.R., Challacombe, S.J. and Hube, B. (2003) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev,* **67,** 400-428.

Petre, B. and Kamoun, S. (2014) How do filamentous pathogens deliver effector proteins into plant cells? *PLoS Biol,* **12,** e1001801.

Petre, B., Kopischke, M., Evrard, A., Robatzek, S. and Kamoun, S. (2016) Cell re-entry assays do not support models of pathogen-independent translocation of AvrM and AVR3a effectors into plant cells. *bioRxiv*. doi: https://doi.org/10.1101/038232.

Revuelta, M.V., van Kan, J.A.L., Kay, J. and ten Have, A. (2014) Extensive expansion of A1 family aspartic proteinases in fungi revealed by evolutionary analyses of 107 complete eukaryotic proteomes. *Genome Biol Evol,* **6,** 1480-1494.

Russo, I., Babbitt, S., Muralidharan, V., Butler, T., Oksman, A. and Goldberg, D.E. (2010) Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature,* **463,** 632-636.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc,* **3,** 1101-1108.

Schornack, S., Huitema, E., Cano, L.M., Bozkurt, T.O., Oliva, R., Van Damme, M., Schwizer, S., Raffaele, S., Chaparro-Garcia, A., Farrer, R., Segretin, M.E., Bos, J., Haas, B.J., Zody, M.C., Nusbaum, C., Win, J., Thines, M. and Kamoun, S. (2009) Ten things to know about oomycete effectors. *Mol Plant Pathol,* **10,** 795-803.

Silva, N.C., Nery, J.M. and Dias, A.L.T. (2014) Aspartic proteinases of *Candida* spp.: role in pathogenicity and antifungal resistance. *Mycoses,* **57,** 1-11.

Simões, I. and Faro, C. (2004) Structure and function of plant aspartic proteinases. *European Journal of Biochemistry,* **271,** 2067-2075.

Sleebs, B.E., Lopaticki, S., Marapana, D.S., O'Neill, M.T., Rajasekaran, P., Gazdik, M., Günther, S., Whitehead, L.W., Lowes, K.N., Barfod, L., Hviid, L., Shaw, P.J., Hodder, A.N., Smith, B.J., Cowman, A.F. and Boddey, J.A. (2014) Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites. *PLoS Biol,* **12,** e1001897.

Torto, T.A., Li, S., Styer, A., Huitema, E., Testa, A., Gow, N.A.R., van West, P. and Kamoun, S. (2003) EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen *Phytophthora*. *Genome Research,* **13,** 1675-1685.

Wang, S., Boevink, P.C., Welsh, L., Zhang, R., Whisson, S.C. and Birch, P.R.J. (2017) Delivery of cytoplasmic and apoplastic effectors from *Phytophthora infestans* haustoria by distinct secretion pathways. *New Phytol***,** 1-11.

Wawra, S., Djamei, A., Albert, I., Nürnberger, T., Kahmann, R. and van West, P. (2013) *In vitro* translocation experiments with RxLR-reporter fusion proteins of Avr1b from *Phytophthora sojae* and AVR3a from *Phytophthora infestans* fail to demonstrate specific autonomous uptake in plant and animal cells. *Mol Plant Microbe Interact,* **26,** 528-536.

Wawra, S., Trusch, F., Matena, A., Apostolakis, K., Linne, U., Zhukov, I., Stanek, J., Koźmiński, W., Davidson, I., Secombes, C.J., Bayer, P. and van West, P. (2017) The RxLR motif of the host targeting effector AVR3a of *Phytophthora infestans* Is cleaved before secretion. *Plant Cell,* **29,** 1184-1195.

Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L. and Birch, P.R.J. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature,* **450,** 115 - 118.

Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R.A. and Lamb, C. (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *EMBO J,* **23,** 980-988.

Zuluaga, A.P., Vega-Arreguín, J.C., Fei, Z., Ponnala, L., Lee, S.J., Matas, A.J., Patev, S., Fry, W.E. and Rose, J.K.C. (2016) Transcriptional dynamics of *Phytophthora infestans* during sequential stages of hemibiotrophic infection of tomato. *Mol Plant Pathol,* **17,** 29-41.

Supplemental data

Table S1. Primers used in this study.

Cloning						
Gene	**Vector**	**Restriction sites**	**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
PiAP10	pMCherryN	*Age*I-*Nhe*I	PiAP10-mC-F	ACCGGTATGGTGCGCGTAATCGCT	PiAP10-mC-R	GCTAGCAAGGCGCTCCAGGGTGT
	pFLAG-ATS	*Eco*RI-*Kpn*I	PiAP10-dom-F	TTGAATTCATGCATCATCATCATCATCATACGCA CTACACATGGGTGTACG	PiAP10-dom-R	CGCCGGTACCAGCGTCCACAAAGCCAACACG
	pGWB5	-	PiAP10-pGWB5-F	CACCATGGTGCGCGTAATCGCT	PiAP10-pGWB5-R	AAGGCGCTCCAGGGTGT
PiAP11	pMCherryN	*Age*I-*Pac*I	PiAP11-mC-F	ACCGGTATGACGTCACGGTGCG	PiAP11-mC-R	TTAATTAAGCCTTCCTCTTCTTCTCGGA
	pFLAG-ATS	*Xho*I-*Kpn*I	PiAP11-dom-F	TTGTCGAGATGCATCATCATCATCATCATACA CACTACGCTGAAATTCACTTAGG	PiAP11-dom-R	CACGTGGTACCCGACTCGGCGAAGCCCAC
	pGWB5	-	PiAP11-pGWB5-F	CACCATGACGTCACGGTGCG	PiAP11-pGWB5-R	GCCTTCCTCTTCTTCTCGGA
PiAP12	pMCherryN	*Pac*I-*Nhe*I	PiAP12-mC-F	TTAATTAAATGCCGCTCGGTGCG	PiAP12-mC-R	GCTAGCCTTGGGTTCGTCTTCCATCA
	pFLAG-ATS	*Eco*RI-*Bgl*II	PiAP12-dom-F	TTGAATTCATGCATCATCATCATCATCATTCGCACA CGATTCAGGTGACAATCG	PiAP12-dom-R	GCTTAGATCTGGCTTCGGCGATTCCCACGC
	pGWB5	-	PiAP12-pGWB5-F	CACCATGCCGCTCGGTGC	PiAP12-pGWB5-R	CTTGGGTTCGTCTTCCATCA
Expression analysis						
Gene			**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
PiActA			PiActin-F	CATCAAGGAGAAGCTGACGTACA	PiActin-R	GACGACTCGGCGGCAG
PiAP10			PiAP10-RT-F	GCAACTCTGGAGTCGTGTCA	PiAP10-RT-R	GTGGTTGTCCACCTTCGACT
PiAP11			PiAP11-RT-F	AACAATTCGTCGGTGGAGTC	PiAP11-RT-R	CAAACCATCACACCAAGTGC
PiAP12			PiAP12-RT-F	CCGTGCGTAGACTGTGAGAA	PiAP12-RT-R	CGAAGATGGAGTCTGGGTGT

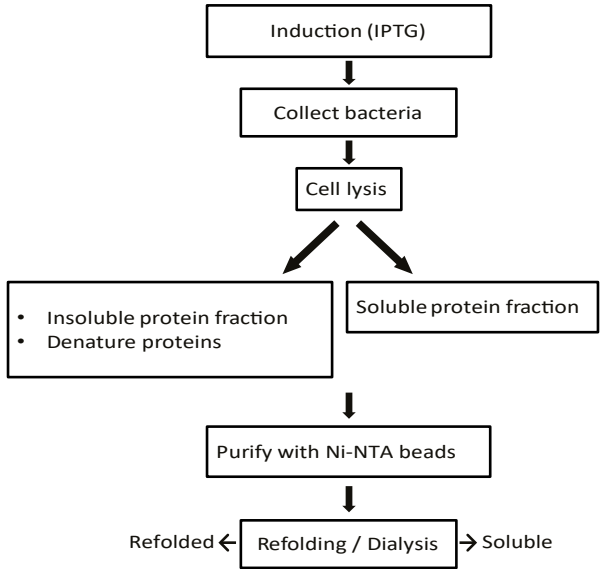

Figure S1. Pipeline for heterologous protein production in *Escherichia coli*. Details of protein production and purification are described in the Materials and methods section.

Chapter 4

Phytophthora infestans AP5, an aspartic protease with a G-protein-coupled receptor domain, plays a role in sporulation and host colonization

Charikleia Schoina, D. Johan van den Hoogen, Natalie Verbeek-de Kruif, Francine Govers and Klaas Bouwmeester

4

Abstract

Late blight disease caused by the oomycete pathogen *Phytophthora infestans* is a significant constraint to potato and tomato production. Asexual reproduction is a key step in the disease cycle of *P. infestans*. Environmental sensing is important to receive signals that stimulate spore germination and host colonization. The *P. infestans* genome contains multiple genes encoding proteins with unique domain architectures. This study focusses on *P. infestans* AP5, an oomycete-specific protease that consists of an aspartic protease (AP) domain and a G-protein-coupled receptor (GPCR) domain. We examined the involvement of PiAP5 in *P. infestans* fitness and virulence by phenotypic characterisation of transformants with enhanced or reduced *PiAP5* expression. *PiAP5* overexpression in *P. infestans* affected colony growth and reduced sporangia production. Sporangia showed an aberrant phenotype in germination and were unable to produce zoospores. *PiAP5* silencing also reduced *P. infestans* sporulation significantly and impacted lesion development. Activity assays showed that recombinant produced PiAP5 has no protease activity against general substrates. These data show that balanced expression of *PiAP5* is essential for proper development of infectious propagules and successful host colonization.

Keywords: Late blight disease, GPCR-bigrams, G-protein-coupled receptors, proteolytic enzyme, pathogenesis, plant-pathogen interactions, Stramenopiles, oomycetes

Introduction

Plant pathogenic oomycetes, such the late blight pathogen *Phytophthora infestans,* cause severe crop diseases that lead to major economic losses. Other well-known oomycetes that are important crop pathogens are *P. sojae*, *P. capsici*, and various *Aphanomyces* and *Pythium* species. During their asexual cycle, plant pathogenic oomycetes produce propagules called sporangia that can either germinate directly or release motile zoospores to infect the plant. Sensing and responding to environmental stimuli is a key step in host cell detection and the initiation of infection. G-protein-coupled receptors (GPCRs), commonly consisting of seven transmembrane (TM) domains, act as important sentinels for a wide range of environmental cues. Inactivated GPCRs form complexes with the heterotrimeric G-protein subunits α, β and γ. The G-protein complex dissociates upon ligand binding to the GPCR domain, thereby activating two distinct intracellular signaling pathways, i.e. the cAMP and phosphatidylinositol pathway, ultimately leading to significant changes in cell function (Kobilka, 2007, Tuteja, 2009, Schafer & Blaxall, 2017). In *P. infestans*, the three subunits of the heterotrimeric G-protein were found to be involved in vegetative growth, sporulation and/or virulence (Latijnhouwers & Govers, 2003, Latijnhouwers *et al.*, 2004).

Comparative analysis revealed that oomycetes, including *Phytophthora* species, possess multiple proteins with unique domain architectures that are absent in other eukaryotes (Seidl *et al.*, 2011, Judelson & Ah-Fong, 2010). An example of such a distinct domain combination are the GPCR-PIPKs (GKs) that harbor a N-terminal GPCR domain and a C-terminal phosphatidylinositol phosphate kinase (PIPK) domain (Bakthavatsalam *et al.*, 2006). Overexpressing *PiGK4* in *P. infestans* resulted in aberrant sporangia development and cleavage, but did not impair virulence. In contrast, *PiGK4*-silenced transformants showed no changes in sporangia size and shape, but were negatively affected in virulence (Hua *et al.*, 2013, Bakthavatsalam *et al*., 2006).

Another protein with a novel domain architecture is PiAP5, one of the 12 aspartic proteases (APs) that can be identified in *P. infestans* (Kay *et al.*, 2011). PiAP5 is an AP-GPCR; unlike PiGK4, it has the GPCR domain at the C-terminus while the accessory AP domain is at the N-terminus. Based on its predicted membrane topology and the presence of a signal peptide, it seems that the AP domain is located extracellularly. APs are proteolytic enzymes that are named after the two aspartate residues in their catalytic domain. APs can be linked to multiple cellular processes, and have been shown to play key roles in stress response and biological fitness of diverse organisms including several fungal pathogens (Dash *et al.*, 2001). Another well-studied example of an AP is Plasmepsin V (PMV) of the malaria pathogen *Plasmodium falciparum*, which cleaves PEXEL effectors prior to their translocation into red blood cells (Boddey *et al.*, 2010, Sleebs *et al.*, 2014). Other examples of APs involved in virulence are ASP5 from the protozoan parasite *Toxoplasma gondii* and several secreted aspartic proteases from *Candida* yeasts (Hammoudi *et al.*, 2015, Monod & Zepelin, 2002, Silva *et al.*, 2014).

In this study we investigated the functional role of the AP-GPCR protein PiAP5 in *P. infestans* development and virulence. For this purpose, we generated multiple *P. infestans* transgenic lines in which *PiAP5* expression is either overexpressed or silenced. These were subsequently examined for growth characteristics and phenotypic changes in pathogenicity. In addition, we expressed a truncated *PiAP5* gene in *Escherichia coli,* and isolated the recombinant protein comprising the aspartic protease domain of PiAP5 for assaying its protease activity against general substrates and the *P. infestans* RXLR effector AVR4.

Results

AP-GPCRs are highly conserved and exclusively found among oomycetes species

AP-GPCRs were firstly identified in three *Phytophthora* species, i.e. *P. infestans, P. ramorum* and *P. sojae* (Kay *et al*., 2011). Here, we questioned whether homologs of AP5 are present in other organisms. Homology searches identified AP-GPCRs in diverse oomycetes, e.g. *Albugo, Phytophthora* and *Saprolegnia* species (Fig. 1A). In contrast, homologs were not found in other bikont genera - such as ciliates and diatoms - or in species belonging to more distantly related lineages, including plants and unikonts. Results show that *AP-GPCRs* are highly conserved single copy genes in oomycete species, all encoding protein bigrams that consist of an aspartic protease (AP) domain and a GPCR domain (Fig. 1B). These data suggest that AP-GCPRs arose *de novo* in oomycetes, and originate from a common ancestor of the three major oomycete orders (Saprolegniales, Pythiales and Peronosporales).

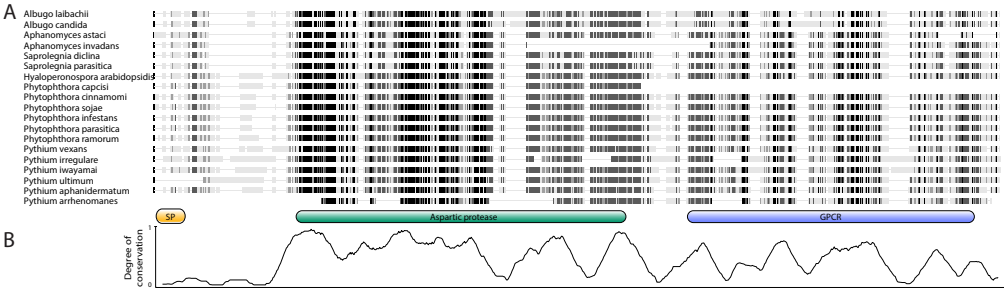

Figure 1. AP-GPCRs are strongly conserved in oomycetes. (A): Multiple sequence alignment of full length AP-GPCR amino acid sequences of different oomycete species. PcAP5 lacks the C-terminal GPCR domain, which is likely due to a misannotation in the *P. capsici* genome. (B): Degree of conservation of oomycete AP-GPCRs included in this study. The degree of conservation was calculated as a sliding moving average over 25 positions on the multiple sequence alignment, only taking full-length sequences into account. The approximate locations of the signal peptide (SP), aspartic protease domain, and GPCR domain are indicated with boxes.

PiAP5 expression is upregulated in infection propagules

As a first step towards unravelling the role of PiAP5, we monitored expression in different *P. infestans* life stages and during potato leaf infection (Fig. 2A). *PiAP5* was found to be upregulated at least 1.5-fold in all pre-infection stages, i.e. sporangia, zoospores and germinating cysts, when compared to the expression level in mycelium. During potato infection, *PiAP5* is expressed at a constant level with relatively minor peaks in expression at 6 and 36 hpi (Fig. 2B). These results show that *PiAP5* expression is predominantly upregulated during asexual development, suggesting potential roles in pre-infection stages.

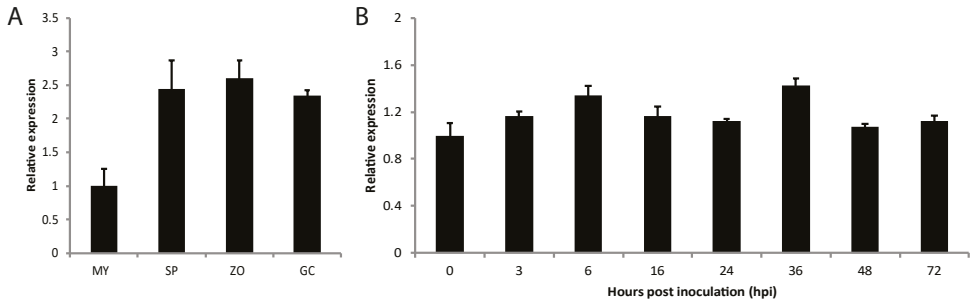

Figure 2. Expression profile of *PiAP5* during life stages and potato colonization. (A): *PiAP5* expression in different *P. infestans* life stages (MY: mycelium, SP: sporangia, ZO: zoospores, GC: germinating cysts). Expression values were calculated relative to that in mycelium. (B): Expression of *PiAP5* during infection of potato leaves (cv. Désirée). Expression values at each time point were calculated relative to the gene expression at 0 hpi. *PiActA* expression was used as endogenous control. Data are representing averages ± SD of three biological repeats.

Alteration of native *PiAP5* expression in *P. infestans* results in aberrant phenotypes

We extended our analysis by monitoring morphological characteristics of *P. infestans* transformants that are either enhanced or silenced in *PiAP5* expression. Five *PiAP5*-overexpressing (PiAP5-OX) and three *PiAP5*-silenced lines (PiAP5-Sil) were obtained (Fig. 3A). PiAP5-OX lines displayed aberrant morphological phenotypes in comparison to the control lines mCh22 and mCh25 (Table 1). Colonies of PiAP5-OX lines have a cotton-like appearance with aerial and strong branching mycelium, and three transformants are reduced in colony growth (Fig. 3B). PiAP5-OX lines are also hampered in their asexual development with reduced amounts of sporangia and no zoospore production (Table 1). The sporangia of PiAP5-OX lines were found to be affected both in size and shape, and germinated in an unorthodox way, i.e. with thicker and immediately branching germ tubes (Fig. 3B).

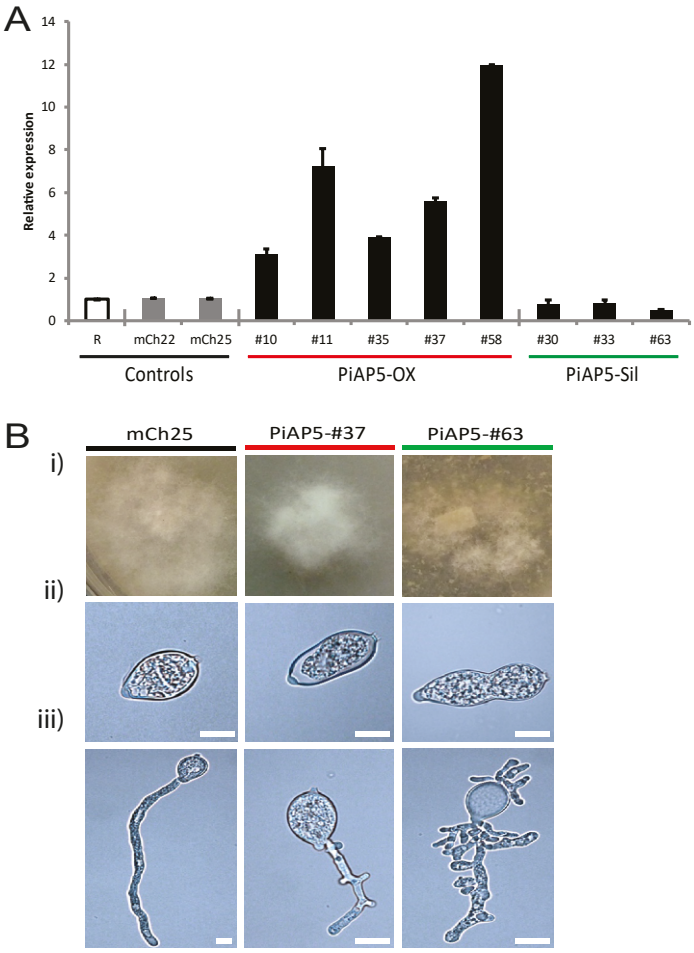

Figure 3. *PiAP5* overexpression and silencing in *P. infestans* results in aberrant growth phenotypes. (A): *PiAP5* expression values calculated relative to transcript level in the recipient isolate (R). *PiActA* expression was used as endogenous control. Bars show averages of two biological repeats. *PiAP5*-overexpression (PiAP5-OX) and -silenced (PiAP5-Sil) lines are underlined in red and green, respectively. (B): Morphological alterations of colony growth (i), sporangial shape (ii) and sporangia germination (iii) of representative PiAP5-OX and -Sil transformants. Quantitative phenotypic data are summarized in Table 1. Bars represent 10 µm (ii) and 20 µm (iii).

The morphological changes observed in PiAP5-Sil lines strongly resemble those of the PiAP5-OX lines. PiAP5-Sil lines are not only impaired in mycelial growth and development (Table 1), but also impacted in sporangia production. Moreover, PiAP5-Sil lines have odd-shaped sporangia that are even more severely affected in their germination (Fig. 3B). Unlike PiAP5-OX lines, the sporangia of PiAP5-Sil lines were able to release zoospores, although in significantly lower amounts than the control lines (Table 1, Fig. 3B). Taken together, these results indicate that alteration of *PiAP5* expression significantly impacts *P. infestans* growth and sporulation.

Table 1. Phenotypic characteristics of PiAP5 transformants

Transformant	Transformation effect	Colony size (mm²)	Sporangia (sp/cm²)	Zoospores (zsp/ml)
AP5-OX #10	Overexpression	250.7 ± 25.1	52.3 ± 19.3	-
AP5-OX #11		352.8 ± 15.3	64.5 ± 10.2	-
AP5-OX #35		171.6 ± 18.6	59.1 ± 17.2	-
AP5-OX #37		890.4 ± 75.1	90.35 ± 17.3	-
AP5-OX #58		760.9 ± 106.3	92.5 ± 16.3	-
AP5-Sil #30	Silencing	143.1 ± 23.8	142.2 ± 12.1	$2*10^3$
AP5-Sil #33		281.3 ± 72.3	121.3 ± 16.4	$1*10^3$
AP5-Sil #63		197.9 ± 26.9	164.2 ± 17.1	$0.6*10^3$
mCh22	Control	809.2 ± 21.2	203.2 ± 16.2	$1*10^5$
mCh25		844.3 ± 24.2	210.3 ± 10.2	$0.9*10^5$

PiAP5-OX and -Sil lines are impaired in virulence

To determine whether alteration of *PiAP5* expression affects *P. infestans* virulence, we performed infection assays with the PiAP5-OX and -Sil lines on potato leaves. Since the PiAP5-OX transformants did not produce zoospores, we performed all inoculations with sporangia. Inoculation with both PiAP5-OX and PiAP5-Sil lines resulted in significantly smaller lesions compared to the controls (Fig. 4A). All transformed lines were also severely impacted in their infection efficiency with at least 50% reduction (Fig. 4B). Based on these results we conclude that alteration of *PiAP5* expression significantly reduces *P. infestans* virulence. To investigate if PiAP5 directly affects host cells we transiently expressed *PiAP5* in *N. benthamiana* by *Agrobacterium*-mediated transformation. No visible cell death was observed in agroinfiltrated leaves that express *PiAP5*, although the level of ion leakage was higher than that of the negative control (Fig. 5A). The cell death-inducing protein CRN2 was used as a positive control in our conductivity assays. Inoculation assays on agroinfiltrated leaves revealed that *in planta* expression of *PiAP5* has no significant effect on *P. infestans* lesion development (Fig. 5B).

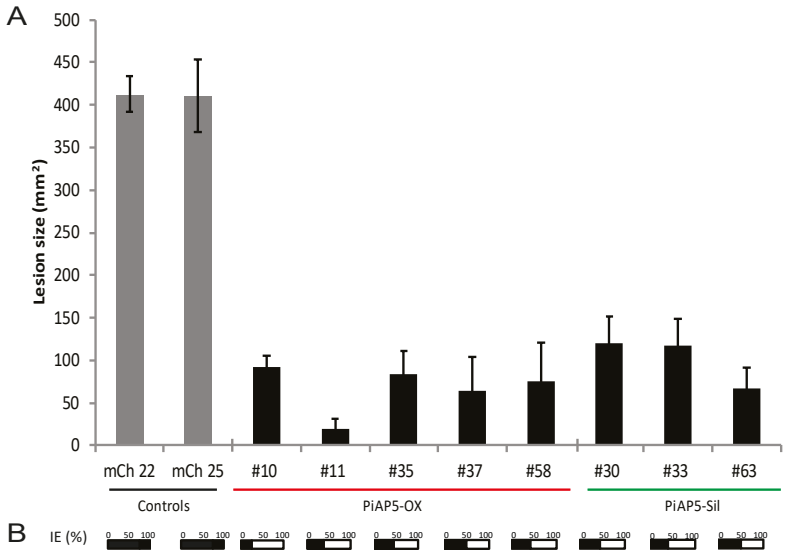

Figure 4. PiAP5-OX and -Sil transformants are impaired in virulence. (A): Lesion sizes on leaves of potato cv. Désirée at 5 dpi. Data represent averages of three biological repeats (n=18) with error bars indicating ± SD. (B): Bar plots representing infection efficiencies in percentages (n=30).

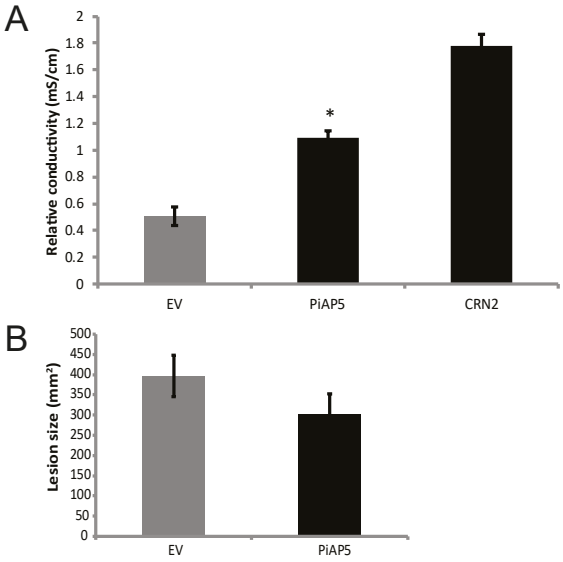

Figure 5. Expression of *PiAP5* in *N. benthamiana* is not impacting the virulence of *P. infestans*. (A): Average lesion size on *N. benthamiana* leaves transiently expressing *PiAP5* at 5 dpi with *P. infestans* isolate T35-3. Data are an average of three biological repeats (n=30). Error bars indicate standard deviation (± SD). Statistical analysis (Student's *t*-test, p<0,05) showed no significant differences between two datasets. (B): Ion leakage measurements of *N. benthamiana* leaves transiently expressing *PiAPs* at 5 dpi. Data are an average of three biological repeats (n=15). Error bars indicate standard deviation (± SD). Asterisks indicate significant differences compared to the empty vector (EV) control (Student's *t*-test, p<0,05).

PiAP5 has no protease activity against general substrates

To determine the protease activity of PiAP5, we heterologously produced the aspartic domain of PiAP5 in *E. coli*. PiAP5 was tested in zymography assays for protease activity against general substrates, i.e. gelatin and casein. Gelatin is a mixture of collagen-derived proteins, whereas casein is a family of related phosphoproteins derived from milk. As a positive control we included trypsin, a serine protease with proteolytic activity. Results suggest that PiAP5 has no proteolytic activity against the general substrates while trypsin does (Fig. 6A). We additionally performed activity assays using recombinantly produced versions of the *P. infestans* RXLR effector AVR4 as substrate. In these assays no enzymatic activity was detected against native PiAVR4 ($AVR4^{RFLR}$) nor its version that is mutated in the RXLR-motif ($PiAVR4^{AAAA}$) (Fig. 6B). Taken together, it can be concluded that PiAP5 has no protease activity against the tested substrates.

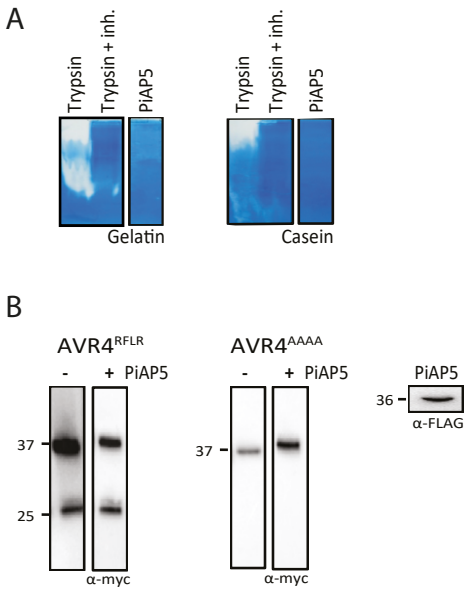

Figure 6. Recombinant PiAP5 is incapable of cleaving general substrates or the RXLR-effector AVR4. (A): In-gel zymography with refolded PiAP5 against the substrates gelatine (left panel) and casein (right panel). Proteolytic activity of trypsin was used as positive control. Gels were stained with Coomassie Brilliant Blue to detect discoloration due to enzyme activity. (B): Activity assay of PiAP5 using $AVR4^{RFLR}$ (left panel) and $AVR4^{AAAA}$ (middle panel) as substrates. AVR4 and PiAP5 (right panel) were detected by immunoblotting using α-myc and α-FLAG antibodies, respectively. Numbers left of the panels depict protein sizes in kDa.

Discussion

Thus far, most studies have been focusing on aspartic proteases in mammalian pathogens and their involvement in virulence, but their role in plant pathogens is largely understudied. Here, we focus on PiAP5, a novel protein that comprises an N-terminal aspartic protease (AP) domain and a C-terminal GPCR domain. Comparative genomics revealed that AP5 homologs are present in oomycetes of different genera, but absent in other eukaryotic species. AP-GPCR is thus oomycete-specific, and arose *de novo* in oomycetes.

The domain architecture of PiAP5 suggests both roles in ligand recognition and enzymatic signaling. Topology prediction suggests that the AP domain is located extracellularly. In order to validate the topology of PiAP5, we performed experiments with *E. coli* strains expressing different truncated GPCR transmembrane segments fused to a β-lactamase reporter enzyme. The results were, however, not conclusive (data not shown).

Expression analysis showed that *PiAP5* has its highest expression in infectious propagules (i.e. sporangia, zoospores and germinating cysts), while its expression remains stable throughout infection of potato. This suggests that PiAP5 is active during early stages of infection. To functionally characterize PiAP5, we generated *P. infestans* transformants with either enhanced or reduced levels of *PiAP5* expression (PiAP5-OX and PiAP5-Sil lines, respectively). In both cases, most transformed lines showed a significant reduction in mycelial growth, in combination with aberrant phenotypes associated with asexual development. The observed phenotypes, however, could not be linked to the level of *PiAP5* expression in the individual transformants. Sporangia were malformed in both PiAP5-OX and PiAP5-Sil lines. Hyphae emerging from germinated sporangia were strongly branched, and this effect was the most severe in silenced lines. These results suggest that PiAP5 is involved in fitness of the pathogen and plays a role in the development of infectious propagules.

To determine whether the transformed lines are able to establish infection, we performed disease assays on potato leaves. Since PiAP5-OX lines do not produce zoospores, inoculations were performed with sporangia. Results show that the infection efficiency and lesion formation of all *PiAP5* transformants is strongly reduced, implying that PiAP5 plays a role in virulence. The reduction in virulence can be caused by the impaired growth and sporulation of the pathogen. Similar findings have been observed for another *P. infestans* GPCR-bigram, i.e. the GPCPR-PIPK GK4. Overexpression of *GK4* affects hyphal elongation, spore germination and sporangia cleavage, while silencing resulted in reduced virulence (Hua *et al*., 2013). Since GPCRs function upstream of heterotrimeric G-proteins that act as molecular signaling switches, it can be hypothesized that the observed effects are due to dysregulated downstream signaling. Silencing of *P. infestans gpa1,* encoding the G-protein α subunit, was found to affect the motility of zoospores and pathogen virulence. Likewise,

silencing of the G-protein β subunit (*gpb1*) results in denser mycelial growth and severe reduction of sporangia production (Latijnhouwers & Govers, 2003, Latijnhouwers *et al*., 2004). Further experiments are needed to unravel the role of the individual AP or GPCR domains.

A recombinant produced version of PiAP5 lacking the GPCR domain showed no enzymatic activity against general substrates, nor against PiAVR4, an RXLR effector that was shown to be cleaved by the clade 5 aspartic proteases PiAP10 and PiAP12 (Chapter 4). Although this could imply that PiAP5 is not active as an AP, it cannot be excluded that the produced protein is improperly refolded and as such, inactive. Another explanation could be that the proteolytic activity of PiAP5 is highly substrate-specific.

Overall our data suggests that PiAP5 is involved in *P. infestans* growth and asexual development. Altering native *PiAP5* expression - either by gene silencing or overexpression - severely affects spore formation, germination, and pathogen virulence, suggesting the need for a balanced PiAP5 level to function properly. However, it is not clear yet whether the observed change in virulence is a direct cause of *PiAP5* overexpression and silencing, or whether it is due to the impaired phenotype of the *P. infestans* transformants.

Materials & Methods

Identification and analysis of AP-GPCRs

To identify AP-GPCRs, we applied the iterative search strategy as described by van den Hoogen *et al*. (2018), including text-based searches in the UniProt database using keywords and protein domain identifiers. Protein sequences of previously annotated AP-GPCRs (i.e. PiAP5s of *P. infestans*, *P. sojae* and *P. ramorum*; Kay *et al*., 2011) were used as queries for BLAST searches. Additional homology searches were performed on the HMMER webserver using default settings. Protein sequences were analysed using the bioinformatic tools SMART, Pfam, SignalP and TMHMM. The degree of conservation among amino acid sequences was calculated using the AMAS method in Jalview (van den Hoogen *et al*., 2018).

Transformation of *P. infestans*

Genomic DNA of *P. infestans* isolate 88069 was used as template for the amplification of full-length *PiAP5*. Amplicons were obtained using Phusion high-fidelity polymerase and primers that generate restriction sites (Table S1). PCR fragments were digested with *Age*I and *Nhe*1 restriction enzymes and ligated into plasmid pMCherryN, resulting into construct p*PiAP5-mC*. Transformation to *P. infestans* isolate T35-3 and subsequent characterization of *PiAP5*-silenced (PiAP5-Sil) and -overexpressing (PiAP5-OX) transformants was performed as described in Chapter 3.

Recombinant PiAP5 purification and enzymatic activity assays.

A DNA fragment encoding the aspartic domain of PiAP5 (AA_{91-414}) was PCR-amplified from cDNA of *P. infestans* isolate 88069 using primes encoding *Eco*RI/*Kpn*I restriction sites and C-terminal 6His tags (Table S1). The obtained amplicon was inserted in plasmid pFLAG-ATS by restriction enzyme digestion. The resulting construct p*FLAG-PiAP5-6His* was transformed to *E. coli* strain BL-21. Recombinant PiAP5 was purified and immunodetected following procedures described in Chapter 3.

The enzymatic activity of PiAP5 was investigated by in-gel zymography and *in vitro* activity assays as conducted by Hassani *et al*., (2014) and Boddey *et al*., (2010), respectively (Chapter 3).

Agroinfiltration assays in *N. benthamiana*

For transient expression assays, the full-length coding sequence of PiAP5 was introduced by TOPO cloning in plasmid pENTR/D-TOPO, and subsequently in the binary plasmid pGWB5 by LR recombination. The resulting construct p35s-*PiAP5*-GFP was transformed by electroporation into *A. tumefaciens* strain AgI1. Agroinfiltrations of *N. benthamiana* leaves were executed as described in Chapter 3. Infiltrated plants were kept in a climate chamber at 21°C and at 70% relative humidity. *P. infestans* disease assays and electrolyte leakage measurements on agroinfiltrated *N. benthamiana* leaves were performed as previously described (Chapter 3).

Phenotypic analysis of PiAP5 transformants

Mycelial growth and sporangia production of *P. infestans* PiAP5-OX and -Sil transformants was determined 12 days after inoculating mycelial plugs (5 mm diameter) on RS agar plates. Sporangia germination was induced by overnight incubation at 18°C, and subsequently observed by light microscopy. Zoospore release was induced by placing equal amounts of sporangia in 1 ml ice-cold water for 3h. Detached potato leaves of cv. Désirée were inoculated with 10 µl droplets containing $1*10^4$ sporangia/ml. Leaves were incubated in a climate room at 18°C and high humidity. Lesions were measured at 5 days post-inoculation, and infection efficiencies and lesion sizes were calculated according to Vleeshouwers *et al*. (1999). Native expression of *PiAP5* was determined in different *P. infestans* life stages (mycelium, sporangia, zoospores and germinating cysts) and during potato infection by qRT-PCR using gene-specific primers (Table S1).

References

Bakthavatsalam, D., Meijer, H.J.G., Noegel, A.A. and Govers, F. (2006) Novel phosphatidylinositol phosphate kinases with a G-protein coupled receptor signature are shared by *Dictyostelium* and *Phytophthora*. *Trends microbiol,* **14,** 378-382.

Boddey, J.A., Hodder, A.N., Gunther, S., Gilson, P.R., Patsiouras, H., Kapp, E.A., Pearce, J.A., de Koning-Ward, T.F., Simpson, R.J., Crabb, B.S. and Cowman, A.F. (2010) An aspartyl protease directs malaria effector proteins to the host cell. *Nature,* **463,** 627-631.

Dash, C., Ahmad, A., Nath, D. and Rao, M. (2001) Novel bifunctional inhibitor of xylanase and aspartic protease: implications for inhibition of fungal growth. *Antimicrob. Agents Chemother,* **45,** 2008-2017.

Hammoudi, P.-M., Jacot, D., Mueller, C., Di Cristina, M., Dogga, S.K., Marq, J.-B., Romano, J., Tosetti, N., Dubrot, J., Emre, Y., Lunghi, M., Coppens, I., Yamamoto, M., Sojka, D., Pino, P. and Soldati-Favre, D. (2015) Fundamental roles of the Golgi-associated *Toxoplasma* aspartyl protease, ASP5, at the host-parasite interface. *PLoS Pathog,* **11,** e1005211.

Hassani, K., Shio, M.T., Martel, C., Faubert, D. and Olivier, M. (2014) Absence of metalloprotease GP63 alters the protein content of *Leishmania* exosomes. *PLoS ONE,* **9,** e95007.

Hua, C., Meijer, H.J.G., de Keijzer, J., Zhao, W., Wang, Y. and Govers, F. (2013) GK4, a G-protein-coupled receptor with a phosphatidylinositol phosphate kinase domain in *Phytophthora infestans*, is involved in sporangia development and virulence. *Mol Microbiol,* **88,** 352-370.

Judelson, H. and Ah-Fong, A. (2010) The kinome of *Phytophthora infestans* reveals oomycete-specific innovations and links to other taxonomic groups. *BMC Genomics,* **11,** 700.

Kay, J., Meijer, H.J.G., ten Have, A. and van Kan, J.A.L. (2011) The aspartic proteinase family of three *Phytophthora* species. *BMC Genomics,* **12**.

Kobilka, B.K. (2007) G protein coupled receptor structure and activation. *BBA - Biomembranes,* **1768,** 794-807.

Latijnhouwers, M. and Govers, F. (2003) A *Phytophthora infestans* G-protein β subunit is involved in sporangium formation. *Eukaryot Cell,* **2,** 971-977.

Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G.A.A., van West, P. and Govers, F. (2004) A Gα subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. *Mol Microbiol,* **51,** 925-936.

Monod, M. and Zepelin, M.B.-v. (2002) Secreted aspartic proteases as virulence factors of *Candida* species. *Biol Chem,* **383,** 1087-1093.

Schafer, A.E. and Blaxall, B.C. (2017) G protein coupled receptor-mediated transactivation of extracellular proteases. *Journal of Cardiovascular Pharmacology,* **Publish Ahead of Print**.

Seidl, M.F., Van den Ackerveken, G., Govers, F. and Snel, B. (2011) A domain-centric analysis of oomycete plant pathogen genomes reveals unique protein organization. *Plant Physiol,* **155,** 628-644.

Silva, N.C., Nery, J.M. and Dias, A.L.T. (2014) Aspartic proteinases of *Candida* spp.: role in pathogenicity and antifungal resistance. *Mycoses,* **57,** 1-11.

Sleebs, B.E., Lopaticki, S., Marapana, D.S., O'Neill, M.T., Rajasekaran, P., Gazdik, M., Günther, S., Whitehead, L.W., Lowes, K.N., Barfod, L., Hviid, L., Shaw, P.J., Hodder, A.N., Smith, B.J., Cowman, A.F. and Boddey, J.A. (2014) Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites. *PLoS Biol,* **12,** e1001897.

Tuteja, N. (2009) Signaling through G protein coupled receptors. *Plant Signal Behav,* **4,** 942-947.

van den Hoogen, D.J., Meijer, H.J.G., Seidl, M.F. and Govers, F. (2018) The ancient link between G-protein-coupled receptors and C-terminal phospholipid kinase domains. *mBio,* **9,** e02119-02117.

Supplemental data

Table S1. Primers used in this study.

Cloning					
Gene	**Vector**	**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
PiAP5	pMCherryN	PiAP5-mC-F	ACCGGTATGCGTCTCGGTCTGCTC	PiAP5-mC-R	GCTAGCATTTGGTCCCATGAGACGC
	pFLAG-ATS	PiAP5-dom-F	TTGAATTCATGCATCATCATCATCATCATCAGT TCTTTGGCCCCATCGCC	PiAP5-dom-R	TTGAATTCATGCATCATCATCATCATCATCAGTT CTTTGGCCCCATCGCC
	pGWB5	PiAP5-pGWB5-F	CACCATGCGTCTCGGTCTGCTC	PiAP5-pGWB5-R	ATTTGGTCCCATGAGACGC
Expression analysis					
Gene		**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
PiActA		PiActin-F	CATCAAGGAGAAGCTGACGTACA	PiActin-R	GACGACTCGGCGGCAG
PiAP5		PiAP5-RT-F	GCAACTCTGGAGTCGTGTCA	PiAP5-RT-R	GTGGTTGTCCACCTTCGACT

Chapter 5

Phytophthora infestans small phospholipase D-like proteins elicit plant cell death and promote virulence

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*Equal contribution

This chapter has been submitted for publication

5

Abstract

Successful invasion of host tissue by (hemi-)biotrophic plant pathogens is dependent on modifications of the host plasma membrane to facilitate two-way transfer of proteins and other compounds. Haustorium formation and establishment of extrahaustorial membranes is likely dependent on a variety of enzymes that modify membranes in a coordinated fashion. Phospholipases, enzymes that hydrolyze phospholipids, have been implicated as virulence factors in several pathogens. The oomycete *Phytophthora infestans* is a hemibiotrophic pathogen that causes potato late blight. It possesses different classes of phospholipase D (PLD) proteins including small PLD-like proteins with and without signal peptide (sPLD-likes and PLD-likes, respectively). Here we studied the role of sPLD-like-1, sPLD-like-12 and PLD-like-1 in the infection process. They are expressed in expanding lesions on potato leaves and during *in vitro* growth with the highest transcript levels in germinating cysts. When expressed *in planta* in the presence of the silencing suppressor P19, all three, elicited local cell death, a response that was visible at the microscopic level and was strongly boosted by the presence of calcium. Moreover, inoculation of leaves expressing the small PLD-like genes resulted in increased lesion growth and more sporangia but this was abolished when mutated PLD-like genes were expressed with non-functional PLD catalytic motifs (HKD). These results show that the three small PLD-likes are catalytically active and suggest that their enzymatic activity is required for promoting virulence, possibly by executing membrane modifications to support growth of *P. infestans* in the host.

Keywords: phospholipids, phospholipases, late blight disease, calcium, signal peptide, oomycete

Introduction

Successful plant colonization by pathogens is the result of a complex interplay between the two partners and depends on the strict regulation of a variety of genes or the modification of cellular components like enzymes, structural proteins or lipids. For biotrophic pathogens that form haustoria for uptake of nutrients from the host and delivery of effectors to the host, modifications of the host plasma membrane and the establishment of extrahaustorial membranes is crucial. These pathogens presumably, exploit certain enzymes to modify membranes in a coordinated fashion and as such these enzymes play a role as virulence factors. An example is phospholipase D (PLD), an enzyme that hydrolyzes structural phospholipids resulting in the production of phosphatidic acid (PA), a second messenger that acts as mediator in many cellular processes. Phospholipases have been implicated as virulence factors in several pathogens (Köhler *et al.*, 2006, Ghannoum, 2000).

The oomycete *Phytophthora infestans* is the causal agent of late blight on potato and tomato, and also known as the Irish potato famine pathogen. It is a hemibiotrophic pathogen that penetrates leaves after breaching the plant cuticle and then expands as intracellular mycelium while forming haustoria in mesophyll cells. *P. infestans* possesses a large repertoire of PLDs divided over six subfamilies, including two families of small PLD-likes with signal peptide (referred to as secreted PLDs or sPLD-likes) (Meijer *et al.*, 2011). Their function is still unknown but the finding that PLD activity can be recovered from liquid medium harboring *P. infestans* mycelium suggests that these small PLDs are secreted and potentially capable of modifying host tissue (Meijer *et al*., 2011, Meijer *et al.*, 2014).

Phospholipids, the substrates of PLDs, are ubiquitous components of membranes and are functionally involved in a disproportionally large number of cellular processes. Besides regulating membrane-bound enzymatic activities, they act as anchors to recruit and translocate proteins containing a phospholipid-binding module and play essential roles in membrane trafficking and defense signaling (Meijer & Munnik, 2003, Behnia & Munro, 2005, Lemmon, 2003, Testerink & Munnik, 2011). Phosphatidic acid (PA), the resultant of PLD activity, is an important intermediate in lipid biosynthesis and a signaling molecule involved in amongst others, vesicle formation and transport, cytoskeleton organization, protein transport, signal transduction and mitosis (Testerink & Munnik, 2005, Testerink & Munnik, 2011, Zhang *et al.*, 2012). Direct application of PA to plant leaves results in pathogenesis-related gene expression and cell death (Andersson *et al.*, 2006, Park *et al.*, 2004, Testerink & Munnik, 2011).

Eukaryotic PLDs usually have two catalytic conserved motifs, each with the core sequence "HxKxxxxD" referred to as the HKD1 and HKD2 motif (IPR001736). These catalytic sites

can be surrounded by accessory regulatory domains as is the case in the two major PLD families PXPH-PLDs and C2-PLDs, which both have a phospholipid-binding domain at the N-terminus, PXPH and C2, respectively (Selvy *et al.*, 2011). PXPH-PLDs are ubiquitous in eukaryotes whereas C2-PLDs only occur in plants. Another PLD subfamily consisting of φ-type PLDs is widespread in eukaryotes, prokaryotes and viruses. These lack the canonical regulatory domains and many bear signal peptides (Tang *et al.*, 2016, Beligni *et al.*, 2015, Liu *et al.*, 2010). *Phytophthora* spp. have six PLD subfamilies (Meijer *et al.*, 2005, Meijer & Govers, 2006, Meijer *et al*., 2011) that are well conserved among oomycetes with some variations in gene numbers (Levesque *et al.*, 2010, Baxter *et al.*, 2010, Kemen *et al.*, 2011, Jiang *et al.*, 2013, Sharma *et al.*, 2015). Four single copy genes encoding a PXPH-PLD, sPLD-like-type-A (sPLD-like-1; φ-type), PXTM-PLD and TM-PLD represent four subfamilies. The latter two are oomycete-specific PLDs that have transmembrane regions as accessory domains (Meijer & Govers, 2006). The two remaining subfamilies comprise three PLD-like genes and nine sPLD-like genes of type B, respectively. The PLD-likes are homologous to the sPLD-like-type-B's but lack a signal peptide (Meijer *et al*., 2011). Because all PLD-likes and sPLD-likes (also referred to as (s)PLD-likes) are relatively small (maximum 68 kDa) they are also collectively referred to as small PLD-likes. PXPH-PLD, PXTM-PLD and sPLD-like-A have two perfect catalytic motifs, but the others do not. In TM-PLD, HKD2 is changed in HKN and in the PLD-likes and the sPLD-like-B's HKD1 is modified in ether HKL, HKT, HKA or HKR *(*Meijer *et al*., 2011). Such imperfect HKD motifs are also found in distinctly related PLD-likes from actinomycetes, in particular *Janibacter* spp. and *Kineococcus radiotolerans*. Despite the aberrant HKD1 motif, the PLD-likes and sPLD-like-B's share other typical PLD motifs with PXPH-PLDs and C2-PLDs that are located in between the HKD1 and HKD2 motif (Meijer *et al.*, 2011), pointing to similarities in catalytic activities.

In several bacterial pathogens PLDs are implicated as major virulence determinants. For example, in *Corynebacterium pseudotuberculosis* PLDs are critical for dissemination of this pathogen from the infection site to the lymph nodes (McKean *et al.*, 2007) and disruption of a PLD gene in *Acinetobacter baumannii* and *Helicobacter pylori* strongly diminished pathogenicity (Jacobs *et al.*, 2010, Sitaraman *et al.*, 2011). Also, in *Klebsiella pneumoniae* a PLD acts as a virulence factor, presumably by controlling the bacterial membrane lipid composition (Lery *et al.*, 2014). In fungi, PLD involvement in pathogenicity was demonstrated in *Candida albicans, Purpureocillium lilacinum* and *Coccidioides posadasii* (Dolan *et al.*, 2004, Hube *et al.*, 2001, Yang *et al.*, 2015, Lajoie & Cordes, 2015).

We previously reported the recovery of significant levels of PLD activity in liquid medium supporting the *in vitro* growth of *P. infestans.* The activity was dependent on the nutrient content of the medium suggesting that the pathogen senses the environment and adapts accordingly (Meijer *et al*., 2011, Meijer *et al*., 2014). Likewise, it can be hypothesized that the pathogen senses its host and is triggered to secrete PLD proteins to execute host membrane modification. In this study, we report that small PLD-like genes are expressed

in germinating cysts and during plant infection. We then tested the effect of small PLD-likes on *Nicotiana benthamiana* and found that, on the one hand they elicit cell death in a calcium-dependent manner and on the other hand promote the virulence of *P. infestans*. Mutations in the catalytic HKD motifs strongly reduced the cell death responses and abolished virulence promotion, demonstrating that the enzymatic activity of the PLDs is the major determinant. These data strengthen the idea that small PLD-likes act as pathogenicity factors in *P. infestans*.

Results

Differential expression of small PLD-likes during development and leaf colonization

We selected PLD-like-1, sPLD-like-1 and sPLD-like-12, representing each small PLD-like class in *P. infestans* (Meijer *et al*., 2011, Haas *et al*., 2009). The expression of *PLD-like-1*, *sPLD-like-1* and *sPLD-like-12* was determined by qRT-PCR in four developmental stages and during *in planta* growth. For *sPLD-like-1*, expression levels were similar in mycelium, sporangia, zoospores and germinating cysts with less than 2-fold variation between the four stages (Fig. 1A). Instead, the expression of *PLD-like-1* and *sPLD-like-12* was highest in germinating cysts with a 23.3- and 2.3-fold increase, when compared to the mycelial stage, respectively (Fig. 1A). Upon inoculation of potato leaves with *P. infestans* zoospores and during the subsequent disease development, the expression of *PLD-like-1* and *sPLD-like-1* started to increase at 2 days post inoculation (dpi), peaked at 6 dpi and thereafter declined (Fig. 1B). The expression of *sPLD-like-12* started to increase earlier, at 1 dpi, and also peaked earlier, at 3 dpi, before declining (Fig. 1B).

These results show that each of the three small PLD-likes has its own distinct expression pattern with *PLD-like-1* showing the highest relative increase in expression in germinating cysts and even higher in expanded lesions. In contrast, *sPLD-like-12* shows a more modest increase in germinated cysts and peaks relatively high shortly after inoculation, in an early stage of infection. Of the three small PLD-likes, *sPLD-like-1* shows the least strong fluctuation in expression but nevertheless, similar to *PLD-like-1*, its expression peaks at 6 dpi and gradually increases during lesion expansion.

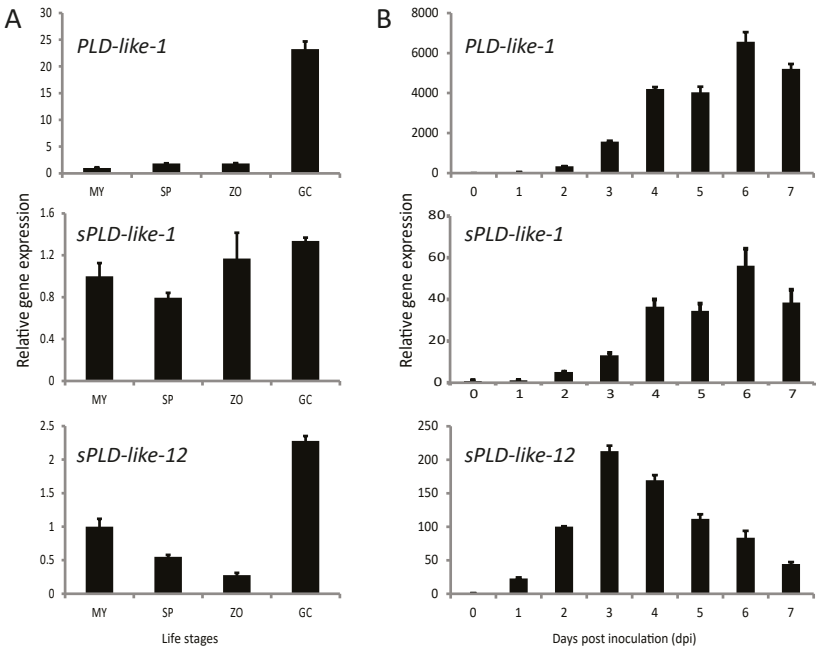

Figure 1. Expression profiles of small PLD-like genes of *Phytophthora infestans* during asexual development (A) and growth *in planta* after inoculation on potato leaves (B). Gene expression was analysed by qRT-PCR relative to the expression in mycelium (set at 1), with the *P. infestans ActA* as endogenous control. Error bars represent standard error of two biological repeats. MY: mycelium; SP: sporangia; ZO: zoospores; GC: germinating cysts.

5

Expression of small PLD-likes *in planta* results in local cell death

The three small PLD-likes genes from *P. infestans* were expressed in *N. benthamiana* leaves by agroinfiltration with *A. tumefaciens* strain Agl1 containing the binary vector pGRAB with the appropriate insert. When infiltrated with Agl1 carrying pGRAB with PLD-like-1, sPLD-like-1 or sPLD-like-12 the leaves showed no obvious response when compared to the negative controls, empty vector (EV) or GUS (Fig. 2). In contrast, the positive control, pGRAB carrying the *PiNPP1* gene, which encodes a general necrosis-inducing elicitor, caused tissue collapsed as reported previously (Kanneganti *et al.*, 2006, Wang *et al.*, 2015).

Experiments were repeated in the presence of P19, a viral protein that is known to function as a silencing suppressor. Co-infiltration of P19 with either PLD-like-1, sPLD-like-1 or sPLD-like-12 resulted in cell death within 6 days. Fig. 2 shows the response visualized by microscopy and observed as autofluorescence at 7 days after infiltration. Although this cell death response was relatively weak when compared to the positive control PiNPP1, no response was observed when the negative controls were co-infiltrated with P19. Also the number of spots reminiscent of cell death fluctuated per individual leaf, but was consistently distinguishable from the empty vector control (n>20).

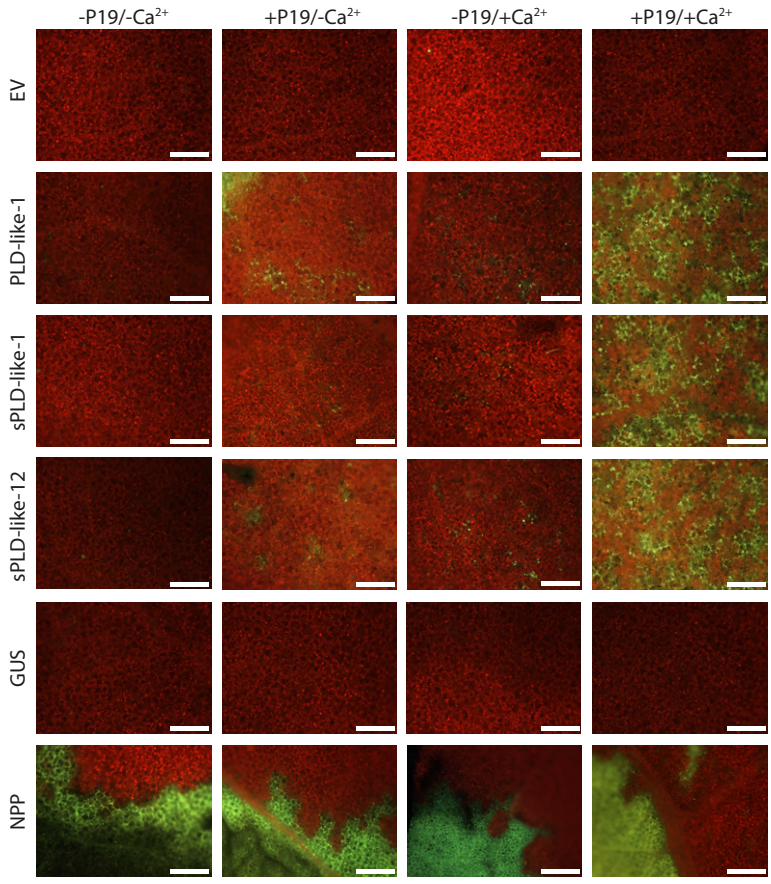

Figure 2. Small PLD-likes induce cell death in *Nicotiana benthamiana.* Epifluorescence pictures of leaves seven days after agroinfiltration with *Agrobacterium tumefaciens* Agl1 carrying small PLD-like constructs (PLD-like-1, sPLD-like-1 and sPLD-like-12) and control constructs (EV, GUS and NPP). Autofluorescence of dead cells is depicted in yellow-green and that of living cells in red. Cell death is enhanced by co-expression of the silencing suppressor P19 (+P19) and in the presence of calcium (+Ca^{2+}; infiltration of 2 mM $CaCl_2$ 24 hours after agroinfiltration). Experiments were repeated three times (n=24). Scale bars represent 500 µm.

PLD-induced cell death is more pronounced in the presence of calcium

Several PLDs, derived from a variety of organisms, require calcium for full activity. The optimum calcium concentration ranges from micromolar to millimolar levels depending on the type of PLD (Meijer & Munnik, 2003, Testerink & Munnik, 2011, Li *et al.*, 2009). To test whether the three *P. infestans* small PLD-likes are calcium-dependent we re-infiltrated the leaves with 2 mM $CaCl_2$ one day after agroinfiltration with Agl1 strains carrying the PLD constructs. In a pilot experiment, in which we tested a range of $CaCl_2$ concentrations, we determined 2 mM as the optimal concentration. With calcium as stimulating factor, local cell death was visible in leaves expressing the small PLD-likes but not in leaves infiltrated with the negative control EV. The cell death response was similar to that observed upon

co-infiltration with P19 (Fig. 2). However, combining the calcium treatment with the P19 silencing suppressor significantly boosted the cell death response (Fig. 2). Despite this boost the tissue collapse was still less prominent than in leaves infiltrated with NPP1.

To investigate whether calcium is the only ion capable of stimulating cell death we also tested the activity of magnesium, manganese and zinc. One day after co-agroinfiltration (PLD constructs and P19) leaves were re-infiltrated with 2 mM $CaCl_2$, $Ca(NO_3)_2$, $MnSO_4$, $MgCl_2$ or $ZnCl_2$. Microscopic examination revealed that calcium-based salts provoked cell death, whereas non-calcium salts were ineffective (Fig. 3). Apparently, the capacity of the small PLD-likes to induce cell death is enhanced specifically by calcium. To further investigate the importance of calcium, the infiltrations were repeated in the presence of lanthanum chloride. La^{3+} is a potent Ca^{2+}-channel blocker and acts as a calcium antagonist. In the presence of 100 µM $LaCl_3$, the cell death boosted by 2 mM $CaCl_2$ was strongly reduced (Fig. S1). In conclusion the cell death-inducing activity of the small PLD-likes is highly dependent on the presence of calcium.

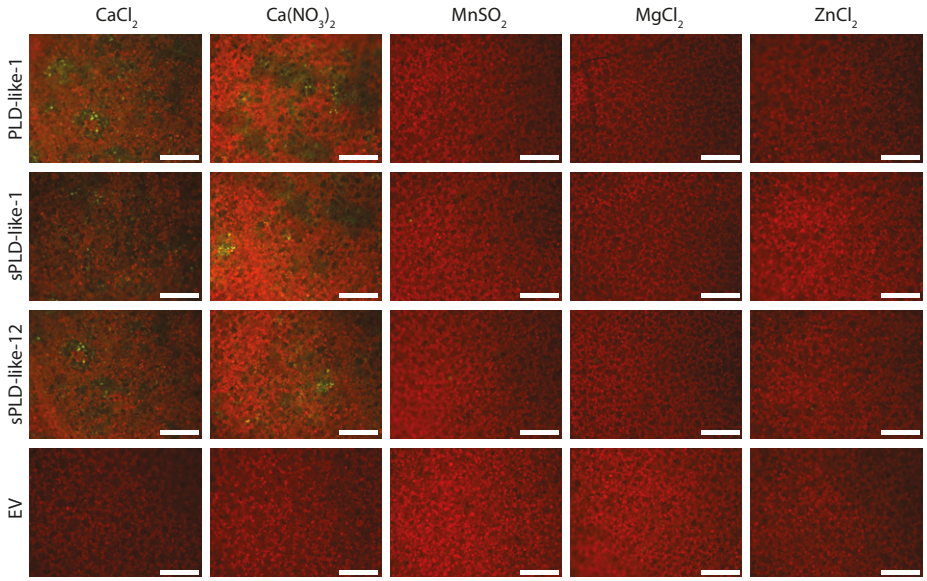

Figure 3. Cell death induced by small PLD-likes is enhanced in the presence of calcium. Epifluorescence pictures of *Nicotiana benthamiana* leaves seven days after agroinfiltration with *Agrobacterium tumefaciens* Agl1 carrying small PLD-like constructs (PLD-like-1, sPLD-like-1 and sPLD-like-12) and the control construct EV. One day after agroinfiltration the leaves were infiltrated with 2 mM solutions containing the different ions as indicated in the figure. Dead cells are depicted in green and living cells in red. Experiments were repeated three times. (n=24). Scale bars represent 500 µm.

The signal peptide is essential for the cell death-inducing activity of sPLD-likes

Based on signal peptide prediction programs, a highly supported signal peptide is predicted for sPLD-like-12 whereas for sPLD-like-1 the prediction is relatively weak (Meijer *et al*., 2011). To test whether the signal peptides of the two sPLD-likes are essential for cell death-inducing activity, truncated sPLD-like constructs were generated, in which we deleted the signal peptide (mSP). Agroinfiltration of these mSP constructs failed to induce cell death, even in the presence of P19 and calcium (Fig. 4). To find further evidence that secretion is essential for activity, we infiltrated sPLD-like constructs in which the authentic signal peptide was replaced by the signal peptide of the extracellular pathogenesis-related protein PR1 (Pfitzner & Goodman, 1987). This resulted in local cell death responses comparable to those observed for wild-type (s)PLD-likes (Fig. 4). These results suggest that the weakly predicted signal peptide in sPLD-like-1 is indeed functional as signal peptide. With respect to sPLD-like-12, the strong effect of the signal peptide was less anticipated. This type B sPLD-like shares over 50% sequence identity with PLD-like-1 (Meijer *et al*., 2011) and, as shown above, the latter induces cell death while lacking a signal peptide.

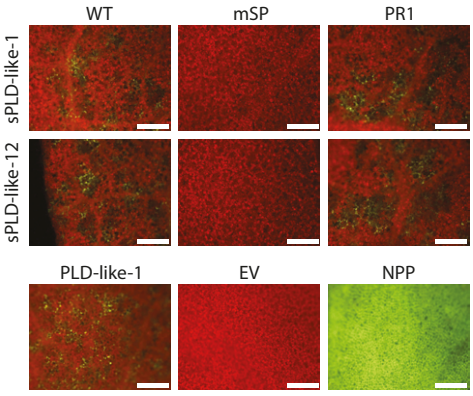

Figure 4. The secretion of sPLD-like-1 and sPLD-like-12 is required for cell death induction. Epifluorescence pictures of *Nicotiana benthamiana* leaves seven days after agroinfiltration with *A. tumefaciens* AgI1 carrying wild-type sPLD-like-1 and sPLD-like-12 constructs (WT), constructs lacking the signal peptide (mSP) or with the signal peptide replaced by the signal peptide from tobacco PR1 (PR1), and control constructs (PLD-like-1, EV and NPP). P19 was co-expressed and 2 mM $CaCl_2$ was infiltrated 24 hours after agroinfiltration. Dead cells are depicted in green and living cells in red. Experiments were repeated three times. (n=24). Scale bars represent 500 µm.

Induction of cell death is dependent on intact catalytic motifs

Amino acid substitutions in the catalytic domain of PLDs have proven to be destructive for their catalytic activity (Sung *et al.*, 1997). To test whether the cell death-inducing activity of each of the small PLD-likes depends on the HKD motif, all key amino acids were independently mutated (Table 1). In the case of sPLD-like-1 both HKD motifs are according to the core sequence. PLD-like-1 and sPLD-like-12 lack the aspartate residue (D) of HKD1. Therefore, we substituted the amino acids according to the positions in the core HKD motif plus one additional amino acid. In PLD-like-1 an adjacent aspartate (D188) residue was selected for mutation because it was hypothesized to replace the arginine at position 184 (R184). Similarly, in sPLD-like-12, the aspartate residue at position 261 (D261) was selected for mutation as it could be the substitute for the alanine located at position

253 (A253). Agroinfiltration of the substitution constructs in the absence or presence of P19/Ca^{2+} did not reveal any activity. Cell death was undetectable or at least extremely limited and comparable to the negative control. In contrast, the wild-type small PLD-likes, used as positive controls, induced local cell death as expected (Fig. 5, Table 1).

The lack of cell death-inducing activity for all modified small PLD-likes urged us to reconsider if the amino acid substitutions could have an effect on the stability of the proteins. Based on the nature of the substitutions it is unlikely that they affect the overall protein structure (Sung *et al*., 1997). For detecting the proteins, we added a 3HA tag to the C-terminus of the PLDs and checked whether the activity of the three wild-type PLDs is affected by the presence of the tag. Agroinfiltration confirmed that all three 3HA-tagged wild-type small PLD-likes induced cell death at similar levels as the non-tagged versions in the presence of Ca^{2+} (Fig. 6A). Proteins isolated from total leaf extracts, agroinfiltrated with the constructs but in the absence of Ca^{2+} to avoid cell death, were then analyzed for the presence of HA-tagged proteins by Western blot analyses. PLD-like-1-3HA was detected but sPLD-like1-3HA and sPLD-like-12-3HA remained undetected (Fig. S2C). The latter suggests that the HA-tagged proteins are either present at extreme low levels, unstable during the extraction procedure or that the tag was cleaved during protein processing. We failed to isolate tagged (s)PLD-likes from the apoplastic fluid of agroinfiltrated leaves.

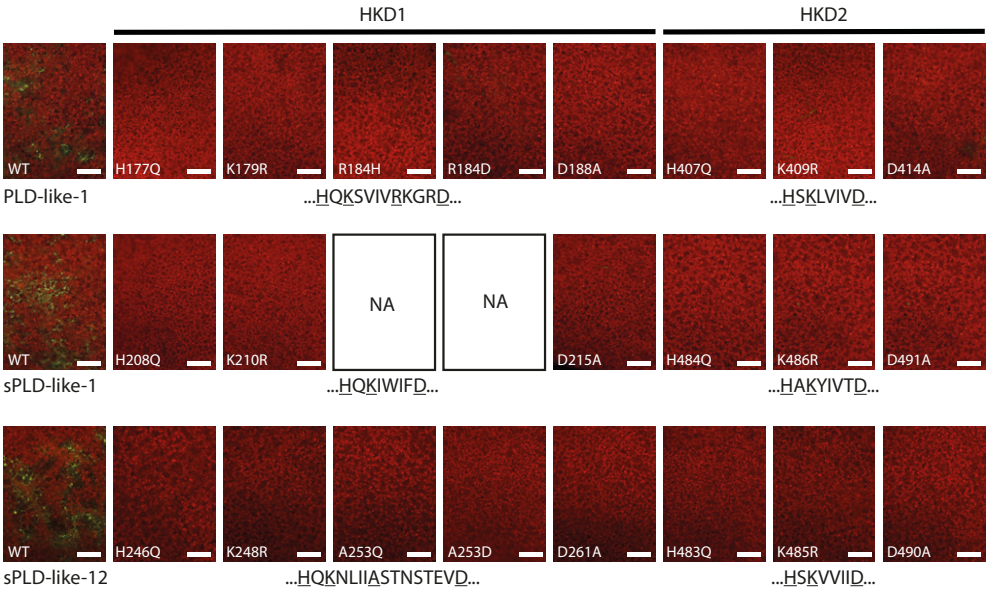

Figure 5. Mutations in the HKD motifs of the small PLD-likes abolish cell death-inducing activity. Epifluorescence pictures of *Nicotiana benthamiana* leaves seven days after agroinfiltration with *Agrobacterium tumefaciens* AgI1 carrying constructs with mutated versions of the small PLD-likes. P19 was co-expressed and 2 mM $CaCl_2$ was infiltrated 24 hours after agroinfiltration. Dead cells are depicted in yellow-green and living cells in red. Experiments were repeated three times (n=24). Scale bars represent 500 µm. NA= Not applicable.

Subsequently, substitution constructs of PLD-like-1 fused to a 3HA tag were tested for their cell death-inducing capacity. None of the substitution constructs induced cell death upon agroinfiltration in the presence of P19 and calcium whereas for five out of nine the corresponding protein was detected in extracts of infiltrated leaves (Fig. S2B, D). Since the HKD versions of sPLD-like-1 and sPLD-like-12 harbor similar amino acid substitutions, we anticipate that most of the mutated PLDs are produced albeit at extremely low levels similar to what was found for the wild-type PLD-likes. Nevertheless, there was no cell death and this is a strong indication that the catalytic activity of the small PLD-likes is important for inducing cell death.

Table 1. Amino acid substitutions in the HKD1 and HKD2 catalytic motifs of the small PLD-likes and the effect of these substitutions on cell death induction and virulence.

	HKD-1	HKD-2	cell death	virulence
PLD-like 1	..HQKSVIVRKGRD..	..HSKLVIVD..	+	+
H177Q	..QQKSVIVRKGRD..	..HSKLVIVD..	-	-
K179R	..HQRSVIVRKGRD..	..HSKLVIVD..	-	-
R184H	..HQKSVIVHKGRD..	..HSKLVIVD..	-	-
R184D	..HQKSVIVDKGRD..	..HSKLVIVD..	-	-
D188A	..HQKSVIVRKGRA..	..HSKLVIVD..	-	-
H407Q	..HQKSVIVRKGRD..	..QSKLVIVD..	-	-
K409R	..HQKSVIVRKGRD..	..HSRLVIVD..	-	-
D414A	..HQKSVIVRKGRD..	..HSKLVIVA..	-	-
sPLD-like 1	..HQKIWIFD..	..HAKYIVTD..	+	+
H208Q	..QQKIWIFD..	..HAKYIVTD..	-	-
K210R	..HQRIWIFD..	..HAKYIVTD..	-	-
D215A	..HQKIWIFA..	..HAKYIVTD..	-	-
H484Q	..HQKIWIFD..	..QAKYIVTD..	-	-
K486R	..HQKIWIFD..	..HARYIVTD..	-	-
D491A	..HQKIWIFD..	..HAKYIVTA..	-	-
sPLD-like 12	..HQKNLIIASTNSTEVD..	..HSKVVIID..	+	+
H246Q	..QQKNLIIASTNSTEVD..	..HSKVVIID..	-	-
K248R	..HQRNLIIASTNSTEVD..	..HSKVVIID..	-	-
A253Q	..HQKNLIIQSTNSTEVD..	..HSKVVIID..	-	-
A253D	..HQKNLIIDSTNSTEVD..	..HSKVVIID..	-	-
D261A	..HQKNLIIASTNSTEVA..	..HSKVVIID..	-	-
H483Q	..HQKNLIIASTNSTEVD..	..QSKVVIID..	-	-
K485R	..HQKNLIIASTNSTEVD..	..HSRVVIID..	-	-
D490A	..HQKNLIIASTNSTEVD..	..HSKVVIIA..	-	-

In planta expression of small PLD-likes promotes *P. infestans* growth and sporulation

To investigate whether or not small PLD-likes have a role in virulence of *P. infestans* we tested the effect of heterologous expression of small PLD-likes in *N. benthamiana* on lesion growth. Leaves were agroinfiltrated with the various PLD constructs described above. After two days, leaves were detached and inoculated with *P. infestans* strain 14-3-GFP (Du *et al.*, 2015). Lesions on leaves agroinfiltrated with the wild-type small PLD-likes were significantly larger than those agroinfiltrated with EV (one-way ANOVA, $P<0.05$, Fig. 6A, B). This was accompanied by an enhanced sporulation as more sporangia were produced compared to the control (Fig. 6C). Deletion of the signal peptide of sPLD-like-1 and sPLD-like-12 resulted in the loss of both lesion growth promotion and enhanced sporulation, whereas replacement by the PR1 signal peptide partially restored it (Fig. 6B, C). Also, the HKD substitution constructs failed to promote lesion growth and sporangia production; the effects were in line with those of the controls (EV and non-infiltrated leaves; Fig. 6B, C). Similarly, all HA-tagged small PLD-likes, both wild-type and mutants, behaved as their non-tagged counterparts (Fig. 6B, C). Strikingly, the growth promotion and enhanced sporulation was also observed when the agroinfiltrations were performed in the absence of P19 and/or calcium (Fig. S3), conditions that boosted the (s)PLD-likes with respect to their cell death-inducing activity. It could well be that *P. infestans* modulates the leaf environment to create optimal conditions for its own secreted enzymes, including its endogenous sPLD-likes. The (s)PLD-likes expressed in the plant cell via agroinfiltration may also benefit from this modulation and hence no longer need the extra boost of calcium and/or P19.

Altogether, these results suggest that the sPLD-likes enter the secretory pathway in order to execute their growth-promoting function and that in all three small PLD-likes a functional HKD motif is required to promote growth and sporulation.

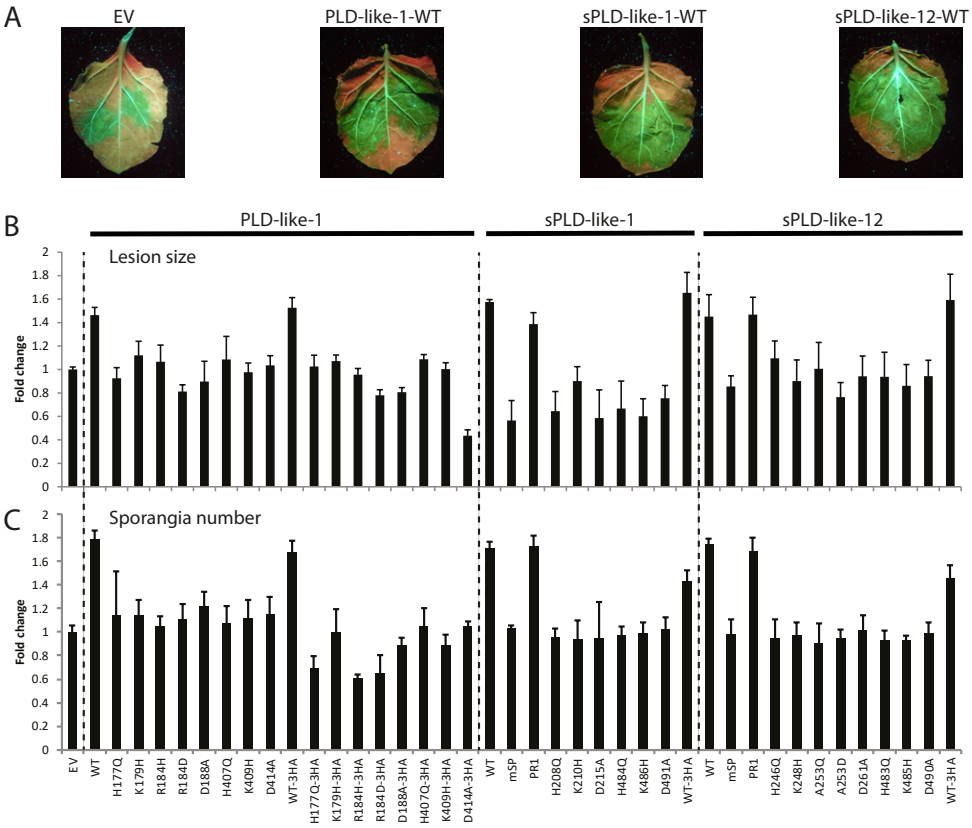

Figure 6. Small PLD-likes promote *Phytophthora infestans* growth and sporulation. (A) Representative UV pictures of agroinfiltrated *Nicotiana benthamiana* leaves infected with *Phytophthora infestans* 14-3-GFP, seven days post inoculation. (B) Lesion size and (C) sporulation of *Phytophthora infestans* on leaves infiltrated with various constructs of small PLD-likes, calculated as fold change in lesion size or amount of sporangia respectively, compared to those on leaves infiltrated with the control construct (EV). P19 was co-expressed and 2 mM $CaCl_2$ was infiltrated 24 hours after agroinfiltration. Experiments were repeated three times. Error bars represent standard deviation (n=24).

Discussion

In this study, we tested the hypothesis that small PLD-likes produced by oomycetes act as virulence factors. Incentives for this study were findings that PLDs and secreted PLDs have been implicated as major virulence determinants in bacteria and fungi (McKean *et al*., 2007, Jacobs *et al*., 2010, Sitaraman *et al*., 2012, Dolan *et al*., 2004, Hube *et al*., 2001, Lery *et al*., 2014, Yang *et al*., 2015, Lajoie & Cordes, 2015) and the fact that oomycetes have an extended repertoire of PLDs including subfamilies with novel oomycete-specific PLDs and potentially secreted PLDs (Meijer *et al*., 2011, Meijer & Govers, 2006).

For our experiments we selected three candidates out of the 13 small PLD-likes that were identified in *P. infestans,* each representing a distinct subfamily. sPLD-like-1 is the only sPLD-like type A in *P. infestans* and is quite divergent from all other PLDs. sPLD-like-A genes are widespread in the tree of life and likely share an ancient ancestor. The closest relatives of sPLD-like-1 are found in plants (Meijer *et al*., 2011). sPLD-like-12 is one of the nine members in the sPLD-like-B subfamily in *P. infestans*, while the closely related PLD-like-l belongs to a PLD-like subfamily with three members. Unlike sPLD-like-B's, PLD-likes lack a signal peptide (Meijer *et al*., 2011). All PLD subfamilies are well conserved among oomycetes always with multiple copies of sPLD-like-B's and PLD-likes, albeit with some variations in numbers (Baxter *et al*., 2010, Levesque *et al*., 2010, Kemen *et al*., 2011, Jiang *et al*., 2013, Sharma *et al*., 2015). The clustering of sPLD-like-B's and PLD-likes and the far distance to other PLDs is reminiscent of expanded oomycete-specific protein families whose members have putative functions in virulence or pathogenicity.

A prerequisite for acting as virulence factor is expression of the encoding gene during interaction of the pathogen with its host. Expression of all three small PLD-like genes was found to be upregulated during *in planta* growth in potato leaves. *PLD-like-1*, one of the highest transcribed genes in *P. infestans* (Meijer *et al*., 2011), peaks in a late stage of the interaction, at 6 dpi, concomitantly with *sPLD-like-1*. In contrast, *sPLD-like-12* is induced shortly after inoculation with a peak at 3 dpi presumably functioning in an early stage of infection. Most sPLD-like genes, including *sPLD-like-1* and *sPLD-like-12*, also showed increased expression during tuber infections (Ah-Fong *et al*., 2017). Moreover, during infection of tomato leaves, expression of *PLD-like-1* and *sPLD-like-1* (but not of *sPLD-like-12*) was monitored and also found to be upregulated (Zuluaga *et al*., 2016).

PA, the product of hydrolysis of phospholipids catalyzed by PLD, is a multifunctional second messenger that is frequently generated during stress conditions. To boost the production of PA during infection, pathogens can either hijack PLDs from the host, as for example done by the *Red clover necrotic mosaic virus* in *N. benthamiana* (Hyodo *et al*., 2015) or they can produce their own PLDs that then exploit plant phospholipids as substrate. The finding that strong PLD activity was found in the extracellular medium of *P. infestans* (Meijer *et al*., 2014, Meijer *et al*., 2011) suggests that this pathogen secretes PLDs, instrumental in colonization of the host. To test how the plant responds to pathogen-derived PLDs we expressed the small PLD-like genes *in planta* and monitored the responses. The responses observed as localized cell death was enhanced in the presence of calcium, a well-known stimulator of PLD activity (Meijer & Munnik, 2003, Testerink & Munnik, 2011, Li *et al*., 2009). Replacement of calcium with other ions or addition of the Ca^{2+}-channel blocker La^{3+}, resulted in a strongly reduced cell death showing indirectly that PLD activity is the trigger for the cell death. This was further confirmed by the finding that *in planta* expression of small PLD-like genes with point mutations affecting the HKD motifs, failed to induce cell death. For PLD-like-1 we could demonstrate by Western blot

analyses that this loss of activity is not due to loss of the overall stability of the mutated versions of the protein. Five out of nine HKD mutated versions of PLD-like-1 were found to be stably present in the infiltrated leaves without causing cell death. For sPLD-like-1 and sPLD-like-12 we could not demonstrate this because the 3HA tagged versions of these two small PLD-likes were not detectable on Western blots, neither in total extracts from agroinfiltrated leaves nor in apoplastic fluid. Yet, since most of the mutated PLD-like-1 versions are stable we assume that also the other mutated small PLD-likes are stable; in all cases similar amino acids are changed in motifs that are highly conserved. Moreover, it is well established that such targeted modifications of catalytic sites of PLDs significantly lower phospholipid turnover or simply render the enzyme inactive (Sung *et al*., 1997, Rudolph *et al*., 1999). All together we can conclude that the induction of cell death by the small PLD-likes fully depends on catalytic activity.

The observation that the three small PLD-likes elicited similar responses irrespective of the signal peptide raised the question where the PLD activity resides. Based on the finding that removal of the signal peptide abolished activity one might conclude that in the agroinfiltrated leaves sPLD-like-1 and sPLD-like-12 are secreted in the apoplast, the site where they readily end up during natural infections when secreted by *P. infestans*. The conventional transport of secreted proteins is via the endoplasmic reticulum, a main Ca^{2+} storage organelle (Denecke, 2007). PLD-like-1 is leaderless but could still be secreted via an unconventional secretion pathway. Many organisms have such pathways and also *P. infestans* seems to have it since several proteins lacking a signal peptide were identified in the secretome (Meijer *et al*., 2014). However, there are also arguments in favor of the hypothesis that the small PLD-likes are functional inside plant cells. One argument is the stimulating influence of calcium on all three small PLD-likes, including the one lacking a signal peptide, combined with the inhibitory effect on cell death by La^{3+}, a Ca^{2+}-channel blocker. Since Ca^{2+}-channels are located in the plasma membrane or in intracellular membranes, La^{3+} is not expected to affect PLD activity in the apoplast. Another argument is the occurrence of cell death in one or just a few cells. In general, the cell death response was relatively weak and, in contrast to the full tissue collapse elicited for example by NPP1 (Kanneganti *et al*., 2006, Wang *et al*., 2015) of INF1 (Kamoun *et al*., 1997), cell death was limited to individual cells or small cell clusters spread over the infiltrated area. This suggests that minute quantities of the PLDs are sufficient to induce cell death, thereby disallowing diffusion of proteins to adjacent cells, as would be expected when the PLDs are entering the apoplast. A third argument, although less strong, is that the only 3HA-tagged small PLD-like detectable in total leaf extracts by Western blotting was not detected in apoplastic fluid. In case these arguments hold, the next question is how the PLDs would get into the host. Many pathogens, including *Phytophthora* spp., have the ability to translocate effectors into the host cell where they function as virulence factors mainly by suppressing defense responses. Such effectors have a characteristic RXLR motif

in the N-terminal part that is essential for the translocation. None of the PLDs, including the ones from the other subfamilies not studied here, has such a motif.

To assess the role of the small PLD-likes during host infection, we tested if the presence of higher levels would be beneficial for *P. infestans* growth. This revealed that all three small PLD-likes facilitate *P. infestans* growth, resulting in the development of larger lesions and more sporangia. In contrast, the presence of the HKD mutated versions of the PLDs had no effect, demonstrating that promotion of virulence by the small PLD-likes depends on intact catalytic domains. The presence of calcium and/or the silencing suppressor did not further increase the virulence-promoting activity. Possibly, calcium was not limited because of the local increase in calcium levels that is frequently reported to occur during plant pathogen interactions (Zhang *et al.*, 2014).

The *in vivo* role of small PLD-likes remains to be elucidated. The cell death-inducing and growth-promoting features might turn out to be extrapolations of their enzymatic activity. During infection the plasma membranes of infected plant cells are modified at sites where haustoria are formed and invade the host cell (Micali *et al.*, 2011). These changes in structure and physiology are initiated by the pathogen to promote exchange of proteins and nutrients without provoking immediate cell death. This study strongly supports our hypothesis that small PLD-likes play a role in virulence. We anticipate that under natural conditions they are deployed by *P. infestans* to modulate plant membranes thereby facilitating invasion and colonization. Their activity can result in localized PA production, e.g. on the extrahaustorial membrane or any membrane, or influence membrane curvatures, and hence lead to recruitment and dynamic regulation of the activity of a broad spectrum of molecules (Putta *et al.*, 2016). We assume that as a group the small PLD-likes can synergistically enhance the pathogenic capacity of *P. infestans*.

Experimental procedures

Phytophthora infestans strains and material

Phytophthora infestans strains 88069 and 14-3-GFP were maintained on rye sucrose medium at 18°C. Mycelium, sporangia, zoospores and germinated cysts for RNA isolation were obtained as described previously (Hua *et al.*, 2013) and stored at -80°C.

Plant material and infection assays

Potato (cultivar Bintje, R0) and *N. benthamiana* plants were grown in a greenhouse under standardized conditions. Infected plant material for RNA isolation was obtained by inoculation of leaves detached from six-week-old potato plants with 20 µl of a zoospore suspension (1×10^4 zoospores/ml) of *P. infestans* strain 88069 followed by incubation in

a climate chamber at 18°C and high relative humidity. Leaves were harvested at seven consecutive days post inoculation (dpi), quickly frozen in liquid N_2, and stored at -80°C.

RNA extraction and qRT-PCR

RNA from the *P. infestans* material was extracted using TriZol (Invitrogen). RNA from infected and healthy potato leaves was isolated using the NucleoSpin RNA II RNA extraction kit (Macherey-Nagel) following the procedures described by the manufacturer. To remove genomic DNA contamination, total RNA was treated with DNaseI followed by a phenol/chloroform extraction. For qRT-PCR, first-strand cDNA was synthesized from 2 µg of total DNAse-treated RNA using oligodT and SuperScript first-strand synthesis system (Invitrogen). PCR was performed with an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR-GREEN Master mix (Bio-Rad) (95°C for 5 min; 40 cycles of 30 s at 95°C, 30 s at 60°C and 20 s at 72°C; followed by 10 min at 72°C). Gene specific primers are listed in Table S1. Each qRT-PCR was performed in triplicate, with three biological repeats and gene expression was normalized according to *P. infestans* actin A (*PiActA*) expression (Du *et al*., 2015, Hua *et al*., 2013).

Plasmid construction

Primers used for cloning, generating mutations and tagging PLD genes are listed in Table S1. Small PLD-like genes were amplified from genomic DNA isolated from *P. infestans* strain 88069 (Haas *et al*., 2009, Meijer *et al*., 2011). Amplification was performed with proofreading *Pfu* polymerase (Promega). Fusion genes (PR1/HA-tagged) and mutated versions were produced by overlap PCR. Fragments were purified from gel and cloned into pGEM-T Easy vectors (Promega) that were transformed into *E. coli* strain DH5. Inserts were sequenced using standard M13 primers (Macrogen Europe) and cloned into the binary vector pGRAB (Whisson *et al.*, 2007) that were transformed into *A. tumefaciens* Agl1 strain.

Agroinfiltration of *Nicotiana benthamiana*

Agroinfiltration assays, each repeated at least three times, were carried out with *A. tumefaciens* Agl1 strains essentially as described by Van Poppel *et al.* (2008). The final optical density (OD_{600}) of the bacterial culture used for infiltration was 1.0. For co-infiltration bacterial cultures were mixed in a 1:1 ratio till a final OD_{600} of 1.0. Leaves of 4- to 6-week-old *N. benthamiana* plants were infiltrated at the lower side and then the area was marked. Positive and negative controls were Agl1 strains carrying *NPP1* (Wang *et al*., 2015), the empty vector (EV) or *GUS* (Du *et al*., 2015), respectively. Infiltrations with $CaCl_2$, $Ca(NO_3)_2$, $MnSO_4$, $MgCl_2$, $ZnCl_2$ and/or $LaCl_3$ were executed 24 h after agroinfiltration covering the same area as the agroinfiltration.

Responses were monitored between 3 to 6 days after agroinfiltration. For fluorescence microscopy a Nikon 90i epifluorescence microscope was used equipped with a digital imaging system (Nikon DS-5Mc camera, Nikon NIS-AR software). Autofluorescence was detected using a GFP filter cube (GFP-LP, EX 460-500, DM 505, BA 510). Images were taken randomly from each infiltrated area. For protein isolation agroinfiltrated *N. benthamiana* leaves were harvested 3 days after agroinfiltration. For infection of the agroinfiltrated leaves, the leaves were detached 2 days after agroinfiltration, inoculated with *P. infestans* strain 14-3-GFP (20 µl of 1×10^4 zoospores/ml) and incubated as described above. Lesion size was determined by measuring the horizontal and vertical diameter of each lesion and then calculating the lesion area in mm^2. To estimate the number of sporangia produced on each lesion, one leaf disc of 0.9 cm diameter was submerged in 1 ml MQ water, in a 1.5 ml Eppendorf tube. After vortexing for about 1 minute, sporangia were counted using a haemocytometer.

Protein extraction and immunoblotting

Leaves were grinded in liquid nitrogen in the presence of RIPA extraction buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% IPEGAL (NP-40), 0.5% sodium deoxycholate, 0.1% SDS). The samples were boiled for 5 min in SDS-loading buffer before being loaded on a 12% SDS/PAGE gel. After electrophoresis the proteins were transferred to Immune-Blot polyvinylidene difluoride membranes (Bio-Rad). For detection of HA-tagged proteins, the membranes were incubated for 1 h at room temperature with HRP-conjugated HA-antibodies diluted 1:5000 in PBS-T with 5% skimmed milk (Promega). The HA-protein signals were detected using West Femto Chemiluminescent Substrate (Thermo Scientific). Pictures were taken by UV imaging in a ChemiDoc MP system (Bio-Rad laboratories). The proteins blotted on the membranes were stained with Coomassie Brilliant Blue R 250 to check if the amount of protein loaded in each lane is in the same range.

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References

Ah-Fong, A.M.V., Shrivastava, J. and Judelson, H.S. (2017) Lifestyle, gene gain and loss, and transcriptional remodeling cause divergence in the transcriptomes of *Phytophthora infestans* and *Pythium ultimum* during potato tuber colonization. *BMC Genomics,* **18,** 764.

Andersson, M.X., Kourtchenko, O., Dangl, J.L., Mackey, D. and Ellerström, M. (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. *Plant J,* **47,** 947-959.

Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A.M.V., Anderson, R., Badejoko, W., *et al.* (2010) Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science,* **330,** 1549-1551

Behnia, R. and Munro, S. (2005) Organelle identity and the signposts for membrane traffic. *Nature,* **438,** 597-604.

Beligni, M.V., Bagnato, C., Prados, M.B., Bondino, H., Laxalt, A.M., Munnik, T. and Ten Have, A. (2015) The diversity of algal phospholipase D homologs revealed by biocomputational analysis. *J Phycol,* **51,** 943-962.

Denecke, J. (2007) Endoplasmic Reticulum In: *Handbook of plant science.* (Roberts, K., ed.). Chichester, West Sussex, England; Hoboken, NJ, USA: Wiley, pp. 223-226.

Dolan, J.W., Bell, A.C., Hube, B., Schaller, M., Warner, T.F. and Balish, E. (2004) *Candida albicans* PLD1 activity is required for full virulence. *Med Mycol,* **42,** 439-447.

Du, Y., Mpina, M.H., Birch, P.R.J., Bouwmeester, K. and Govers, F. (2015) *Phytophthora infestans* RXLR effector AVR1 interacts with exocyst component Sec5 to manipulate plant immunity. *Plant Physiol,* **169,** 1975–1990.

Ghannoum, M.A. (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev,* **13,** 122-143.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H.Y, Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T., *et al.* (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature,* **461,** 393 - 398.

Hua, C., Meijer, H.J.G., de Keijzer, J., Zhao, W., Wang, Y. and Govers, F. (2013) GK4, a G-protein-coupled receptor with a phosphatidylinositol phosphate kinase domain in *Phytophthora infestans*, is involved in sporangia development and virulence. *Mol Microbiol,* **88,** 352-370.

Hube, B., Hess, D., Baker, C.A., Schaller, M., Schäfer, W. and Dolan, J.W. (2001) The role and relevance of phospholipase D1 during growth and dimorphism of *Candida albicans*. *Microbiology,* **147,** 879-889.

Hyodo, K., Taniguchi, T., Manabe, Y., Kaido, M., Mise, K., Sugawara, T., Taniguchi, H. and Okuno, T. (2015) Phosphatidic acid produced by phospholipase D promotes RNA replication of a plant RNA virus. *PLoS Pathog,* **11,** e1004909.

Jacobs, A.C., Hood, I., Boyd, K.L., Olson, P.D., Morrison, J.M., Carson, S., Sayood, K., Iwen, P.C., Skaar, E.P. and Dunman, P.M. (2010) Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infect Immun,* **78,** 1952-1962.

Jiang, R.H.Y., de Bruijn, I., Haas, B.J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Bulone, V., Díaz-Moreno, S.M., *et al.* (2013) Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. *PLoS Genet,* **9**, e1003272.

Kamoun, S., van West, P., de Jong, A.J., de Groot, K.E., Vleeshouwers, V.G.A.A. and Govers, F. (1997) A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol Plant Microbe Interact.,* **10,** 13-20.

Kanneganti, T.-D., Huitema, E., Cakir, C. and Kamoun, S. (2006) Synergistic interactions of the plant cell death pathways induced by *Phytophthora infestans* Nep1-Like protein PiNPP1.1 and INF1 elicitin. *Mol Plant Microbe Interact.,* **19,** 854-863.

Kemen, E., Gardiner, A., Schultz-Larsen, T., Kemen, A.C., Balmuth, A.L., Robert-Seilaniantz, Bailey, K., Holub, E., Studholme, D.J., Maclean, D., *et al.* (2011) Gene gain and loss during evolution of obligate parasitism in the white rust pathogen of *Arabidopsis thaliana*. *PLoS Biol,* **9,** e1001094.

Köhler, G.A., Brenot, A., Haas-Stapleton, E., Agabian, N., Deva, R. and Nigam, S. (2006) Phospholipase A(2) and phospholipase B activities in fungi. *Biochim Biophys Acta,* **1761,** 1391-1399.

Lajoie, D.M. and Cordes, M.H.J. (2015) Spider, bacterial and fungal phospholipase D toxins make cyclic phosphate products. *Toxicon,* **108,** 176-180.

Lemmon, M.A. (2003) Phosphoinositide recognition domains. *Traffic,* **4,** 201-213.

Lery, L.M.S., Frangeul, L., Tomas, A., Passet, V., Almeida, A.S., Bialek-Davenet, S., Barbe, V., Bengoechea, J.A., Sansonetti, P., Brisse, S. and Tournebize, R. (2014) Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biology,* **12,** 41.

Levesque, C.A., Brouwer, H., Cano, L., Hamilton, J.P., Holt, C., Huitema, E., Raffaele, S., Robideau, G.P., Thines, M. and Win, J., *et al*. (2010) Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. *Genome Biol,* **11,** R73.

Li, M., Hong, Y. and Wang, X. (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. *Biochim Biophys Acta - Mol Cell Biol Lipids,* **1791,** 927-935.

Liu, Q., Zhang, C., Yang, Y. and Hu, X. (2010) Genome-wide and molecular evolution analyses of the phospholipase D gene family in Poplar and Grape. *BMC Plant Biology,* **10,** 117-117.

McKean, S.C., Davies, J.K. and Moore, R.J. (2007) Expression of phospholipase D, the major virulence factor of *Corynebacterium pseudotuberculosis*, is regulated by multiple environmental factors and plays a role in macrophage death. *Microbiology,* **153,** 2203-2211.

Meijer and Munnik (2003) Phospholipid-based signaling in plants. *Annu Rev Plant Biol.,* **54,** 265-306.

Meijer, H.J.G. and Govers, F. (2006) Genomewide analysis of phospholipid signaling genes in *Phytophthora* spp.: Novelties and a missing link. *Mol Plant Microbe Interact,* **19,** 1337-1347.

Meijer, H.J.G., Hassen, H.H. and Govers, F. (2011) *Phytophthora infestans* has a plethora of phospholipase D enzymes including a subclass that has extracellular activity. *PLoS ONE,* **6,** e17767.

Meijer, H.J.G., Latijnhouwers, M., Ligterink, W. and Govers, F. (2005) A transmembrane phospholipase D in *Phytophthora*; a novel PLD subfamily. *Gene,* **350,** 173-182.

Meijer, H.J.G., Mancuso, F.M., Espadas, G., Seidl, M.F., Chiva, C., Govers, F. and Sabido, E. (2014) Profiling the secretome and extracellular proteome of the potato late blight pathogen *Phytophthora infestans*. *Mol Cell Proteomics,* **13,** 2101-2113.

Micali, C.O., Neumann, U., Grunewald, D., Panstruga, R. and O'Connell, R. (2011) Biogenesis of a specialized plant–fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. *Cell Microbiol,* **13,** 210-226.

Park, J., Gu, Y., Lee, Y., Yang, Z. and Lee, Y. (2004) Phosphatidic acid induces leaf cell death in *Arabidopsis* by activating the Rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation. *Plant Physiol,* **134,** 129-136.

Pfitzner, U.M. and Goodman, H.M. (1987) Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. *Nucleic Acids Res,* **15,** 4449-4465.

Putta, P., Rankenberg, J., Korver, R.A., van Wijk, R., Munnik, T., Testerink, C. and Kooijman, E.E. (2016) Phosphatidic acid binding proteins display differential binding as a function of membrane curvature stress and chemical properties. *Biochim Biophys Acta - Biomembr,* **1858,** 2709-2716.

Rudolph, A.E., Stuckey, J.A., Zhao, Y., Matthews, H.R., Patton, W.A., Moss, J. and Dixon, J.E. (1999) Expression, characterization, and mutagenesis of the *Yersinia pestis* murine toxin, a phospholipase D superfamily member. *J Biol Chem,* **274,** 11824-11831.

Selvy, P.E., Lavieri, R.R., Lindsley, C.W. and Brown, H.A. (2011) Phospholipase D - enzymology, functionality, and chemical modulation. *Chem Rev,* **111,** 6064-6119.

Sharma, R., Xia, X., Cano, L.M., Evangelisti, E., Kemen, E., Judelson, H., Oome, S., Sambles, C., van den Hoogen, D.J., Kitner, M., Klein,

J., Meijer, H.J.G., Spring, O., Win, J., Zipper, R., Bode, H.B., Govers, F., Kamoun, S., Schornack, S., Studholme, D., Van den Ackerveken, G. and Thines, M. (2015) Genome analyses of the sunflower pathogen *Plasmopara halstedii* provide insights into effector evolution in downy mildews and *Phytophthora*. *BMC Genomics,* **16,** 741.

Sitaraman, R., Israel, D.A., Romero-Gallo, J. and Peek, R.M. (2012) Cell-associated hemolysis induced by *Helicobacter pylori* is mediated by phospholipases with mitogen-activated protein kinase-activating properties. *J Clin Microbiol,* **50,** 1014-1018.

Sung, T.C., Roper, R.L., Zhang, Y., Rudge, S.A., Temel, R., Hammond, S.M., Morris, A.J., Moss, B., Engebrecht, J. and Frohman, M.A. (1997) Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. *EMBO J,* **16,** 4519-4530.

Tang, K., Dong, C.-J. and Liu, J.-Y. (2016) Genome-wide comparative analysis of the phospholipase D gene families among allotetraploid cotton and Its diploid progenitors. *PLoS ONE,* **11,** e0156281.

Testerink, C. and Munnik, T. (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. *Trends Plant Sci,* **10,** 368-375.

Testerink, C. and Munnik, T. (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J Exp Bot,* **62,** 2349-2361.

van Poppel, P.M.J.A., Guo, J., van de Vondervoort, P.J.I., Jung, M.W.M., Birch, P.R.J., Whisson, S.C. and Govers, F. (2008) The *Phytophthora infestans* avirulence gene Avr4 encodes an RXLR-dEER effector. *Mol Plant Microbe Interact,* **21,** 1460-1470.

Wang, Y., Weide, R., Govers, F. and Bouwmeester, K. (2015) L-type lectin receptor kinases in *Nicotiana benthamiana* and tomato and their role in *Phytophthora* resistance. *J Exp Bot,* **66,** 6731-6743.

Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L. and Birch, P.R.J. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature,* **450,** 115 - 118.

Yang, F., Abdelnabby, H. and Xiao, Y. (2015) The role of a phospholipase (PLD) in virulence of *Purpureocillium lilacinum* (*Paecilomyces lilacinum*). *Microb Pathog,* **85,** 11-20.

Zhang, L., Du, L. and Poovaiah, B.W. (2014) Calcium signaling and biotic defense responses in plants. *Plant Signal Behav,* **9,** e973818.

Zhang, Q., Lin, F., Mao, T., Nie, J., Yan, M., Yuan, M. and Zhang, W. (2012) Phosphatidic acid regulates microtubule organization by interacting with MAP65-1 in response to salt stress in *Arabidopsis*. *Plant Cell,* **24,** 4555-4576.

Zuluaga, A.P., Vega-Arreguín, J.C., Fei, Z., Ponnala, L., Lee, S.J., Matas, A.J., Patev, S., Fry, W.E. and Rose, J.K.C. (2016) Transcriptional dynamics of *Phytophthora infestans* during sequential stages of hemibiotrophic infection of tomato. *Mol Plant Pathol,* **17,** 29-41.

Supplemental data

Table S1. Primers used in this study.

Cloning				
Gene	**Forward primer**	**Sequence (5′-3′)**	**Reverse primer**	**Sequence (5′-3′)**
PLD-like-1	PLDL1-H177Q-F	CGTCGCACCAGCAGAAGTCG	PLDL1-H177Q-R	CGACTTCTGCTGGTGCGACG
	PLDL1-K179R-F	CACCACCAGAGGTCGGTGATC	PLDL1-K179R-R	GATCACCGACCTCTGGTG GTG
	PLD1-R184D-F	GTGATCGTGGATAAGG GCCGTG	PLD1-R184D-R	CACGGCCCTTATCCACGAT CACC
	PLDL1-D188A-F	GGGCCGTGCTCTCGTCGC	PLDL1-D188A-R	CGAGAGCACGGCCCTTACG
	PLDL1-H407Q-F	GTACATCCAGAGTAAGCTGG	PLDL1-H407Q-R	GCTTACTCTGGATGTACAGC
	PLDL1-K409R-F	CACAGTAGGCTGGTGATTG	PLDL1-K409R-R	CACCAGCCTACTGTGGATG
	PLDL1-D414A-F	GATTGTCGCCGACGTGTAC	PLDL1-D414A-R	CACGTCGGCGACAATCAC
	PLD1-F-HA	CGTTGAGGGGGATGACGGG TTAATTAACATC	PLD1-R-HA	GATGTTAATTAACCCGTCAT CCCCCTCAACG
sPLD-like-1	sPLD1-H208Q-F	GCGGTATTTTACAGCAGAA GATATGG	sPLD1-H208Q-R	CCATATCTTCTGCTGTAAA TACCGC
	sPLD1-K210R-F	GTATTTTACATCAGAGGA TATGGATCTTTGAC	sPLD1-K210R-R	GTCAAAGATCCATATCCTCT GATGTAAAATAC
	sPLD1-D215V-F	GATATGGATCTTTGTCGAC AGTCAC	sPLD1-D215V-R	GTGACTGTCGACAAAGATC CATATC
	sPLD1-H484Q-F	GCGTGAATCAGGCCAAG TACATTG	sPLD1-H484Q-R	CAATGTACTTGGCCT GATTCACGC
	sPLD1-K486R-F	GTGAATCATGCCAGGTACATT GTCAC	sPLD1-K486R-R	GTGACAATGTACCTGGCAT GATTCAC
	sPLD1-D491A-F	CATTGTCACCGTCACTC GCGTG	sPLD1-D491A-R	CACGCGAGTGACGGT GACAATG
	PR1-sPLD1-F	GCAATTTCACGAGGAGT GAAGCGGGCACGGCAA GAGTGGGATATTAC	sPLDL1-R	GCCGCGGCCGCTTAC GATTCAGCAGATACAAT
	sPLDl-mSP-F	CGGAATTCATGCGCTTCA CTCCTCGTGAA		
	sPLD1-F-HA	GAAATTGTATCTGCTGAATC GGGGTTAATTAACATC	sPLD1-R-HA	GATGTTAATTAACCCCGATT CAGCAGATACAATTTC

Table S1. *Continued*

Cloning				
Gene	**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
sPLD-like-12	sPLDL12-H246Q-F	CAACTCACCAGCAGAAGAATC	sPLDL12-H246Q-R	GATTCTTCTGCTGGTGAGTTG
	sPLDL12-K248R-F	CCAGAGGAATCTAATCATAGC	sPLDL12-K248R-R	GCTATGATTAGATTCCTCTGG
	sPLD-12-A253D-F	CTAATCATAGACTCTACCAATTC GACG	sPLD-12-A253D-R	CGTCGAATTGGTAGAGTCTAT GATTAG
	sPLDL12-D261A-F	GGTGGCTCAACACCCTG	sPLDL12-D261A-R	GTGTTGAGCCACCTCCG
	sPLDL12-H483Q-F	CATCCAGTCAAAAGTTGTGATC	sPLDL12-H483Q-R	CAACTTTTGACTGGATGTA-CAG
	sPLDL12-K485R-F	CACTCAAGAGTTGTGATCATC	sPLDL12-K485R-R	CACAACTCTTGAGTGGATG
	sPLDL12-D490R-F	GATCATCGCCGACGTGTAC	sPLDL12-D490R-R	CACGTCGGCGATGATCAC
	PR1-sPLD12-F	CGACTGACTGTGGAGA GAATTGAGGGCACG GCAAGAGTGGGATATTAC	sPLD12-R	GCCGCGGCCGCCTACGT-GCAG GTGTCCTGTG
	sPLD12-mSP-F	GCCACTAGTATGGTTCGCCATC TAGG		
	sPLD12-F-HA	CCACAGGACACCTGCACGGGG TTAATTAACATC	sPLD12-R-HA	GATGTTAATTAACCCCGTG-CAG GTGTCCTGTGG
Expression analysis				
Gene	**Forward primer**	**Sequence (5'-3')**	**Reverse primer**	**Sequence (5'-3')**
ActA	ActA-F	CATCAAGGAGAAGCTGAC GTACA	ActA-R	GACGACTCGGCGGCAG
PLD-like-1	qPLD1-F	CCAGAAGTTCATGGAGG CGAC	qPLD1-R	GCCGTCGTCGTGCGCAGC
sPLD-like-1	qsPLD1-F	TGGGGATATTTCTACACCA CGG	qsPLD1-R	GCAGATACAATTTCCTC CAAGCTT
sPLD-like-12	qsPLD12-F	CTGCCAACATCATTGACGA	qsPLD12-R	TAAACGGCATAGCGTCCAA

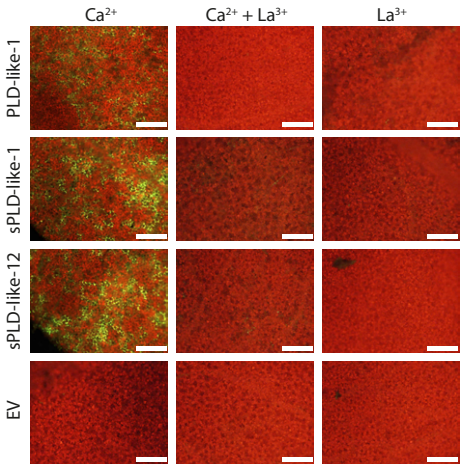

Figure S1. The calcium antagonist lanthanum blocks the cell death-promoting effect of calcium. Epifluorescence pictures of *Nicotiana benthamiana* leaves seven days after agroinfiltration with *Agrobacterium tumefaciens* Agl1 carrying small PLD-likes constructs (PLD-like-1, sPLD-like-1 and sPLD-like-12) and a control construct EV. P19 was co-expressed and 2 mM $CaCl_2$ and/or 100 µM $LaCl_2$, were infiltrated 24 hours after agroinfiltration. Dead cells are depicted in yellow-green and living cells in red. Experiments were repeated three times. Scale bars represent 500 µm.

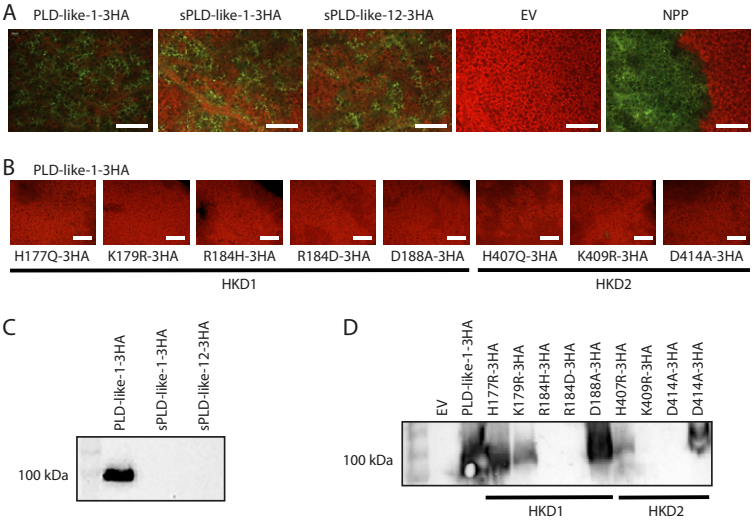

Figure S2. HA-tagged versions of small PLD-likes induce cell death in *Nicotiana benthamiana*. (A) Epifluorescence pictures of *Nicotiana benthamiana* leaves, seven days after agroinfiltration with *Agrobacterium tumefaciens* Agl1 carrying 3HA-tagged versions of small PLD-likes constructs (PLD-like-1, sPLD-like-1 and sPLD-like-12) and the control constructs EV and NPP. (B) Epifluorescence pictures of *Nicotiana benthamiana* leaves expressing 3HA-tagged versions of PLD-like-1 with intact or mutated HKD motifs at 7 days post infiltration. P19 was co-expressed and 2 mM $CaCl_2$ and/or 100 µM $LaCl_2$, were infiltrated 24 hours after agroinfiltration. Dead cells are depicted in green and living cells in red. Experiments were repeated three times. Scale bars represent 500 µm. (C) Western blot of small PLD-likes at three days post infiltration. (D) Western blot of PLD-like-1 with altered HKD motifs, three days post infiltration. Proteins were detected with anti-HA antibody.

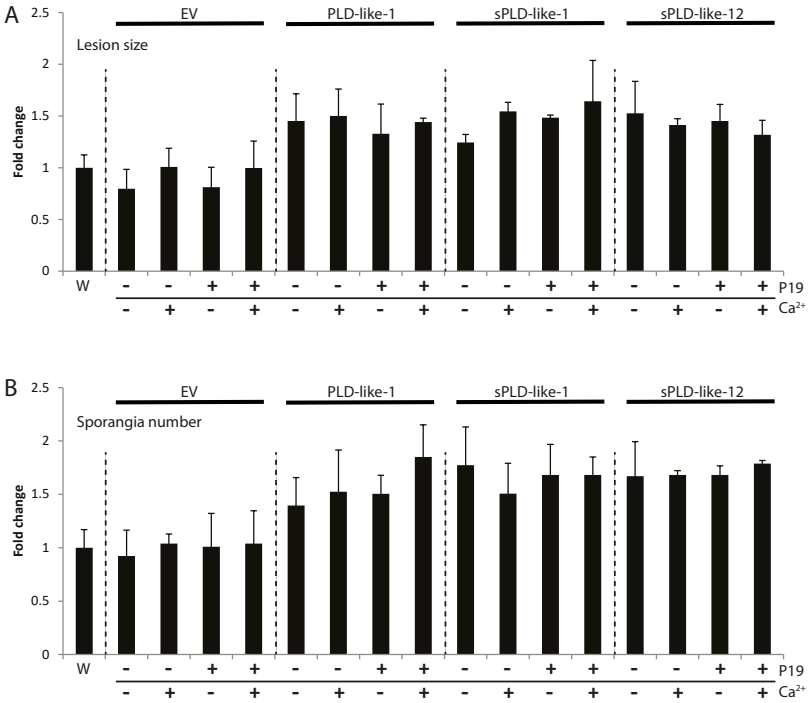

Figure S3. Co-infiltrations with $CaCl_2$ and P19 do not play a role on the promotion of lesion size and sporulation of *Phytophthora infestans* on infiltrated *Nicotiana benthamiana* leaves. (A) Growth and (B) sporulation of *Phytophthora infestans* on leaves infiltrated with small PLD-likes, calculated as fold change in lesion size or amount of sporangia respectively, compared to those on leaves infiltrated with the control construct (EV). Experiments were repeated three times. Error bars represent standard deviation (n=24).

Chapter 6

Infection of a tomato cell culture by *Phytophthora infestans*; a versatile tool to study *Phytophthora*-host interactions

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*Equal contribution

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6

Abstract

The oomycete *Phytophthora infestans* causes late blight on potato and tomato. Despite extensive research, the *P. infestans*-host interaction is still poorly understood. To find new ways to further unravel this interaction we established a new infection system using MsK8 tomato cells. These cells grow in suspension and can be maintained as a stable cell line that is representative for tomato. MsK8 cells can host several *Phytophthora* species pathogenic on tomato. Species not pathogenic on tomato could not infect. Microscopy revealed that 16 hours after inoculation up to 36 percent of the cells were infected. The majority were penetrated by a germ tube emerging from a cyst (i.e. primary infection) while other cells were already showing secondary infections including haustoria. In incompatible interactions, MsK8 cells showed defense responses, namely reactive oxygen species production and cell death leading to a halt in pathogen spread at the single cell level. In compatible interactions, several *P. infestans* genes, including RXLR effector genes, were expressed and in both, compatible and incompatible interactions tomato genes involved in defense were differentially expressed. Our results show that *P. infestans* can prosper as a pathogen in MsK8 cells; it not only infects, but also makes haustoria and sporulates, and it receives signals that activate gene expression. Moreover, MsK8 cells have the ability to support pathogen growth but also to defend themselves against infection in a similar way as whole plants. An advantage of MsK8 cells compared to leaves is the more synchronized infection, as all cells have an equal chance of being infected. Moreover, analyses and sampling of infected tissue can be performed in a non-destructive manner from early time points of infection onwards and as such the MsK8 infection system offers a potential platform for large-scale omics studies and activity screenings of inhibitory compounds.

Keywords: MsK8, disease, infection, defense responses, gene expression, reactive oxygen species (ROS), cell death, microscopy

Introduction

Plants are constantly facing potential microbial attackers that can cause disease, such as bacteria, fungi and oomycetes. In order to fend off pathogens and circumvent infection, plants have developed several defense mechanisms like cell wall thickening, reactive oxygen species (ROS) production and programmed cell death. These plant immune responses are often initiated by plasma membrane-spanning surface receptors as well as cytoplasmic receptors recognizing pathogen-derived molecules. Furthermore, timely transcriptional regulation of genes involved in pathogen recognition and genes involved in plant defense play an important role (Tyler & Rouxel, 2012, Wirthmueller *et al.*, 2013). The pathogen-derived molecules include effectors secreted by the pathogens to promote infection (Stam *et al.*, 2014), and microbe-associated molecular patterns (MAMPs) that are recognized by plants and trigger defense responses (Wirthmueller *et al*., 2013, Mott *et al.*, 2014). Well-known examples of MAMPs are the bacterial flagellin, fungal chitin and oomycete elicitins such as INF1 (Dodds & Rathjen, 2010). Induced defence responses can enable the plant to circumvent infection.

Plant diseases caused by oomycetes such as downy mildews and *Phytophthora* spp. cause large losses in crop production and substantial damage in natural habitats. The genus *Phytophthora* includes over a hundred species, of which, some have a limited host range while others have a much broader host range (Kroon *et al.*, 2011). *Phytophthora infestans* and *Phytophthora sojae* are two well-studied species with a narrow host range. *P. infestans* causes late blight disease and only infects potato and tomato. *P. sojae* causes stem and root rot and has just one host, soybean. In contrast, *Phytophthora capsici* has a very broad host range comprising more than 200 plants, mainly *Solanaceae* and *Cucurbitaceae* spp. Similarly, *Phytophthora palmivora* can infect many different plants species, including oil palm, cocoa, and several vegetable crops like tomato. *Phytophthora* parasitica is another broad host range pathogen infecting the foliage, fruits or roots of plant species in more than 250 genera (Kamoun *et al.*, 2015). A typical class of effectors secreted by *Phytophthora* spp. are the so called RXLR effectors. They all have a conserved RXLR motif that has a function in translocating these effectors into the host cells (Petre & Kamoun, 2014). For several RXLR effectors it has been shown that they are involved in suppressing plant defense and/or promoting infection (Whisson *et al.*, 2016, Tyler & Rouxel, 2012, Oh *et al.*, 2010, Oliva *et al.*, 2010).

So far, studies exploring the *P. infestans*-host interaction are mainly based on infections of leaves of potato and tomato, as well as *N. benthamiana* (Kamoun *et al.*, 1998). Leaf infections resulting from drop inoculations have the advantage that the development of the expanding lesions and the lesion growth rate can be followed over time. The disadvantage however, is that an expanded lesion is composed of different zones, ranging from a biotrophic zone at the edge of the lesion to a necrotrophic zone in the center. This

zonation makes it difficult to collect material of only one specific infection stage (van West *et al.*, 1998). Moreover, due to the thickness of the leaves with a multilayer of cells and the autofluorescence of chlorophyll, microscopy studies are quite challenging. In analogy with studies on mammalian systems where *in vitro* cell cultures are frequently used as model for the *in vivo* situation, one could try to eliminate the disadvantages of *in planta* studies by using an *in vitro* system. Single cells could allow a more synchronized infection process and are better accessible for microscopy.

In several studies focused on plant-microbe interactions, cell suspension cultures have been utilized instead of whole plants. Cell suspensions have also been successfully used as a model system to study signaling pathways or the effect of exogenous compounds on plant cells. A very popular stable plant cell line maintained in suspension is the tobacco cell line BY-2 (Kato *et al.*, 1972) that has been used to study plant responses to biotic stress. For example, upon inoculation with zoospores of different *P. nicotianae* strains it was found that reactive oxygen species (ROS) were produced by the BY-2 cells in an incompatible interaction with a non-pathogenic strain (Able *et al.*, 2000, Able *et al.*, 2001). BY-2 cells were also used to study the potential of a non-pathogenic *Streptomyces* sp. as biocontrol agent against *Pectobacterium* spp. (Baz *et al.*, 2012). Another example is the use of *Arabidopsis* cell suspension cultures for transcriptomic analysis with a focus on phosphoinositide-dependent phospholipase C (PI-PLC) regulated gene expression (Ruelland *et al.*, 2014). Tomato cell suspension cultures have been used to study responses to abiotic stress factors. Exposure to low oxygen activated fermentative metabolism and sugar alcohol synthesis while inhibiting the activity of the tricarboxylic acid (TCA) cycle and the biosynthesis of metabolites such as organic acids, amino acids and sugars . Aimé *et al*. (2008), used cell suspensions from the tomato cultivar Montfavet to study expression of genes encoding Pathogenesis-Related (PR) proteins upon inoculation with a pathogenic strain of the soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* and a biocontrol strain of *F. oxysporum* and found a lower PR gene expression in the presence of the biocontrol strain compared to the pathogenic strain. A similar pattern was found in intact tomato plants pointing towards priming as mode of action of the biocontrol strain. Rice cell suspensions have been used to study responses upon treatment with an elicitor from the rice blast fungus *Magnaporthe oryzae* and this revealed that the elicitor causes metabolic alterations in the rice cells (Takahashi *et al.*, 2008).

Cell suspensions have also been used to study plant responses to *Phytophthora* spp. For example, parsley cells were used to monitor cell death and changes in cell structural components upon infection with *P. infestans* (Naton *et al.*, 1996). Moreover, potato cell suspensions established from stems and microtubers from the cultivar Bintje were used to study the responses to *P. infestans* culture filtrates, such as acidification of the cell culture medium and lipoxygenase induction (Val *et al.*, 2008). In a recent study, the induction of defense responses to various pathogens (*P. infestans*, *Verticillium dahliae*,

Spongospora subterranea, and *Colletotrichum coccodes*) was studied using potato and *Arabidopsis* cells suspensions. It was shown that treatments with elicitors or zoospores from *P. infestans*, as well as exposure to other pathogens, induce alkalinisation of medium, ROS production and induction of defense related genes (Moroz *et al.*, 2017). In addition, defense responses, including ROS production, defense gene expression and MAPK activation were studied in the interaction between *P. capsici* and *Capsicum chinense* cell suspensions (Nakazawa-Ueji *et al.*, 2009).

It should be noted that apart from studies with BY-2 cell line, all of the studies described above made use of unstable cell lines with a limited life span. Unlike many human and animal cell lines, plant cells cannot easily be maintained as stable cell lines. Exceptions are the tobacco BY-2 cell line and the tomato MsK8 cell line that was utilized in this study. The MsK8 cell line (hereafter referred to as MsK8 cells) is a stable cell suspension culture that was developed as a cell line representative for tomato (Koornneef *et al.*, 1987). The majority of tomato genotypes are not amenable for generation of *in vitro* suspension cultures and several crosses and backcrosses were needed to find a line that had the desired characteristics. A cross between *Solanum lycopersicum* VF11 and K93 (F3 generation of *S. lycopersicum* x *S. peruvianum*) led to the selection of one plant in the F1 generation named MsK93. Two backcrosses with the parental line VF11 resulted in a F3 generation with one line named MsK8 that could be maintained as cell suspensions. So far, MsK8 cells have been used for monitoring defense responses upon treatment with pathogen elicitors, including chitin and the flagellin peptide flg22, and the cells have shown similar defense responses as in intact tomato plants (Sánchez-Vallet *et al.*, 2013, Felix *et al.*, 1999, Felix *et al.*, 1993).

The aim of this study was to develop an *in vitro* infection system that offers consistent, synchronized infections by *P. infestans* and to test its suitability for microscopy and histochemical studies. For this purpose, the stable tomato cell suspension culture MsK8 was tested as a host for *Phytophthora*. Tomato is a natural host of *P. infestans* and therefore MsK8 cells were preferred rather than tobacco BY-2 cells. MsK8 cells were inoculated with different *Phytophthora* spp. and strains, and the infection process was monitored over time by microscopy and histochemical staining. Detailed studies of the different *P. infestans* infection stages were performed in order to estimate the levels of infection and the resemblance to intact leaf infection. Furthermore, defense responses of the MsK8 cells upon inoculation were studied, such as ROS production and induction of expression of several defense-related genes.

Results & Discussion

Tomato cells growing in suspension can host *Phytophthora infestans*

In order to investigate if MsK8 cells are susceptible to *P. infestans*, a fluorescent strain was used for inoculation and the infection process was monitored by microscopy (Table S1). Zoospores from *P. infestans* 14-3-GFP were mixed with MsK8 cells in a 1:1 ratio. This mixture was placed on a shaking platform in the dark at RT to provide optimal conditions for both the pathogen and the host. Infection progress was monitored at different time points. Germinating cysts were found at 3 hours post inoculation (hpi) while appressorium formation and initial penetrations occurred at 6 hpi (primary infections) (Fig. 1A, B). At 16 hpi, hyphae from infected cells were observed to invade neighboring cells forming secondary infections, where formation of haustoria and relocation of the nucleus to the infection point were observed (Fig. 1A, C). At 48 hpi formation of sporangiophores and sporangia was observed, suggesting successful infection of MsK8 cells and completion of the life cycle (Figs 1A, S1A). In order to determine the optimal inoculum density for obtaining the maximum number of infected cells, a range of zoospore concentrations was used. A concentration of 10^5 zoospores/ml gave the highest percentages of infected cells (Table S2). In addition to *P. infestans* strain 14-3-GFP, two other *P. infestans* strains (IPO-C and T20-2) were used as inoculum (Table S1). It appeared that also these two strains are capable of infecting the MsK8 cells. To compare the three strains, the infection efficiency was quantified. To this end, infected cells, i.e. cells with primary (first penetration into a single cell) and secondary infections (hypha expanding from infected to neighbouring cells), as well as cells containing haustoria were counted (Table 1). It was found that around 36% of the cells were infected by *P. infestans* 14-3-GFP. From the infected cells, 73% had primary infections and 26% secondary infections, from which 21% contained haustoria. Cells inoculated with strain IPO-C showed a lower percentage of infected cells (25%), but the percentages of primary and secondary infections were similar to those obtained upon inoculation with strain 14-3-GFP. MsK8 cells inoculated with strain T20-2 showed the lowest percentage of infected cells, around 16%, with a lower percentage of infected cells harbouring haustoria (Table 1). In all cases, formation of sporangia was observed at 48 hpi.

In infected potato and tomato leaves infection is usually established at 48 hpi and sporulation is first observed after 3-4 days post inoculation (van West *et al*., 1998, Zuluaga *et al*., 2016). In the MsK8 cells, however, the infection progress was clearly faster (Fig. 1A) suggesting that MsK8 cells can be more easily penetrated by *P. infestans*, likely due to the lack of a cuticle and a differentiated epidermal cell layer. In conclusion, *P. infestans* is able to consistently successfully infect the MsK8 cells and complete its life cycle in these host cells. This justifies further analyses of the MsK8 cell suspension line for its potential as alternative infection system for *P. infestans*.

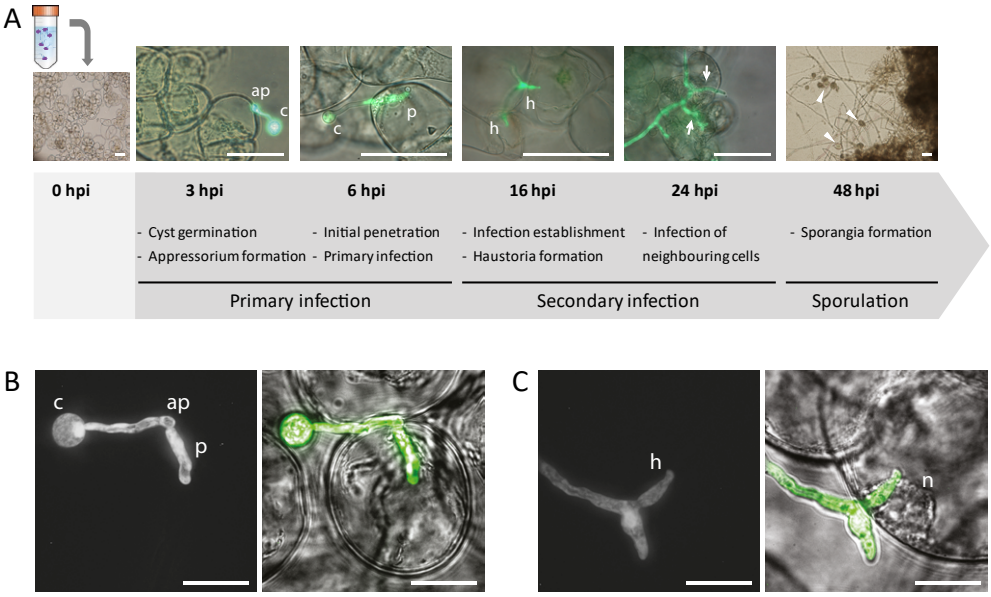

Figure 1. MsK8 cells are successfully infected by *Phytophthora infestans*. (A) Timeline of MsK8 cells infected with *P. infestans* strain 14-3-GFP. At time point 0 zoospores were added to MsK8 cells. Bars represent 100 µm. (B, C) Microscopic images (left panels: epifluorescent, right panels: bright field). (B) Primary infection and penetration of a MsK8 cell at 6 hpi. (C) Fully developed haustorium in an infected cell and relocation of the nucleus to the penetration point at 16 hpi. ap: appressorium; c: cyst; h: haustorium; hpi: hours post inoculation; n: nucleus; p: penetration peg. Arrows and arrowheads point to secondary infections and sporangia, respectively. Bars represent 50 µm.

Tomato cells growing in suspension can host other *Phytophthora* species but not all

To determine whether MsK8 cells can be infected by other *Phytophthora* spp., MsK8 cells were inoculated with strains from four other *Phytophthora* species namely *P. capsici, P. palmivora*, *P. sojae* and *P. parasitica* (Table S1). Inoculations with *P. capsici* LT263 showed that germinating cysts are capable to attach and penetrate the cell. Eventually however, infection did not proceed, despite the fact that tomato is a host for this strain (Wang *et al.*, 2015). Based on microscopic observations LT263 attempted to infect the cells, suggesting that this strain is less aggressive on MsK8 cells or that it cannot circumvent the induced defense mechanisms of MsK8 cells. To further investigate this, two other *P. capsici* strains were used that can also infect tomato plants. Strain LT3239 was not capable of infecting MsK8 cells, but strain LT51 was. The latter was able to establish infection, form haustoria and develop sporangia while the percentages of cells showing primary or secondary infection or haustoria were similar to those obtained after *P. infestans* inoculation (Table 1, Figure S1B). Also, inoculation with *P. palmivora* strain GFP3 resulted in successful infection and with similar percentages of infected cells. *P. palmivora* is not listed as a typical tomato pathogen but has been reported to infect tomato fruits (Akinrefon, 1969). Apparently the

MsK8 cells are easy victims for *P. palmivora*; the infection with *P. palmivora* was progressing faster than the infection with *P. infestans* with the first penetrations already observed at 3 hpi as opposed to 6 hpi with *P. infestans*. Formation of haustoria (7%) was observed at 12 hpi while at 16 hpi most cells had secondary infections (73%). Similar to *P. infestans*, *P. palmivora* could complete its life cycle in the MsK8 cells, producing sporangia after 36 hpi (Figure S1C). Unlike *P. palmivora,* the *P. sojae* and *P. parasitica* strains tested here were not able to infect the MsK8 cells (Table 1). For *P. sojae* this is not surprising because this species has a very narrow host range. *P. parasitica* is known to cause tomato root rot (Le Berre *et al.*, 2008) but this might be strain dependent. Zoospores from *P. sojae* strain P6497 and *P. parasitica* strain H1111 did encyst and germinate but there was no attachment to the MsK8 cells.

These results show that the *in vitro* infection system is suitable for studying interactions with other *Phytophthora* spp. besides *P. infestans* and moreover, that the MsK8 cells have retained their capacity to distinguish between different *Phytophthora* spp. and even between strains thus behaving similar to intact tomato plants with respect to host specificity.

Table 1. Infection efficiency of various *Phytophthora* species and strains on MsK8 cells. The efficiency was quantified by determining the percentage of infected cells at 16 hours post inoculation. For each sample a total of 500 cells was monitored in triplicate.

%	*P. infestans*			*P. capsici*			*P. palmivora*	*P. sojae*	*P. parasitica*
	14-3-GFP	IPO-C	T20-2	LT263	LT3239	LT51	GFP3	P6497	H1111
Infected MsK8 cells[a]	36.4 ± 1.2	25.4 ± 0.4	16.6 ± 2.3	7.0[e] ± 4.5	0	34.0 ± 3.4	39.4 ± 1.3	0	0
Primary infection[b]	73.6 ± 3.6	73.2 ± 4.0	52.0 ± 2.5	-	-	55.3 ± 2.8	26.4 ± 2.1	-	-
Secondary infection[c]	26.4 ± 1.4	26.8 ± 2.7	48.0 ± 2.6	-	-	44.7 ± 1.1	73.6 ± 2.6	-	-
Cells containing haustoria[d]	21.4 ± 1.1	11.8 ± 1.8	7.1 ± 0.9	-	-	7.0 ± 0.6	7.1 ± 1.5	-	-

[a] Cells that had been penetrated by *Phytophthora* were counted as infected.

[b] Infected cells due to primary infection i.e. penetrated by germ tubes emerging from cysts.

[c] Infected cells due to secondary infection i.e. penetrated by hyphae expanding from a neighbouring infected cell.

[d] Infected cells containing haustoria.

[e] Attachment of hyphae and scarce initial penetrations with no further growth of hyphae in the cell.

Defense responses in MsK8 cells can differentiate a compatible from an incompatible interaction

To investigate how the MsK8 cells respond to exposure to *Phytophthora*, defense responses were examined. ROS accumulation and cell death are two defense mechanisms employed by the plant during the interaction with a potential pathogen (Torres *et al.*, 2006). First, the viability of the MsK8 cells at 20 hpi was analyzed by propidium iodine (PI) staining. It was found that the three *P. infestans* strains and the *P. capsici* strain LT51 that are compatible with the MsK8 cells, do not induce cell death. In contrast, *P. sojae* P6497 and *P. capsici* LT263, two strains that are not able to infect, did induce cell death (Fig. 2A). At 20 hpi with *P. sojae*, 72% of the cells were dead, and this is in line with the microscopic observations that clearly revealed an incompatible interaction (Fig. 2A). However, also in the compatible interaction of MsK8 cells with *P. palmivora*, a high percentage of cell death was observed, namely in 79% of the cells, and this was also visible by microscopy.

When measuring electrolyte leakage, which is an indirect method to quantify cell death, MsK8 cells inoculated with *P. sojae* at 20 and 24 hpi showed higher conductivity when compared to the mock treated cells indicating cell death. An increase in electrolyte leakage was also observed upon inoculation with *P. palmivora* from 20 hpi onwards, but to a lesser extent. However, MsK8 cells inoculated with the other *Phytophthora* spp. did not show a significant difference in conductivity compared to the mock (Figure S2A), despite the fact that some of the interactions were clearly incompatible when analysed by microscopy and showed (partial) cell death.

ROS production is another marker for activation of defense responses and can be quantified using the xylenol orange assay. Measurements at different time points post inoculation revealed that inoculation of MsK8 cells with *P. sojae* leads to a peak in ROS production at 3.5 hpi. Similarly, Able *et al.* (2000) observed cell death and ROS production in incompatible interactions between BY-2 cells and avirulent strains of *P. parasitica* var. *nicotianae*. Also, in MsK8 cells inoculated with *P. palmivora* GFP3 and *P. capsici* LT263 we observed accumulation of ROS but at lower levels (Fig. 2B). Although the interaction with *P. palmivora* was compatible, the higher percentage of dead cells and the higher levels of ROS production compared to *P. infestans*, could be due to the fact that the infection process is faster and *P. palmivora* enters the necrotrophic stage earlier. At the time of measurement, the *P. palmivora*-MsK8 interaction probably had already entered the necrotrophic stage, as noticed by the microscopic observations and the cell death measurements.

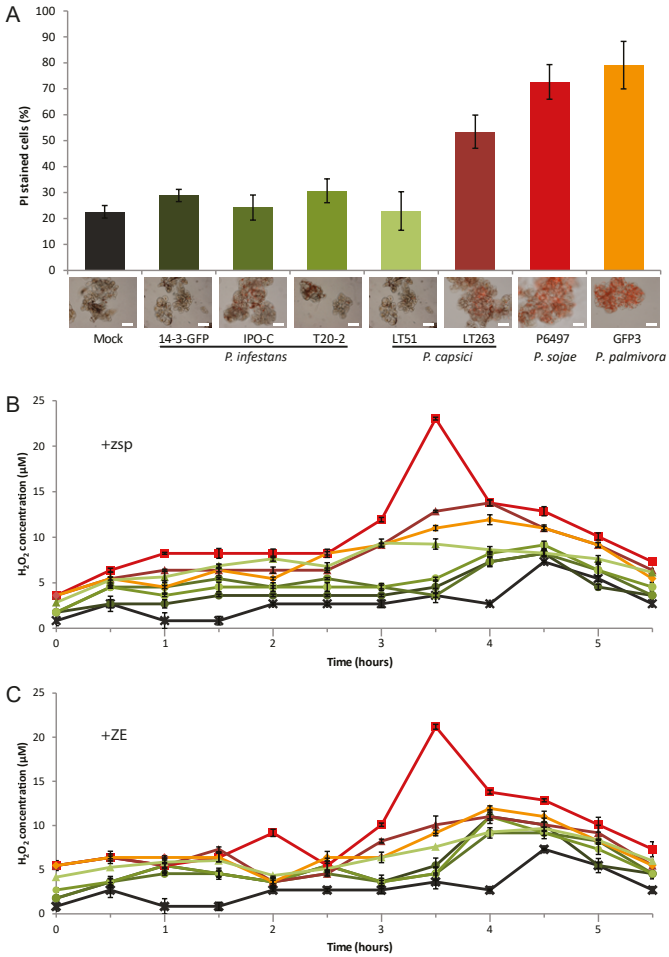

Figure 2. Responses of MsK8 cells to inoculation with different *Phytophthora* spp. and strains. (A) Percentage of MsK8 cells in suspension showing cell death at 16 hpi. Cell death was quantified by counting the cells stained with propidium iodine (PI), as shown in the microscopy images, and the total number of cells (set at 100%). Bars represent 200 µm. (B, C) ROS production by MsK8 cells measured at different time points (X-axis) after (B) inoculation with *Phytophthora* zoospores or (C) after treatment with zoospore exudate (ZE). ROS production was quantified by measuring the H_2O_2 concentration with the xylenol orange assay. Colors of the bars (A) and lines (B, C) correspond to a specific strain and species as indicated in (A). Error bars represent standard deviation (n=3).

The fact that *P. parasitica,* a species that is able to infect tomato plants, did not cause successful infection of MsK8 cells, urged us to evaluate the fitness and virulence of the strain used in this study. *P. parasitica* also infects tobacco (Meng *et al.*, 2014, Kebdani *et al.*, 2010) and therefore we used *P. parasitica* strain H1111 to inoculate the tobacco cell suspension BY-2. While, H1111 was unable to cause infection of MsK8 cells, it was able to infect BY-2 cells; 37% of the cells became infected and haustoria were formed in 5% of the

infected cells (Fig. 3A). These percentages are similar to those obtained in the *P. infestans* - MsK8 interaction (Table 1). Furthermore, *P. parasitica* was able to complete its life cycle and produce sporangia from 48 hpi onwards (Figs 3B-D). Cell death measurements showed an increase of dead cells upon inoculation with *P. parasitica*. This increase was higher for MsK8 compared to BY-2 cells at 16 hpi, depicting a difference between successful and unsuccessful infection (Fig. 3E). Measurements of ROS production showed higher amounts of H_2O_2 in inoculated MsK8 cells at 3.5 hpi compared to inoculated BY-2 cells, indicating a stronger defense response in MsK8 cells when challenged with the pathogen (Fig. 3F).

In summary, based on cell death and ROS production the defense responses of MsK8 cells are indicative of a compatible or incompatible interaction, depending on the *Phytophthora* species. The high levels of ROS production and cell death in the incompatible interaction with *P. sojae,* indicate recognition of the pathogen. On the other hand, in the compatible interaction with *P. infestans* the ROS production was much lower and this is in agreement with the hemi-biotrophic lifestyle of the pathogen.

Responsiveness of MsK8 cells to *Phytophthora* spp. zoospore exudates

In order to examine if *Phytophthora* zoospores release molecules that induce defense responses in MsK8 cells, the cells were mixed with zoospore exudates (ZE) and responses were measured by quantifying ROS production and electrolyte leakage. As upon zoospore treatment, ROS production by MsK8 cells was the highest at approximately 3.5 hours after mixing with ZE of *P. sojae* P6497 and reached similar levels (Fig. 3C). Electrolyte leakage measurements did not show an increase in conductivity for any of the samples of MsK8 cells treated with ZEs when compared to mock treated cells, not even with ZE of *P. sojae* strain P6497 of which the zoospores, when mixed with MsK8 cells, caused an increase in conductivity (Figure S2B). Overall, treatments of MsK8 cells with ZE of *Phytophthora* spp. showed levels of cell death and ROS accumulation similar to zoospore treatments, suggesting that the presence of the pathogen is not prerequisite for induction of defense responses. In other studies, treatments with MAMPs such as chitin, induced defense responses in Msk8 cells (Felix *et al.*, 1991). Furthermore, culture filtrates from *P. infestans* and a species of the bacterium *Streptomyces* induced changes in pH in potato cells and ROS production in BY-2 cells, respectively (Baz *et al*., 2012, Val *et al*., 2008).

A

Infected BY-2 cells (%)	37.0 ± 2.8
Primary infection	69.1 ± 3.4
Secondary infection	30.9 ± 5.1
Cells with haustoria	5.2 ± 4.7

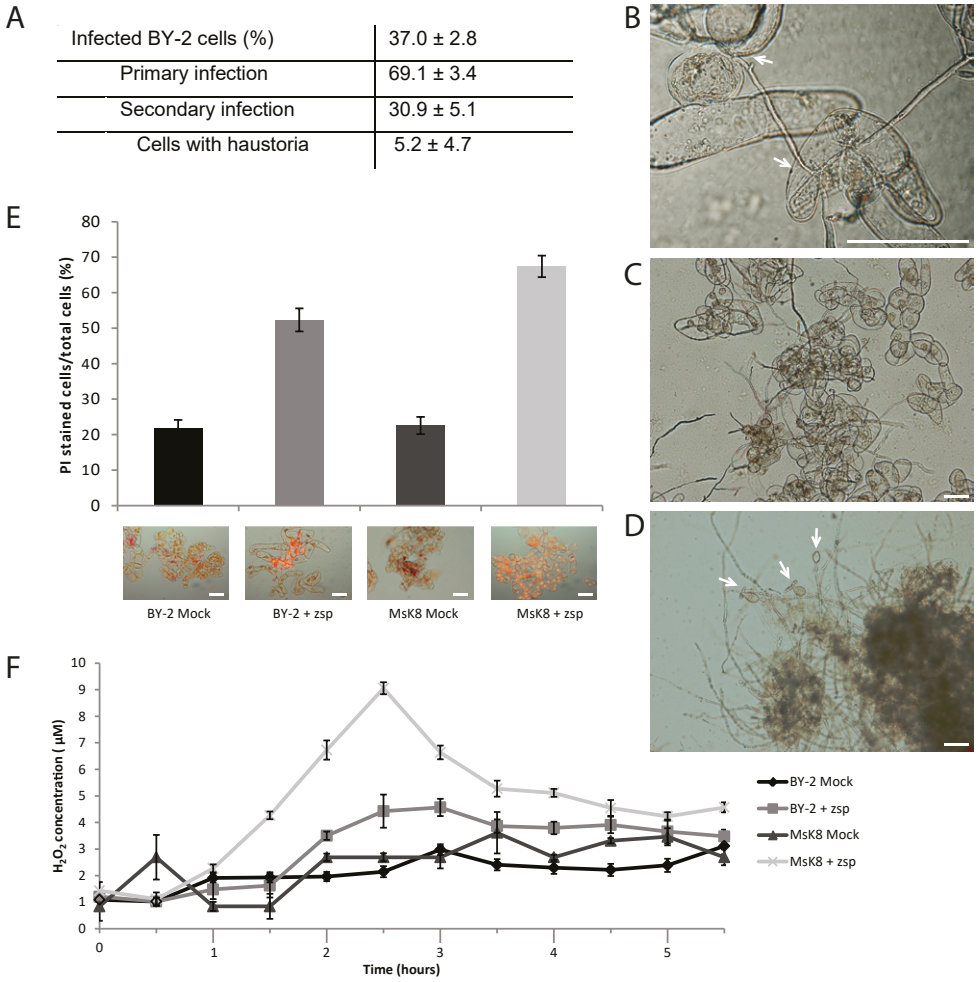

Figure 3. *Phytophthora parasitica* H1111 is able to infect BY-2 cells. (A) Quantification of *P. parasitica* H1111 infections on BY-2 cells at 16 hpi (n=3, 500 cells/sample). Cells that have been penetrated by *Phytophthora* hyphae were counted as infected. (B, C, D) Microscopic images of BY-2 cells penetrated by *P. parasitica* hyphae at 6 hpi (B), secondary infection at 16 hpi (C) and formation of sporangia at 48 hpi (D). Arrows point to the sites of penetration (B) or sporangia (D). Bars represent 100 µm. (E) Percentages of BY-2 and MsK8 cells in the cell suspension showing cell death at 16 hpi. Cell death was quantified by counting the cells stained with PI (as shown in the microscopic images) and the total number of cells (set at 100 %). Bars in images represent 200 µm. (F) ROS production by MsK8 and BY-2 cells measured at different time points (X-axis) after inoculation with *P. parasitica* zoospores. ROS production was quantified by measuring the H_2O_2 concentration with the xylenol orange assay. Error bars represent standard deviation (n=3).

The most abundant protein secreted by *P. infestans* is the elicitin INF1, a small protein of 98 amino acids classified as a MAMP that elicits cell death in several *Nicotiana* spp. (Kamoun *et al.*, 1998). INF2B is larger but shares the canonical elicitin domain with INF1 (Jiang *et al.*,

2006). In the wild potato species *S. microdontum*, elicitin recognition is mediated by the receptor-like protein ELR (Du *et al.*, 2015). To study the response of MsK8 cells to elicitins the cells were exposed to the full length INF1 and the elicitin domain of INF2B proteins. The responsiveness was measured by monitoring medium alkalinization. No pH shift was observed upon treatment with INF1 or INF2B (Figure S3A). On the other hand, tobacco BY-2 cells showed a response to both INF1 and INF2B (Figure S3B) demonstrating that the protein preparations used do have elicitor activity. To verify the responsiveness of MsK8 cells to other MAMPs, they were treated with the flagellin peptide flg22 and this resulted in a pH shift (Figure S3C). These findings confirmed previous studies by Felix *et al.* (1999) who also observed medium alkalinization in MsK8 cells upon exposure to flg22. In conclusion, the MsK8 cells do respond to flg22 but not to elicitins and this resembles the response of intact tomato plants to these MAMPs (Felix *et al*., 1999, Kamoun *et al*., 1998).

Activation of *Phytophthora* genes during interaction with MsK8 cells

In order to investigate how *Phytophthora* responds upon encountering MsK8 cells as potential host we analysed the expression of several *Phytophthora* genes in time up to 36 hpi. These included a number of RXLR effector genes as well as genes that are proposed as marker genes for subsequent infection stages, namely *HMP1*, *NPP1* and *CDC14* (Jupe *et al.*, 2013); Table S3).

The qRT-PCR analyses showed that in all three *P. infestans* strains (14-3-GFP, IPO-C and T20-2) the haustorium-specific gene *HMP1* (Avrova *et al.*, 2008) reaches the highest expression level at 16 hpi after which expression continues but decreases. *P. palmivora* GFP3 only shows *HMP1* expression at 16 hpi (Figs 4A, S4). This is in accordance with the microscopic observations showing the formation of haustoria at that time point. However, also in *P. capsici* LT263 and *P. sojae* P6497 that are both incompatible with MsK8 cells, *HMP1* is expressed and in *P. sojae* P6497 the expression peaks even earlier than in *P. infestans* and *P. palmivora,* i.e. at 6 hpi, the earliest time point measured (Fig. 4). In these incompatible interactions, there are no haustoria formed indicating that *HMP1* expression does not necessarily correlate with haustoria formation. In fact, Avrova *et al*. (2008) who first identified the gene in *P. infestans,* showed that *HMP1* is also highly expressed in germinating cysts and appressoria. This likely explains why we see *HMP1* expression in these incompatible interactions where, as described above, the cysts do germinate and make attempts to penetrate the MsK8 cells. Overall, the *HPM1* expression profiles that we observe in compatible interactions with MsK8 cells are in line with the profiles observed by others in *P. infestans* and *P. capsici* infected leaves with upregulation early during infection concurrently with haustoria formation and downregulation afterwards (Avrova *et al*., 2008, de Vries *et al.*, 2016). We are not aware of studies in which *HPM1* expression has been monitored in incompatible interactions or non-host interactions.

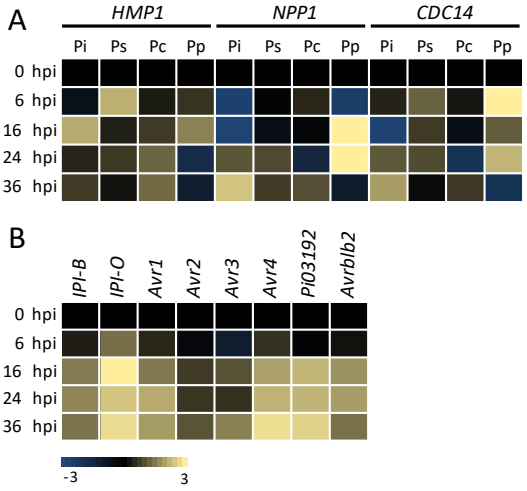

Figure 4. Expression profiling of *Phytophthora* genes during infection. (A) Expression of stage-specific genes *HMP1*, *NPP1* and *CDC14* upon inoculation of MsK8 cells with zoospores of *P. infestans* 14-3-GFP (Pi), *P. capsici* LT263 (Pc), *P. palmivora* GFP3 (Pp) and *P. sojae* P6497 (Ps). (B) Expression of *IPI-B* and various *P. infestans* RXLR effector genes upon inoculation of MsK8 cells with zoospores of *P. infestans* 14-3-GFP. Expression levels were determined by qRT-PCR and the values at each time point were calculated relative to the expression level at time point 0 (0 hpi). Expression of the actin gene *ActA* was used as endogenous control.

The *NPP1* gene that encodes a necrosis-inducing protein, is upregulated at later time points than *HPM1*. Expression in the three tested *P. infestans* strains and in *P. palmivora* GFP3 that are compatible with MsK8 cells, is higher than in *P. capsici* LT263 and *P. sojae* P6497 that do not infect MsK8 cells (Figs 4A, S4). In *P. palmivora* GFP3 expression is already high at 16 hpi, possibly due to the faster infection progress and in accordance with the cell death that is more pronounced in the *P. palmivora*-MsK8 interaction (Figs 4A, S4). In infected tomato leaves a comparable *NPP1* expression pattern was observed with no or very low expression in early stages and upregulation in later stages (de Vries *et al*., 2016, Abrahamian *et al*., 2016, Zuluaga *et al*., 2016, Jupe *et al*., 2013).

Similar to *NPP1, CDC14*, a gene encoding a cell cycle regulator, is expressed at earlier time points (6 hpi) in *P. palmivora* than in *P. infestans* where expression is first observed at 24 hpi (Fig. 4A). At 6 hpi the cysts are germinating and at 24 and 36 hpi there is mycelial growth and formation of sporangia. Ah Fong and Judelson (2003), who first identified *CDC14* as a sporulation-specific gene, reported low expression in vegetative hyphae and high expression in sporangia, zoospores and cysts. As such, this explains the expression patterns that we observe here in infected MsK8 cells and that others have observed in tomato leaves infected with either with *P. infestans* (de Vries *et al*., 2016) or *P. capsici* (Jupe *et al*., 2013). Also the very low expression of *CDC14* in the incompatible interaction of MsK8 with *P. capsici* or *P. sojae* is not suprising since these species do not proceed to the stage where sporulation occurs.

RXLR effectors are virulence proteins produced by *Phytophthora* species and as such important markers for monitoring the suitability of a novel infection system. They do share the RXLR motif but apart from that, they are very diverse in sequence with every species having hundreds of different RXLR effectors each with a specific role in the infection

process (Jiang *et al.*, 2008). Moreover, RXLR effectors are highly variable among isolates, not only with respect to sequence or copy number polymorphism but also expression levels and expression dynamics. Since RXLR effectors are species-specific we limited this analysis to the three *P. infestans* strains included in this study. We selected seven *P. infestans* RXLR effector genes that have been analyzed in previous studies. In infected leaves expression usually occurs early during infection (Vleeshouwers *et al.*, 2011). In line with that, we observed expression at variable levels of all seven RXLR effector genes at 16 hpi in *P. infestans* 14-3-GFP and even earlier, at 6 hpi, in *P. infestans* IPO-C (Figs 4, S4). In the less aggressive T20-2 strain expression of the tested RXLR effector genes is overall lower and peaks later during the interaction with MsK8 cells (24 hpi) (Figure S4). Differences among the three strains in timing of expression and expression level of an individual RXLR effector gene can likely be explained by the dynamics of the gene itself, in addition to the dynamics of the whole RXLR effectome. *Avr2* for example, is regulated at various levels (Gilroy *et al.*, 2011). Analyses of a large set of field isolates revealed presence/absence of the *Avr2* gene, the existence of *Avr2*-like genes, differential expression and sequence polymorphism. Wang *et al.* (2011) showed that the *P. sojae* effectome is a complex network in which the interplay between effectors and the redundancy among effectors determines expression and activity of each individual RXLR effector.

Taken together our results show that in the MsK8 interactions the expression patterns of *Phytophthora* genes characteristic for early (*HMP1*) and later (*NPP1*) infection stages and for growth of the pathogen (*CDC14*), are in accordance with the microscopic observations. Moreover, induction of expression of RXLR effector genes early in the interaction shows that *P. infestans* recognises MsK8 cells as a suitable host.

Expression of defense-related genes in MsK8 cells

In order to monitor how MsK8 cells respond to inoculation with *Phytophthora*, the expression of several defense-related genes was analysed in time up to 36 hpi. This included genes encoding pathogenesis-related (PR) proteins (*PR1A*, *PR1B*, *PR2A*, *PR2B*, and *PR5*), chitinases (*Chi3* and *Chi9*), a hypersensitive response marker (*HSR203J*) and isoforms of a pathogenesis-related subtilase (*P69a/b* and *P69c*) (Table S3). In mock-treated MsK8 cells that were included as control, nearly all tested defense-related genes showed an increase in expression at 6 hpi and in particular the chitinase and subtilase genes continued to be expressed at higher levels up to 36 hpi. Compared to the mock-treated controls MsK8 cells challenged with *P. infestans* 14-3-GFP showed a lower expression of *PR1A, PR5*, *Chi3*, *Chi9*, *HSR203J* and *P69a/b,* at all time points while *PR1B, PR2A* and *PR2B*, expression was the same or slightly upregulated. The expression of *P69c* at 6 hpi was lower than in the mock control but at 16 hpi it was slightly higher (Fig. 5). In MsK8 cells inoculated with zoospores of the other *P. infestans* strains (IPO-C and T20-2), a similar trend was observed (Figure S5). In MsK8 cells inoculated with *P. palmivora* GFP3, the expression of the *PR* genes and

chitinase genes was overall comparable to expression in *P. infestans* infected cells with the exception of a strong upregulation of *PR1A*, *PR2A* and *PR2B* at 36 hpi. Also *P69a/b* and *P69c* showed a higher expression in *P. palmivora* infected cells compared to *P. infestans* infected cells and this was already evident at the earliest time point that was monitored, i.e. 6 hpi (Fig. 5). In MsK8 cells inoculated with *P. capsici* LT263 hardly any changes in expression levels were observed. This is in contrast to MsK8 cells inoculated with *P. sojae* P6497 in which seven out of the nine defense genes analysed here showed a strong upregulation shortly after inoculation, with a peak at 6 hpi. This concerned *PR1B*, *PR2A*, *Chi3, Chi9, HSR203J*, *P69a/b* and *P69c* (Fig. 5).

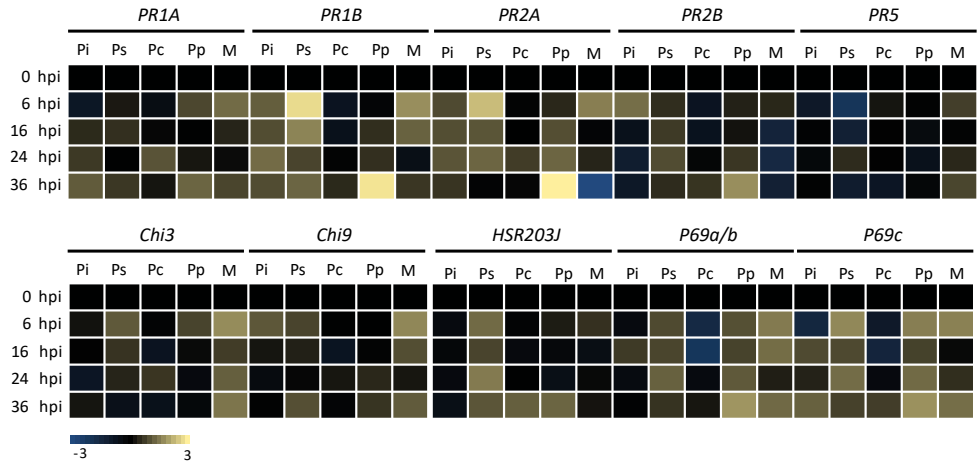

Figure 5. Expression profiling of tomato defense marker genes upon inoculation of MsK8 cells with zoospores of *P. infestans* 14-3-GFP (Pi), *P. sojae* P6497 (Ps), *P. capsici* LT263 (Pc) and *P. palmivora* GFP3 (Pp). Defense genes include genes encoding different pathogenesis-related proteins (PR), chitinases (Chi), a hypersensitivity marker (HSR203J) and isoforms of the subtilase P69 (P69a/b and P69c). Expression levels were determined by qRT-PCR and the values were calculated relative to the expression level at time point 0 (0 hpi). Expression of the tomato *ActA* was used as endogenous control.

To investigate if expression of defense genes is induced by molecules secreted by the pathogen, MsK8 cells were treated with ZE and expression analyses were performed in a similar manner. When comparing the expression patterns between zoospore inoculated MsK8 cells and ZE treated MsK8 cells some differences were observed. Most remarkable was the rather strong and rapid response (within 6 hours) of *PR1A*, *P69a*/b and *P69c* to ZE derived from *P. sojae, P. capsici* and *P. palmivora* but not *P. infestans* (Fig. 5, S6). Another remarkable difference was the increased expression of these three defense related genes as well as *PR1B*, *Chi9* and *HSR203J* upon treatment with ZE from *P. capsici* while inoculation with *P. capsici* zoospores did not increase the expression of these same genes. Furthermore, it was noted that ZE of *P. infestans* 14-3-GFP seems to lack molecules that activate the

defense genes tested here while treatment with ZE of the other two *P. infestans* strains resulted in activation of *PR1B*, *PR2A*, *PR2B* and *Chi3* (Figure S6). This suggests variability among strains or, potentially, among ZE from different strains. All ZE samples were collected by the same procedure. Yet, we have no means to check the composition of the exudates in a straightforward manner. In the ROS production assays and electrolyte leakage measurements the MsK8 cells responded in a similar manner to ZE from all three *P. infestans* strains (Fig. 2, S2A) but with respect to defense gene expression we observe some minor differences.

Overall, our results show that there is differential expression of several defense related genes in MsK8 cells when the cells are challenged with different *Phytophthora* spp. or their ZEs. However, there is no clear correlation between the patterns that we observe and the type of interaction with the MsK8 cells, i.e. compatible versus incompatible. We could speculate that the rapid increase in defense gene expression in MsK8 cells leads to an incompatible interaction as observed upon inoculation with *P. sojae,* a species that is unable to circumvent the defense. However, this does not explain why the interaction between *P. capsici* and Msk8 cells is incompatible because there is no obvious increase in defense gene expression. In literature there are many reports showing expression profiles of defense-related genes during host-pathogen interactions. In cases where compatible and incompatible interactions are compared the overall tendency is a stronger expression in incompatible interactions. It should be noted however, that those studies often concern interactions of one host species with either a virulent or avirulent strain of the same *Phytophthora* spp. and not non-host interactions.

Conclusion

Model pathosystems have been instrumental in extending our knowledge on how *Phytophthora* pathogens infect plants and cause disease. Leaf infection assays are routinely used to dissect the complexity of *Phytophthora*-host interactions. A disadvantage of leaf infections, however, is the occurrence of different infection stages simultaneously. In the *in vitro* infection system established in this study all MsK8 cells growing in suspension have an equal chance of being infected. Defined quantities of *Phytophthora* zoospores can be mixed with a standardized amount of MsK8 cells, and infection can be followed over time by microscopy. Shortly after inoculation initial penetration and primary infection of MsK8 cells were observed and this was followed by a secondary stage including haustorium formation and infection of neighbouring cells. The induced expression of infection-related genes in *P. infestans*, including RXLR effector genes, showed that *P. infestans* recognises MsK8 cells as a suitable host. During incompatible interactions, MsK8 cells mounted early defense responses, including cell death and ROS accumulation. So apart from the ability

to host pathogen growth, MsK8 cells retained their capacity to perceive and counterattack *Phytophthora* pathogens in a similar way as observed in whole plants. MsK8 cells can easily be maintained under controlled conditions in a way that the status of the host tissue is comparable between experiments. The variability among infection assays was found to be minimal. This *in vitro* system accommodates synchronized infection allowing analyses and sampling in a non-destructive manner from early time points of infection onwards and offers a potential platform for large-scale -omics studies and activity screenings of inhibitory compounds.

Materials & Methods

Plant cell suspensions culture

MsK8 (Koornneef *et al*., 1987) and BY-2 cell suspensions (Kato *et al*., 1972) were cultured in Murashige and Skoog (MS) medium including vitamins (Duchefa Biochemie), supplemented with 30 g/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin, which was set to pH 5.7 (MS-30). Cells were routinely grown in 60 ml MS-30 medium in 300 ml Erlenmeyer flasks, placed at 25°C in the dark, on a platform shaking at approximately 100 rpm. To maintain the vigor of the cell suspensions, sub-culturing was performed every 7 days by transferring 10 ml cell suspension to flasks containing 50 ml MS-30 medium.

Phytophthora spp. culturing and inoculum preparation

The *Phytophthora* strains used in this study are listed in Table S1. *P. infestans* strains were grown on rye sucrose agar medium (RSA) at 18°C. *P. capsici*, *P. sojae, P. palmivora* and *P. parasitica* were grown on solid V8 medium at 25°C. *P. infestans* zoospores were isolated from 10 days-old sporulating mycelium which was flooded with 15 ml ice-cold water and thereafter kept at 4°C for 3 hours to induce zoospore release. Zoospores from *P. capsici, P. palmivora* and *P. parasitica* were obtained in a similar way. In particular, 8 days-old mycelium was flooded with 20 ml cold water and kept at room temperature (RT) for 2 hours. Zoospore suspensions were filtered through a sterile 50 µm mesh. Zoospore concentrations were determined using a haemocytometer and adjusted to 10^5 zoospores/ml. To obtain *Phytophthora* zoospore exudates (ZE), zoospore suspensions were pelleted by centrifugation (4.600 x g for 10 min). The supernatant was collected and filtered through a 0.45 µm filter.

Inoculation assays

Inoculations of MsK8 or BY-2 cells with *Phytophthora* zoospore suspensions were performed by mixing a sample of a 6 days-old cell culture (approx. 10^6 cells/mL) with

zoospore suspensions of 10^5 zoospores/ml, in a 1:1 ratio. Inoculated cells were incubated at RT in the dark, shaking at 80-100 rpm. Mock and ZE treatments were performed in a similar way.

Histochemical staining and microscopy

Cell death in treated BY-2 and MsK8 cells, was determined by staining with propidium iodine (PI) (0.05 mg/ml). The value for cell viability was inferred by the number of PI stained cells relative to the total number of cells expressed in percentages. Each measurement consisted of three technical replicates per sample and a total number of 500 cells per replicate. Microscopic observations were performed on a Nikon eclipse 90i epifluorescence microscope equipped with a Nikon DS-U2 digital imaging camera. Fluorescence was captured using a GFP-LP filter (EX 460-500, DM 505). Confocal fluorescence microscopy was performed on a Roper Spinning Disc confocal microscope (Nikon Ti microscope equipped with Yokogawa CSUX1-spinning disc, Photometrics Evolve camera, and Metamorph software) using 491 and 561 nm laser lines and a GFP filter (495–560 nm).

RNA isolation and gene expression analyses

Treated MsK8 cell samples were freeze dried and ground in 1 ml of TRIzol reagent (Invitrogen). Total RNA was extracted with a NucleoSpin RNA Plant kit (Macherey-Nagel Inc.) according to manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA and was reverse transcribed using oligo(dT) primers and M-MLV reverse transcriptase (Promega). Quantitative RT-PCR was performed using an ABI7300 real-time PCR system (Applied Biosystems). Expression of *Phytophthora* or tomato actin genes was used as internal control (Table S3). Primers used are listed in Table S4. Data were analyzed using ABI 7300 SDS 1.3.1.21 software and the comparative C_T (also referred as $2^{-\Delta\Delta C}{}_T$) method (Schmittgen & Livak, 2008).

Electrolyte leakage measurements

Electrolyte leakage was measured on 1 ml samples of treated MsK8 cells. To each sample, 4 ml of water was added and incubated for 2 hours at RT with gentle agitation. Electrolyte leakage was determined by measuring conductivity (mS/cm) using a digital conductivity meter equipped with LabX direct PH 2.1 software (Mettler Toledo). Statistical analysis of the data was conducted by one-way ANOVA ($P < 0.05$) with IBM SPSS statistics 19 software.

ROS measurements

ROS production was determined by measuring H_2O_2 using a modified xylenol orange assay (Gay *et al.*, 1999, Choi *et al.*, 2007). Xylenol orange reagent was freshly prepared according to Choi *et al.* (2007). In particular, 100 µl of treated cells were added to 500 µl

0.2 N HCl and mixed by vortexing for 30 seconds. Subsequently, samples were centrifuged for 5 minutes at 12.000 rpm. Supernatants were collected and mixed with 233 µl 50 mM phosphate buffer (pH 5.7) and 467 µl 0.2 N NaOH. Subsequently, 100 µl were mixed with 1 mL of fresh-prepared xylenol orange reagent and incubated for 30 minutes at RT in the dark. Absorbance was measured at 560 nm using a NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific). To determine the H_2O_2 concentrations in the MsK8 samples, in each measurement a standard curve was made from absorbance values of samples with increasing concentrations of H_2O_2.

Elicitor treatments

For elicitation assays, 2.5 mL of MsK8 or BY-2 cells were incubated for 1 hour at RT with gentle shaking. Subsequently, cells were treated with *P. infestans* recombinant INF1 or INF2B elicitins purified from *E. coli*, or the bacterial MAMP peptide flg22 (Genscript), at different concentrations. Responsiveness of cell suspensions was measured as a pH shift by using a digital pH meter equipped with LabX direct PH 2.1 software (Mettler Toledo). Data were collected up to 30 minutes after elicitor treatment. MQ water was used as a negative control treatment.

Authors contributions

CS and KB performed the experiments. CS, KB and FG designed the research and wrote the paper. All authors read and approved the final manuscript.

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References

Able, A.J., Guest, D.I. and Sutherland, M.W. (2000) Hydrogen peroxide yields during the incompatible interaction of tobacco suspension cells inoculated with *Phytophthora nicotianae*. *Plant Physiol,* **124,** 899-910.

Able, A.J., Guest, D.I. and Sutherland, M.W. (2001) Relationship between transmembrane ion movements, production of reactive oxygen species and the hypersensitive response during the challenge of tobacco suspension cells by zoospores of *Phytophthora nicotianae*. *Physiol Mol Plant P,* **58,** 189-198.

Abrahamian, M., Ah-Fong, A.M., Davis, C., Andreeva, K. and Judelson, H.S. (2016) Gene expression and silencing studies in *Phytophthora infestans* reveal infection-specific nutrient transporters and a role for the nitrate reductase pathway in plant pathogenesis. *PLoS Pathog,* **12,** e1006097.

Ah Fong, A.M.V. and Judelson, H.S. (2003) Cell cycle regulator Cdc14 is expressed during sporulation but not hyphal growth in the fungus-like oomycete *Phytophthora infestans*. *Mol Microbiol,* **50,** 487-494.

Aimé, S., Cordier, C., Alabouvette, C. and Olivain, C. (2008) Comparative analysis of PR gene expression in tomato inoculated with virulent F*usarium oxysporum* f. sp. *lycopersici* and the biocontrol strain *F. oxysporum* Fo47. *Physiol Mol Plant Pathol,* **73,** 9-15.

Akinrefon, O.A. (1969) Biochemical studies on plant tissues infected by *Phytophthora palmivora* (Butl.). *Ann Appl Biol,* **63,** 303-313.

Ampofo-Asiama, J., Baiye, V.M.M., Hertog, M.L.A.T.M., Waelkens, E., Geeraerd, A.H. and Nicolai, B.M. (2014) The metabolic response of cultured tomato cells to low oxygen stress. *Plant Biol,* **16,** 594-606.

Avrova, A.O., Boevink, P.C., Young, V., Grenville-Briggs, L.J., Van West, P., Birch, P.R.J. and Whisson, S.C. (2008) A novel *Phytophthora infestans* haustorium-specific membrane protein is required for infection of potato. *Cell microbiol,* **10,** 2271-2284.

Baz, M., Tran, D., Kettani-Halabi, M., Samri, S.E., Jamjari, A., Biligui, B., Meimoun, P., El-Maarouf-Bouteau, H., Garmier, M., Saindrenan, P., Ennaji, M.M., Barakate, M. and Bouteau, F. (2012) Calcium- and ROS-mediated defence responses in BY2 tobacco cells by nonpathogenic *Streptomyces* sp. *J Appl Microbiol,* **112,** 782-792.

Choi, H.W., Kim, Y.J., Lee, S.C., Hong, J.K. and Hwang, B.K. (2007) Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defense response to bacterial pathogens. *Plant Physiol,* **145,** 890-904.

de Vries, S., von Dahlen, J.K., Uhlmann, C., Schnake, A., Kloesges, T. and Rose, L.E. (2016) Signatures of selection and host-adapted gene expression of the *Phytophthora infestans* RNA silencing suppressor PSR2. *Mol Plant Pathol,* **18,** 110-124.

Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat Rev Genet,* **11,** 539-548.

Du, J., Verzaux, E., Chaparro-Garcia, A., Bijsterbosch, G., Keizer, L.C.P., Zhou, J., Liebrand, T.W.H., Xie, C., Govers, F., Robatzek, S., van der Vossen, E.A.G., Jacobsen, E., Visser, R.G.F., Kamoun, S. and Vleeshouwers, V.G.A.A. (2015) Elicitin recognition confers enhanced resistance to *Phytophthora infestans* in potato. *Nat Plants,* **1,** 1-5.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J,* **18,** 265-276.

Felix, G., Grosskopf, D.G., Regenass, M., Basse, C.W. and Boller, T. (1991) Elicitor-induced ethylene biosynthesis in tomato cells: characterization and use as a bioassay for elicitor action. *Plant Physiol,* **97,** 19-25.

Felix, G., Regenass, M. and Boller, T. (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. *Plant J,* **4,** 307-316.

Gay, C., Collins, J. and Gebicki, J.M. (1999) Hydroperoxide assay with the ferric–xylenol orange complex. *Anal Biochem,* **273,** 149-155.

Gilroy, E.M., Breen, S., Whisson, S.C., Squires, J., Hein, I., Kaczmarek, M., Turnbull, D., Boevink, P.C., Lokossou, A., Cano, L.M., Morales, J., Avrova, A.O., Pritchard, L., Randall, E., Lees, A., Govers, F., van West, P., Kamoun, S., Vleeshouwers, V.G.A.A., Cooke, D.E.L. and Birch, P.R.J. (2011) Presence/absence, differential expression and sequence polymorphisms between PiAVR2 and PiAVR2-like in *Phytophthora infestans* determine virulence on R2 plants. *New Phytol,* **191,** 763-776.

Jiang, R.H.Y., Tripathy, S., Govers, F. and Tyler, B.M. (2008) RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc Natl Acad Sci,* **105,** 4874-4879.

Jiang, R.H.Y., Tyler, B.M., Whisson, S.C., Hardham, A.R. and Govers, F. (2006) Ancient origin of elicitin gene clusters in *Phytophthora* genomes. *Mol Biol Evol,* **23,** 338 - 351.

Jupe, J., Stam, R., Howden, A., Morris, J., Zhang, R., Hedley, P. and Huitema, E. (2013) *Phytophthora capsici*-tomato interaction features dramatic shifts in gene expression associated with a hemi-biotrophic lifestyle. *Genome Biology,* **14,** R63.

Kamoun, S., Furzer, O., Jones, J.D.G., Judelson, H.S., Ali, G.S., Dalio, R.J.D., Roy, S.G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B.M., Grünwald, N.J., Mukhtar, M.S., Tomé, D.F.A., Tör, M., Van Den Ackerveken, G., McDowell, J., Daayf, F., Fry, W.E., Lindqvist-Kreuze, H., Meijer, H.J.G., Petre, B., Ristaino, J., Yoshida, K., Birch, P.R.J. and Govers, F. (2015) The Top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol,* **18,** 413-434.

Kamoun, S., van West, P., Vleeshouwers, V.G., de Groot, K.E. and Govers, F. (1998) Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell,* **10,** 1413-1426.

Kato, K., Matsoumoto, T., Koiwai, S., Mizusaki, S., Nishida, K., Nogushi, M. and Tamaki, E. (1972) Liquid suspension culture of tobaco cells. *in Ferment Technology Today, ed Terui G (Society of Fermentation Technology, Osaka)***,** 689–695.

Kebdani, N., Pieuchot, L., Deleury, E., Panabières, F., Le Berre, J.Y. and Gourgues, M. (2010) Cellular and molecular characterization of *Phytophthora parasitica* appressorium-mediated penetration. *New Phytol,* **185,** 248-257.

Koornneef, M., Hanhart, C.J. and Martinelli, L. (1987) A genetic analysis of cell culture traits in tomato. *Theor Appl Genet,* **74,** 633-641.

Kroon, L.P.N.M., Brouwer, H., de Cock, A.W.A.M. and Govers, F. (2011) The Genus *Phytophthora* Anno 2012. *Phytopathology,* **102,** 348-364.

Le Berre, J.-Y., Engler, G. and Panabières, F. (2008) Exploration of the late stages of the tomato–*Phytophthora parasitica* interactions through histological analysis and generation of expressed sequence tags. *New Phytol,* **177,** 480-492.

Meng, Y., Zhang, Q., Ding, W. and Shan, W. (2014) *Phytophthora parasitica*: a model oomycete plant pathogen. *Mycol,* **5,** 43-51.

Moroz, N., Fritch, K.R., Marcec, M.J., Tripathi, D., Smertenko, A. and Tanaka, K. (2017) Extracellular alkalinization as a defense response in potato cells. *Front Plant Sci,* **8**.

Mott, G.A., Middleton, M.A., Desveaux, D. and Guttman, D.S. (2014) Peptides and small molecules of the plant-pathogen apoplastic arena. *Front Plant Sci,* **5,** 677.

Nakazawa-Ueji, Y.E., Núñez-Pastrana, R., Souza-Perera, R.A., Santana-Buzzy, N. and Zúñiga-Aguilar, J.J. (2009) Mycelium homogenates from a virulent strain of *Phytophthora capsici* promote a defence-related response in cell suspensions from *Capsicum chinense*. *Eur. J. Plant Pathol,* **126,** 403-415.

Naton, B., Hahlbrock, K. and Schmelzer, E. (1996) Correlation of rapid cell death with metabolic changes in fungus-infected, cultured parsley cells. *Plant Physiol.,* **112,** 433-444.

Oh, S.K., Kamoun, S. and Choi, D. (2010) Oomycetes RXLR effectors function as both activator and suppressor of plant immunity. *Plant Pathol J,* **26,** 209-215.

Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T.O., Chaparro-Garcia, A., Segretin, M.E., Stam, R., Schornack, S., Cano, L.M., van Damme, M., Huitema, E., Thines, M., Banfield, M.J. and Kamoun, S. (2010) Recent developments in effector biology of filamentous plant pathogens. *Cell microbiol,* **12,** 705-715.

Petre, B. and Kamoun, S. (2014) How do filamentous pathogens deliver effector proteins into plant cells? *PLoS Biol,* **12,** e1001801.

Ruelland, E., Pokotylo, I., Cantrel, C., Djafi, N., Repellin, A. and Zachowski, A. (2014) Salicylic acid modulates levels of phosphoinositide dependent-phospholipase C substrates and products to remodel the *Arabidopsis* suspension cell transcriptome. *Front Plant Sci,* **5,** 608.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.-J., Thomma, B.P.H.J. and Mesters, J.R. (2013) Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *eLife,* **2,** e00790.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc,* **3,** 1101-1108.

Stam, R., Mantelin, S., McLellan, H. and Thilliez, G. (2014) The role of effectors in nonhost resistance to filamentous plant pathogens. *Front. Plant Sci,* **5,** 582.

Takahashi, H., Matsumura, H., Kawai-Yamada, M. and Uchimiya, H. (2008) The cell death factor, cell wall elicitor of rice blast fungus (*Magnaporthe grisea*) causes metabolic alterations including GABA shunt in rice cultured cells. *Plant Signal Behav,* **3,** 945-953.

Torres, M.A., Jones, J.D.G. and Dangl, J.L. (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol,* **141,** 373-378.

Tyler, B.M. and Rouxel, T. (2012) Effectors of fungi and oomycetes: Their virulence and avirulence functions and translocation from pathogen to host cells. In: *Molecular Plant Immunity.* (Sessa, G., ed.). Wiley-Blackwell, pp. 123-167.

Val, F., Desender, S., Bernard, K., Potin, P., Hamelin, G. and Andrivon, D. (2008) A culture filtrate of *Phytophthora infestans* primes defense reaction in potato cell suspensions. *Phytopathology,* **98,** 653-658.

van West, P., de Jong, A.J., Judelson, H.S., Emons, A.M.C. and Govers, F. (1998) The ipiO gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genet Biol,* **23,** 126-138.

Vleeshouwers, V.G.A.A., Raffaele, S., Vossen, J.H., Champouret, N., Oliva, R., Segretin, M.E., Rietman, H., Cano, L.M., Lokossou, A., Kessel, G., Pel, M.A. and Kamoun, S. (2011) Understanding and exploiting late blight resistance in the age of effectors. *Annu Rev Phytopathol,* **49,** 507-531.

Wang, Q., Han, C., Ferreira, A.O., Yu, X., Ye, W., Tripathy, S., Kale, S.D., Gu, B., Sheng, Y., Sui, Y., Wang, X., Zhang, Z., Cheng, B., Dong, S., Shan, W., Zheng, X., Dou, D., Tyler, B.M. and Wang, Y. (2011) Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell,* **23,** 2064.

Wang, Y., Weide, R., Govers, F. and Bouwmeester, K. (2015) L-type lectin receptor kinases in *Nicotiana benthamiana* and tomato and their role in *Phytophthora* resistance. *J Exp Bot,* **66,** 6731-6743.

Whisson, S.C., Boevink, P.C., Wang, S. and Birch, P.R.J. (2016) The cell biology of late blight disease. *Curr Opin Microbiol,* **34,** 127-135.

Wirthmueller, L., Maqbool, A. and Banfield, M.J. (2013) On the front line: structural insights into plant-pathogen interactions. *Nat Rev Microbiol,* **11,** 761-776.

Zuluaga, A.P., Vega-Arreguín, J.C., Fei, Z., Ponnala, L., Lee, S.J., Matas, A.J., Patev, S., Fry, W.E. and Rose, J.K.C. (2016) Transcriptional dynamics of *Phytophthora infestans* during sequential stages of hemibiotrophic infection of tomato. *Mol Plant Pathol,* **17,** 29-41.

Suplemmental data

Table S1. *Phytophthora* isolates used in this study.

Phytophthora species	Isolate	Origin			References and remarks
		Country	Year	Source	
P. infestans	IPO-C	Belgium	1982	Potato	-
	T20-2 (H20P02)	The Netherlands	1993	#	F1 progeny from a cross between *P. infestans* 80029 and 88133 (Drenth *et al.*, 1994)
	14-3-GFP	#	#	Potato	*GFP*-expressing transformant of *P. infestans* H30P02
P. capsici	LT263	USA	2004	Pumpkin	(Donahoo & Lamour, 2008)
	LT3239	USA	2006	Pumpkin	(Wang *et al.*, 2013)
	LT51	USA	1997	Cucumber	-
P. palmivora	GFP3	#	#	#	*GFP*-expressing transformant of *P. palmivora* P6390 (Vijn & Govers, 2003)
P. sojae	P6497	USA	2001	Soybean	(Förster *et al.*, 1994)
P. parasitica	H1111	Australia	##	Tobacco	(Robold & Hardham, 1998)

not applicable; ## unknown

Table S2. Infection efficiency of *Phytophthora infestans* strain 14-3-GFP on MsK8 cells. The efficiency was quantified by determining the percentage of infected cells at 16 hours post inoculation. For each sample a total of 500 cells was monitored in triplicate.

%	Inoculum concentration (zoospores/mL)				
	10^4	$5x10^4$	10^5	$5x10^5$	10^6
Infected MsK8 cells[a]	17.0 ± 2.8	18.8 ± 3.9	36.4 ± 1.2	32.0 ± 2.8	30.4 ± 3.4
Primary infection[b]	81.1 ± 3.4	80.0 ± 4.2	73.6 ± 3.6	78.7 ± 5.7	74.3 ± 2.4
Secondary infection[c]	18.9 ± 2.1	20.0 ± 2.2	26.4 ± 1.4	21.2 ± 3.3	25.7 ± 1.4
Cells containing haustoria[d]	12.9 ± 1.8	18.1 ± 3.1	21.4 ± 1.1	18.1 ± 2.1	17.1 ± 2.0

[a] Cells that had been penetrated by *Phytophthora* were counted as infected.

[b] Infected cells due to primary infection i.e. penetrated by germ tubes emerging from cysts.

[c] Infected cells due to secondary infection i.e. penetrated by hyphae expanding from a neighbouring infected cell.

[d] Infected cells containing haustoria.

Table S3. Genes selected for expression analysis by qRT-PCR.

Organism	Gene	Function	Reference
Phytophthora spp.	*ActA*	Structural protein	
	HMP1	Haustorial membrane protein	(Avrova *et al*., 2008)
	NPP1	Necrosis-inducing protein	(Qutob *et al.*, 2002)
	CDC14	Cell cycle regulator	(Ah Fong & Judelson, 2003)
	IPI-B	Putative cell wall protein	(Pieterse *et al.*, 1994)
	IPI-O	RXLR effector	(van West et al., 1998)
	Avr1	RXLR effector	(Guo, 2008)
	Avr2	RXLR effector	(Gilroy *et al*., 2011)
	Avr3	RXLR effector	(Armstrong *et al.*, 2005)
	Avr4	RXLR effector	(van Poppel *et al*., 2008)
	Avrblb2	RXLR effector	(Oh *et al.*, 2009)
	PITG_03192 (RD28)	RXLR effector	(McLellan *et al.*, 2013, Oh *et al*., 2009)
Solanum lycopersicum	*ActA*	Structural protein	
	Chi3	Chitinase	(Danhash *et al.*, 1993)
	Chi9	Chitinase	(Danhash *et al*., 1993)
	PR1a	Pathogenesis-related protein	(van Kan *et al.*, 1992)
	PR1b	Pathogenesis-related protein	(van Kan *et al*., 1992)
	PR2a (GlucA)	Glucanase	(van Kan *et al*., 1992)
	PR2b (GlucB)	Beta 1,3-glucanase	(van Kan *et al*., 1992)
	PR5	Pathogenesis-related protein	(Singh *et al.*, 1987, Rodrigo *et al.*, 1993, Anžlovar & Dermastia, 2003)
	HSR203J	Hypersensitive response marker	(Pontier *et al.*, 1998)
	P69 (a, b, c)	Subtilase	(Meichtry *et al.*, 1999)

Table S4. qRT-PCR primers used in this study.

	Gene	Forward primer	Sequence (5′-3′)	Reverse primer	Sequence (5′-3′)
Phytophthora infestans	*ActA*	PiActin-F	CATCAAGGAGAAGCTGACGTACA	PiActin-R	GACGACTCGGCGGCAG
	HMP1	PiHMP1-F	CATGATGGCTGTCATGGTCGGTGAGG	PiHMP1-R	TTAGCTAACATTCAAGCGAGCATGAAG
	NPP1	PiNPP1-F	CAGATCCACATCTCGAACGGCT	PiNPP1-R	CTGTAGCCGTCGATCGTGTTG
	CDC14	PiCDC14-F	GGAAGCGATCGAGTTCCTCCC	PiCDC14-R	TTCTCGACCCGTTCATAGTG
	IPI-B	IPI-B-F	GTAATGCTGCTGTCGGCGCTG	IPI-B-R	GCCGTTCTGCGACGTAGATGTG
	IPI-O	IPI-O-F	CTCGCACTCAGTCCAAGACGG	IPI-O-R	AGCCTTATCGAGATGCTTGGTCC
	Avr1	Avr1-F	CCGTCGTTTATCGAGTCCTTCG	Avr1-R	CGCCAGGCTCGTTCCG
	Avr2	Avr2-F	ATGCGTCTCGCCTACATTTT	Avr2-R	GCATTTGAAAATTAAGGGTGACA
	Avr3	Avr3a-F	CGCCATAAACTTTGCAACCA	Avr3a-R	TGCCGGCTGAATCGTGTAT
	Avr4	Avr4-F	CATCACGACGCTTAAACTCAACGG	Avr4-R	GATACAACTTGACATACTTCAGGAGCG
	Avrblb2	Avrblb2-F	CGTCGCAGCATTCCCAAT	Avrblb2-R	GCCACAGTGTCAGGAGATGTCTT
	PITG_03192	Pi03192-F	CCTGCTCGTCGTCAGTGTGA	Pi03192-R	TCGAAATTCCGGTTATTCATGA
P. capsici	*HMP1*	PcHMP1-F	CATGATGGCAGTCATGGTCGGTGAAG	PcHMP1-R	TTAGCTAACATTGAGGCGGGCATGCAG
	NPP1	PcNPP1-F	CAGCTCCACATCACCAACGGCT	PcNPP1-R	CTCTTCCCGTTCAAATAGTTC
	CDC14	PcCDC14-F	GGAAGCGATTGAGTTCTTGC	PcCDC14-R	TTCTCCACACGCTCAAAGTG
P. sojae	*HMP1*	PsHMP1-F	CATGATGGCCGTCATGGTCGGCGAG	PsHMP1-R	TTAGCTAACATTCAGACGAGCGTGCAG
Solanum lycopersicum	*ActA*	Actin-F	TATGGAAACATTGTGCTCAGTGG	Actin-R	CCAGATTCGTCATACTCTGCC
	Chi3	Chi3 -F	CAATTCGTTTCCAGGTTTTG	Chi3-R	ACTTTCCGCTGCAGTATTTG
	Chi9	Chi9-F	AATTGTCAGAGCCAGTGTCC	Chi9-R	TCCAAAAGACCTCTGATTGC
	PR1a	PR1A-F	TCTTGTGAGGCCCAAAATTC	PR1A-R	ATAGTCTGGCCTCTCGGACA
	PR1b	PR1A-R	ATAGTCTGGCCTCTCGGACA	PR1B-R	GAACCTAAGCCACGATACCA
	PR2a (GlucA)	PR2A-F	TATAGCCGTTGGAAACGAAG	PR2A-R	TGATACTTTGGCCTCTGGTC
	PR2b (GlucB)	PR2B-F	CAACTTGCCATCACATTCTG	PR2B-R	CCAAAATGCTTCTCAAGCTC
	PR5	PR5-F	ATGGGGTAAACCACCAAACA	PR5-R	GTTAGTTGGGCCGAAAGACA
	HSR203J	HSR203J-F	TCCCGTCATTCTTCACTTCC	HSR203J-R	GTTGAAATCGGCGTATTCGT
	P69a/b	P69a/b-F	GTTTGCAGCAGAACATGGGTGTATGGAA	P69a/b-R	CTCCTGCAGCTGTGCTTGCTGTGTGTGTAC
	P69c	P69c-F	GGAATCTCAGCTGAGAGGTC	P69c-R	GAAGCCATGAAAGAACATTGTC

[a]:F:forward primer, R: reverse primer

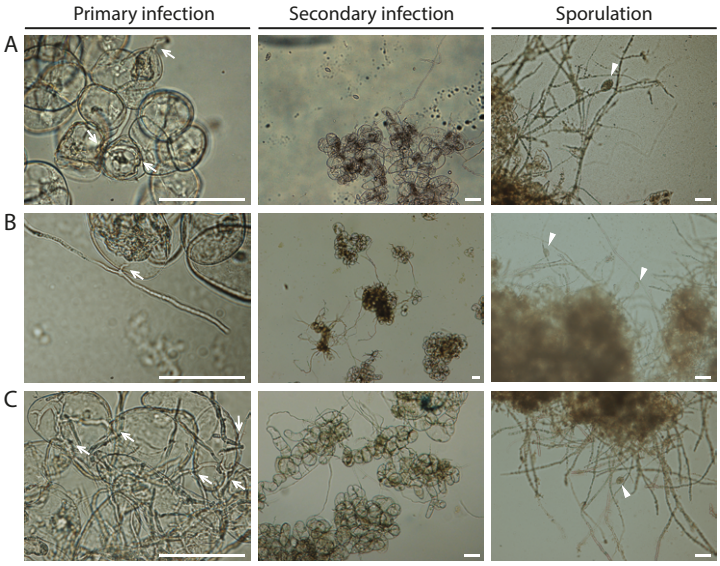


Figure S1. MsK8 cells infected with different *Phytophthora* spp. MsK8 cells were inoculated with **(**A) *P. infestans* 14-3-GFP, (B) *P. capsici* LT51 and (C) *P. palmivora* GFP3. Bright field images showing primary infection at 6 hpi, secondary infection at 16 hpi, and sporangia formation at 48 hpi. Arrows point to the sites of penetration (left panels) or sporangia (right panels). Bars represent 100 µm.

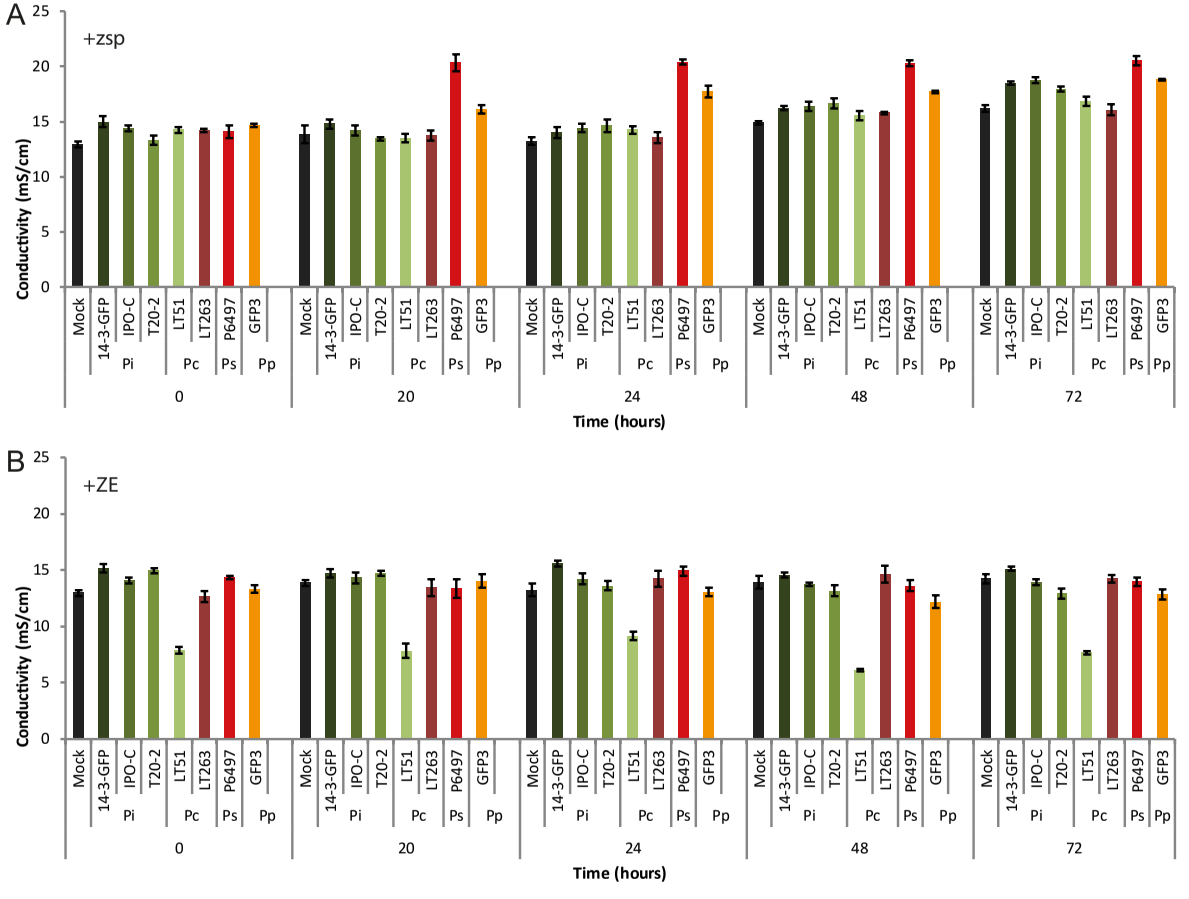


Figure S2. Electrolyte leakage of MsK8 cells (A) upon inoculation with *Phytophthora* zoospores (zsp) or (B) treatment with zoospore exudate (ZE) measured as conductivity at various time points. Colors of the bars represent a specific species and/or strain as indicated and correspond to the colors in Figure 2. Error bars represent standard deviation (n=3).

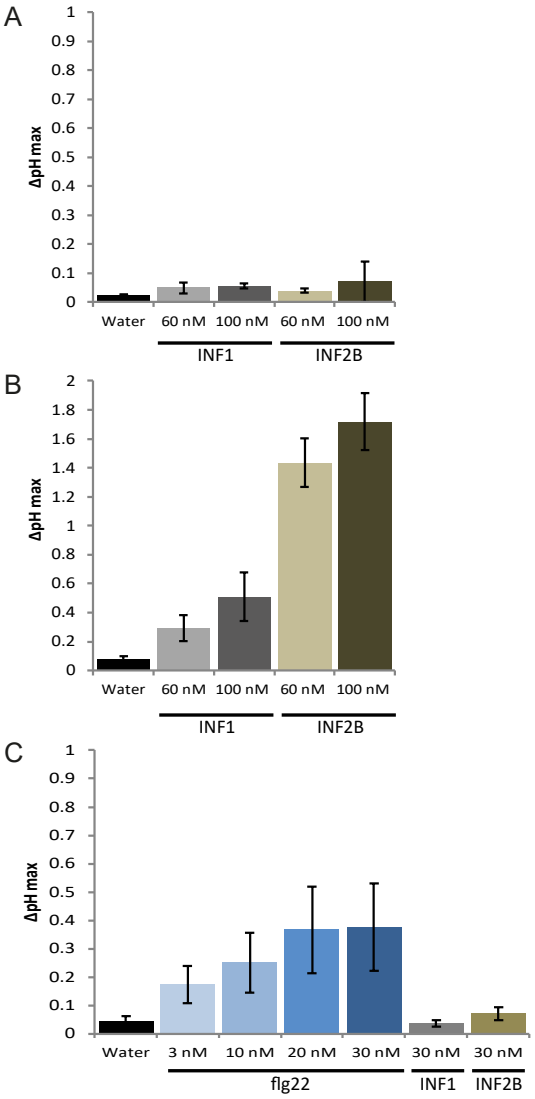


Figure S3. Responsiveness of MsK8 and BY-2 cells to *P. infestans* elicitins. MsK8 cells (A) and BY-2 cells (B), treated with *P. infestans* elicitins INF1 and INF2B. MsK8 cells treated with *P. infestans* elicitins INF1 and INF2B and flg22 (C). pH values were measured every three seconds during 20 minutes. ΔpH max value is the difference between the highest and the lowest pH value measured within 15 minutes after treatment. Error bars represent standard deviation (n=3).

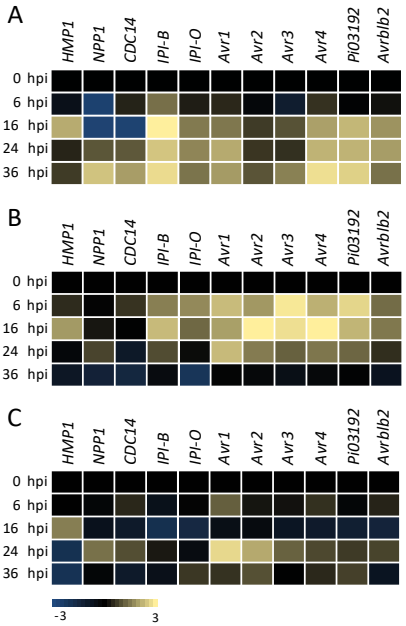


Figure S4. Expression of *P. infestans* genes upon inoculation of MsK8 cells with *P. infestans* 14-3-GFP (A), IPO-C (B) and T20-2 (C). Expression of stage-specific genes *HMP1*, *NPP1* and *CDC14, IPI-B* and various RXLR effector genes upon inoculation of MsK8 cells with zoospores. Expression levels were determined by qRT-PCR and the values at each time point were calculated relative to the expression level at time point 0 (0 hpi). Expression of the actin gene *ActA* was used as endogenous control.

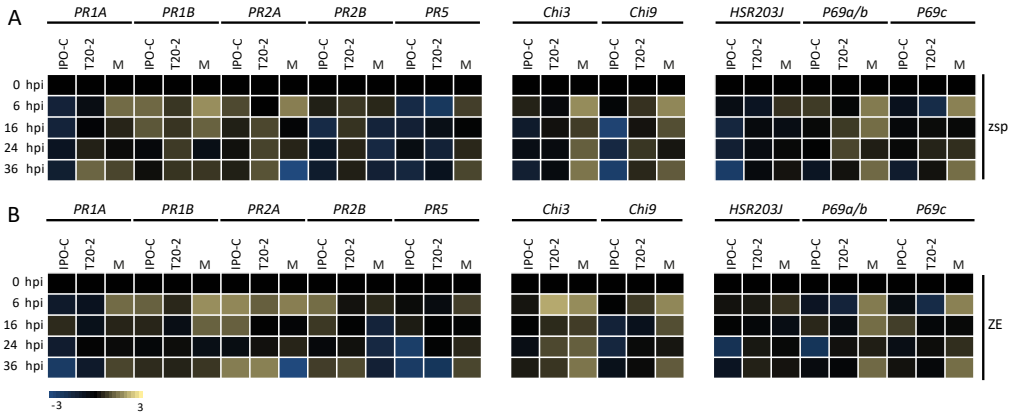


Figure S5. Expression of defense marker genes upon (A) inoculation of MsK8 cells with zoospores (zsp) or(B) treatment with zoospore exudate (ZE) of *P. infestans* strains IPO-C and T20-2. Defense genes include genes encoding pathogenesis-related proteins (PR), chitinases (Chi), a hypersensitivity marker (HSR203J) and isoforms of the subtilase P69 (P69a/b and P69c). Expression levels were determined by qRT-PCR and the values were calculated relative to the expression level at time point 0 (0 hpi). Expression of the tomato *ActA* was used as endogenous control.

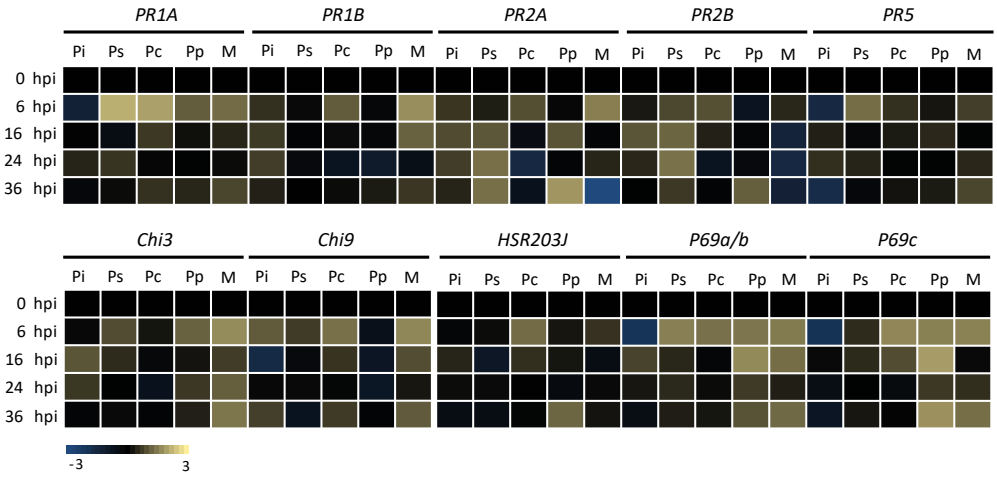


Figure S6. Expression profiling of tomato defense marker genes upon treatment of MsK8 cells with ZE of *P. infestans* 14-3-GFP (Pi), *P. sojae* P6497 (Ps), *P. capsici* LT263 (Pc) and *P. palmivora* GFP3 (Pp). Defense genes include genes encoding pathogenesis-related proteins (PR), chitinases (Chi), a hypersensitivity marker (HSR203J) and isoforms of the subtilase P69 (P69a/b and P69c). Expression levels were determined by qRT-PCR and the values were calculated relative to the expression level at time point 0 (0 hpi). Expression of the tomato *ActA* was used as endogenous control.

Chapter 7

General discussion

7

General discussion

Phytophthora infestans, the notorious late blight pathogen of potato and tomato, is one of the most studied oomycetes. Still, several aspects of its lifestyle and its virulence mechanisms remain to be uncovered. In order to infect the host cells, *P. infestans* utilizes several molecules to modify the host cell walls and membranes to initiate penetration, circumvent the induced plant defense responses and enable subsequent colonization. Besides the hundreds of identified RXLR effectors, of which several have been shown to be involved in virulence, *P. infestans* also has a large repertoire of enzymes. Enzymes are important cellular components that participate in almost every cellular reaction. Previous studies on several mammalian and plant pathogens have indicated that enzymes play a role in virulence. (Cassone *et al.*, 2016, ten Have *et al.*, 2002, Aoki *et al.*, 2011).

The aim of **this thesis** was to investigate certain groups of enzymes potentially involved in virulence of *P. infestans* and to further explore their function or mode of action. We have focused on three different groups of enzymes, namely, metalloproteases (MPs) aspartic proteases (APs) and phospholipase Ds (PLDs). The whole repertoire of *P. infestans* MPs was identified by genome mining and an inventory was created. Their domain architectures were analysed and expression profiles were explored based on RNA-seq data. From the 12 identified APs in *P. infestans*, we selected four members; PiAP10, PiAP11 and PiAP12 are three APs that belong to clade 5 and have homology to the *Plasmodium* Plasmepsin V (PMV), while PiAP5 is an AP with a domain architecture unique to oomycetes (Kay *et al.*, 2011). Of the PLDs, three small PLD-likes representative of different subfamilies i.e. sPLD-like-A, sPLD-like-B's and PLD-likes were selected for functional analysis (Meijer *et al.*, 2011).

Exploiting –omics resources for identifying and selection potential virulence factors

Nowadays, the increasing number of whole genome sequences and the large –omics data that are available can provide input not only for one single gene, but for the compilation of full repertoires of gene families in an organism. From these data, one can gather information about the properties of these families and this can be used to predict the encoded proteins and to select candidates for functional analysis. Several studies describing gene families in oomycetes have been published. Hallmarks are the studies dealing with RXLR effectors and Crinklers, classes of oomycete specific effectors that were only

discovered when the first *Phytophthora* genome sequences were released. Other studies have focused for example, on elicitins, another class of oomycete specific effectors (Jiang *et al.*, 2006), or on signal transduction components like kinases (Judelson & Ah-Fong, 2010) or phospholipid modifying enzymes including the PLDs (Meijer & Govers, 2006, Meijer *et al*., 2011). So far, however, inventories of enzyme families in *Phytophthora* are rather limited. Few studies report the repertoire of carbohydrate-active enzymes, including cell wall-degrading enzymes like glycosylic hydrolases and pectin methylesterases (Ospina-Giraldo *et al.*, 2010, Mingora *et al.*, 2014) while another study made an inventory of APs in three *Phytophthora* species including *P. infestans* (Kay *et al*., 2011).

For the research described in this thesis we made use of the existing inventories of PLDs and APs in *P. infestans* (Meijer *et al*., 2011, Kay *et al*., 2011). For MPs however, no full inventory was available and therefore, our first step was to mine *P. infestans* genome for MPs. We classified the 99 identified genes into 20 MP families based on their predicted MP domains (**Chapter 2**). The large number of genes that were not yet annotated as MPs and the large number of wrong gene models showed the importance of making such systemic inventories. Often, genome assemblies and genome annotations are far from perfect and, although it takes more effort, it pays off to have a complete view of all relevant genes. This helps in a more rational selection of candidates for future studies.

In *Phytophthora* pathogens, many of the genes with putative functions in virulence belong to expanded gene families. A clear example is the NLP (necrosis- and ethylene-inducing-like proteins) in *Phytophthora sojae*. Genome comparisons revealed a strong expansion and diversification of this particular family with more than 20 members in *Phytophthora* species compared to fungal plant pathogens, that contain only a few NLP genes (Tyler *et al.*, 2006). Recently, it was shown that one of the NLPs bind to glycosylinositol phosphorylceramide (GIPC) sphingolipids and form complexes that change the conformation of the toxin. Furthermore, plants with altered GIPC were found to be more tolerant to NLP toxins, with reduced cell death levels upon infiltration with the toxin (Lenarčič *et al.*, 2017). The subfamily of *Phytophthora* sPLD genes also shows hallmarks of expansion with a cluster comprising twelve secreted sPLD-likes and three PLD-likes without signal peptide (Meijer *et al*, 2011). With respect to the MPs, the total number of genes in oomycetes is expanded, compared to other Stramenopile species and more distantly related fungi, suggesting the importance of these enzymes for oomycete pathogens (**Chapter 2**).

The presence of a signal peptide (SP) is a strong indication that the enzyme is secreted (Sperschneider *et al.*, 2015). All three enzyme groups in our studies have members with and members without SPs. Among the 104 MP genes (and pseudogenes) we have identified 29 genes encoding a MP with SP. Only one of the twelve APs lacks a SP (Kay *et al*., 2011). In contrast, PLDs typically lack a SP but in *Phytophthora* there are notable exceptions. Of the sPLD-likes the majority has a SP (Meijer *et al*. 2011). Yet, lack of a SP does not exclude secretion since many proteins are secreted by non-conventional secretion pathways (Ding

et al., 2012, Rabouille *et al.*, 2012). Therefore, we cannot exclude that the MPs or PLDs lacking a SP are secreted and may function in virulence. In fact, the finding that transient expression in *Nicotiana benthamiana* leaves of PLD-like-1. The finding that a small PLD-like lacking a SP, results in cell death and promotes lesion growth suggests that PLD-like-1 is also secreted, be it via an alternative pathway (**Chapter 5**).

The presence of a transmembrane domain (TM) suggests that the protein is associated with, or is integrated in a membrane. One or more TMs were predicted in 17 *P. infestans* MPs (**Chapter 2**), while the majority of the identified PiAPs has one TM, suggesting they are membrane-bound enzymes (**Chapter 1**, Kay *et al*., 2011). In addition, PiAP5 contains seven TMs in the C-terminal part which is reminiscent of a GPCR (G-protein-coupled receptor domain). In the case of PLDs, the majority of genes does not contain a TM, with the exception of PXTM-PLD that has one TM and TM-PLD, that has five TMs preceding the catalytic PLD domain. None of the small PLD-likes has a TM (**Chapter 1, Fig. 1**, Meijer & Govers, 2006).

Enzymes are classified in different families based on their catalytic domains (Rawlings & Morton, 2008, Rawlings *et al.*, 2016). Analysis of all the predicted proteins in *Phytophthora* based on genome analyses, revealed many oomycete-specific domain combinations (Seidl *et al.*, 2011). Examples are the GPCR-PIPKs, combining a GPCR domain with a phosphatidylinositol phosphate kinase domain (PIPK). The GPCR-PIPK family is significantly expanded in oomycetes with 12 members in each *Phytophthora* species. Although initially considered as oomycete-specific, a recent study showed that GPCR-PIPKs occur sporadically in other genera throughout the tree of life (van den Hoogen *et al.*, 2018). Through domain analysis, proteins with unique domain architectures were identified in all three selected enzyme groups. Analysis of the full repertoire of *P. infestans* MPs revealed three members with domain architectures that are unique to *Phytophthora* species (**Chapter 2**). Among the APs, PiAP5 was identified as an oomycete-specific AP-GPCR (van den Hoogen *et al*., 2018).

Expression profiling, shows in which life stages the genes are active, likely leading to the presence of the encoded protein in that stage. Expression profiling of *P. infestans* MP genes based on RNA-Seq, showed that several of them are highly expressed in zoospores and germinating cysts, suggesting a potential involvement in either formation of spores, or in initiation of infection (**Chapter 2**). Expression analysis of small PLD-likes and PiAP genes by qRT-PCRs, showed that they are expressed in all *P. infestans* life stages and that expression is increased in germinating cysts. Furthermore, they are all expressed during infection, but with distinct expression patterns. Expression of small PLD-like genes is induced at later stages of infection, after 3 dpi, while the PiAP gene expression is higher at earlier stages, already at 36 hpi, suggesting that those enzymes are required at different phases of the interaction. PiAPs at the biotrophic phase and small PLD-likes at the transition stage from the biotrophic to the necrotrophic phase (**Chapters 3, 4** and **5**). Analyses of *Phytophthora* spp. transcriptomes have shown that gene families encoding RXLR effectors or cell wall-modifying enzymes are upregulated in infection propagules (zoospores and germinating cysts) and at the

early stages of infection, indicating a highly dynamic transcriptome (Ah-Fong *et al.*, 2017b, Ah-Fong *et al.*, 2017a, Judelson *et al.*, 2008, Haas *et al.*, 2009) (**Chapter 6**). Compilation of inventories of gene families, domain composition analysis as well as exploiting the available transcriptomics data are instrumental for the identification of virulence factors.

Box 1. Expanding the toolbox for functional analysis

Through functional analyses, we have evidence that several *P. infestans* enzymes are involved in virulence. Verification of enzymatic activity of the candidate enzymes, identifying the substrates and the cleavage sites, testing protease inhibitors and their effect during infection, mutating the catalytic sites to study the abolishment of the enzymatic activity or employing the newest molecular techniques like clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) for genome editing in *P. infestans*, would be the following steps. Despite the many studies on *P. infestans* – host interactions, the current, whole plant- or leaf-based systems in use, come also with several practical disadvantages. Lack of synchronization of the infection process, resulting in more than one infection stage on the same leaf, is a disadvantage when the aim is to monitor, for example, the changes in the transcriptome during a particular infection stage. These issues suggested the need for an appropriate infection system. For this purpose, we established a novel infection system using the tomato cell line MsK8 (**Chapter 6**). This system was evaluated through infection assays with several *Phytophthora* species and monitoring of the progress of the disease as well as the defense responses of the cells. Disease progression was easily followed by microscopic observations, and infections were more synchronised compared to leaf infections, while the cells responded in a similar manner whole plants do. We conclude that MsK8 cells can be used for detailed analysis of the interaction by introducing the purified enzymes or their inhibitors in the infection system and monitor the progress, as well as for obtaining large scale –omics data.

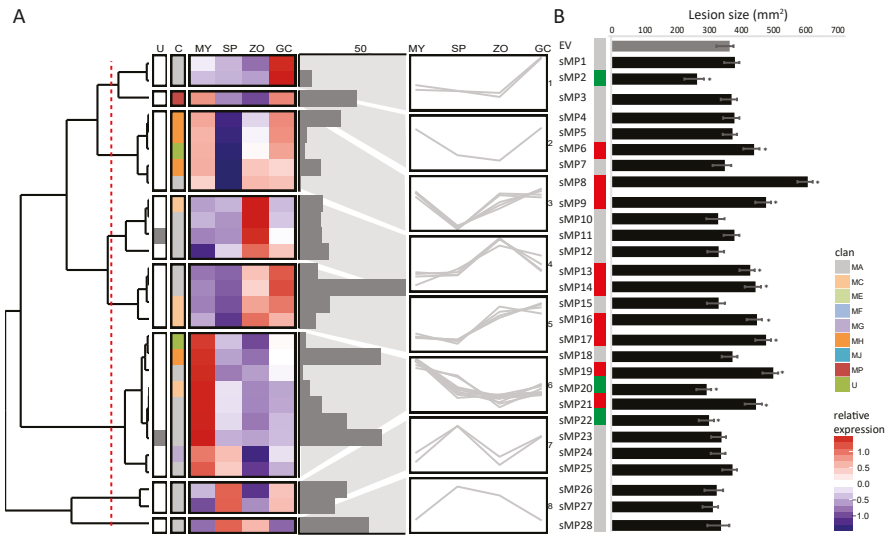
Characterizing *P. infestans* enzymes involved in virulence

Functional characterization is key for the identification of candidate virulence factors. Based on the properties of the encoded protein and the hypotheses of its function, different approaches can be followed, such as transient expression in leaves to monitor effects on plant cells, modification of expression inside the pathogen by DNA transformation resulting in overexpressing (OE) or silenced (Sil) lines or by heterologous production of the enzyme for *in vitro* biochemical analysis. Furthermore, infection assays, in combination with cell biology studies to localize the enzymes inside the pathogen or *in planta*, using plant leaves or cell suspension cultures are also possible (**Box 1**).

The *P. infestans* **MP** repertoire consists of 99 members from different families (**Chapter 2**). Due to the large number of MPs and the lack of information about their potential substrates, we used an approach in which we transiently expressed MP genes in *N. bentamiana* leaves to analyse their involvement in virulence. Several virulence factors such as RXLR effectors are secreted from the pathogen into the host cell to facilitate infection (Anderson *et al.*, 2015, Schornack *et al.*, 2009, Bozkurt *et al.*, 2012). For example, *in planta* expression of the RXLR effector AVR1 promoted lesion growth (Du *et al.*, 2015). As shown in **Box 2**

Box 2. Functional characterization of *Phytophthora infestans* secreted metalloproteases

In order to investigate the potential role of secreted metalloproteases (sMPs) in virulence, we transiently expressed the encoded genes in *Nicotiana benthamiana* leaves and subsequently, performed infection assays, as described in **Chapters 3** and **4**. In short, the coding sequences were amplified on cDNA from *P. infestans* strain 88069 and cloned into the binary vector pGWB5 (**Chapters 3** and **4**). Constructs harbouring *sMPs* were agroinfiltrated in *N. benthamiana* leaves and inoculations were performed one day post infiltration, with *P. infestans* 14-3-GFP zoospores. Lesions were measured at 5 days post inoculation. Furthermore, the expression profiles of sMPs during the different *P. infestans* life stages were deduced from RNA-seq data as described in **Chapter 2**. Our findings showed that all genes that have an effect on virulence are from two clans (MA and MC). Transient expression of nine *sMPs* in *N. benthamiana* leaves positively affected *P. infestans* growth, resulting in significantly larger lesions while three sMPs negatively affected *P. infestans* proliferation (B). The 12 sMPs that affect lesion growth belong to only six families out of the 20 MP families identified in *P. infestans*. Two out of three members of family M79, and all *P. infestans* sMPs from family M12 promoted lesion growth while all M3 MP members hampered lesion growth in *N. benthamiana* leaves. These results provide evidence that these particular sMPs are potential virulence factors. It should be noted that the M12 family (astacins) is related to the sheddase family of matrix metalloproteases (MMPs), with known proteolytic activity (Hartmann *et al.*, 2013). PITG_09851 is a particularly interesting case since it was detected in the extracellular proteome (Meijer *et al.*, 2014) and enhances lesion expansion. Several MP genes clustered together in clusters 3, 4 and 5, having high expression in infectious propagules, i.e. zoospores and/or germinating cysts (A). These results indicate that the 12 sMPs are involved *P. infestans* virulence.



A: Clustering of sMP genes based on expression patterns during the *P. infestans* life cycle. The dendrogram on the left displays the hierarchical clustering of the expression pattern (red-dashed line: cut off). The grey bar (column U), indicates the sMPs with a unique domain architecture. The colour-coded bar (column C) points to the clan in *MEROPS* comprising the MP. The heatmap shows the relative expression of each gene based on the stage-wise, z-score transformed expression values in mycelium (MY), sporangia (SP), zoospores (ZO) and germinating cysts (GC). The bar plot shows the mean transcripts per million (TPM) value for each gene. Line plots on the right display the stage-wise expression profiles of the eight

Box 2. *Continued*

clusters, resulting from the cut off number of genes in each cluster (detailed material and methods in Chapter 2) **B:** Lesion sizes at 5 dpi on infected *N. benthamiana* leaves transiently expressing *sMPs*. Black bars represent average data from three biological repeats (n=20), while the color bar on the left of the graph indicated the effect of the particular sMP on lesion growth, compared to control (EV) (grey: no effect, red: larger lesions, green, smaller lesions) Error bars indicate SD and asterisks indicate significant differences compared to the EV (ANOVA, p<0,05). Data presented in B were generated together with Lysette T. Lacambra.

we transiently expressed 28 genes encoding a MP with a SP (sMPs) in *N. benthamiana* leaves. Overexpression in the leaves did not have any visible effect, such as induction of cell death, as observed with other *P. infestans* proteins that act as virulence factors like RXLR and Crinkler effectors or elicitins (Oliva *et al.*, 2010, Oh *et al.*, 2010, Derevnina *et al.*, 2016). Infection assays showed that the presence of three sMPs in the leaves resulted in smaller lesions when inoculated with *P. infestans* while nine other sMPs promoted lesion growth. These results point out that several sMPs play a role in virulence. However, follow-up studies, such as testing these genes without their SP sequence, silencing them in *P. infestans*, detection of sMPs in host cells or in the apoplast, or biochemical analysis for proteolytic activity, are required to fully understand their function.

For the functional analysis of the selected **PiAPs**, a different approach was followed. We have focused on clade 5 PiAPs, based on their similarity to the *Pl. falciparum* AP, PMV. PMV modifies *Plasmodium* PEXEL effectors prior to their translocation into the host cell and inhibition of PMV activity results in blocking of effector translocation and reduced virulence (Boddey *et al.*, 2010, Russo *et al.*, 2010). PMV has been shown to locate and function in the endoplasmic reticulum (ER) (Boddey & Cowman, 2013, Russo *et al*., 2010). ASP5, an AP from *Toxoplasma gondii* homologous to PMV, is also modifying effector proteins inside the microbe and is located in the Golgi apparatus (Hammoudi *et al.*, 2015). We hypothesized that clade 5 PiAPs could be functioning inside the pathogen, in a similar way as the APs of *Pl. falciparum* and *T. gondii*. Therefore, we preformed infection assays on potato leaves with transgenic *P. infestans* lines in which the selected PiAP genes were either silenced or overexpressed (**Chapter 3**). A similar approach was used for the oomycete-specific AP-GPCR PiAP5, which is predicted to be membrane-bound (**Chapter 4**). Phenotypic analysis of the transformants showed that overexpression or silencing of *PiAP10* or *PiAP12* resulted in reduced growth and sporulation (**Chapter 3**). Alteration of *PiAP5* expression had severe effects on *P. infestans* growth and sporulation resulting in aberrant germination and loss of zoospore production, pointing to a function of this *PiAP* in pathogen fitness (**Chapter 4**). Infection assays showed that, regardless of the *PiAP* expression levels, alteration of *PiAP* expression results in reduced virulence, while overexpression of PiAP10 and PiAP12 induced cell death (**Chapter 3**). Similar findings have been described for *Pl. falciparum* where

blocking of PMV activity by inhibitors impairs its survival and virulence (Russo *et al*., 2010). Growth inhibition was observed upon inhibition of AP activity in fungal pathogens such as *Alternaria* and *Fusarium* (Dash *et al*., 2001). PiAP5 transformants also showed reduced lesion growth and infection efficiency on potato leaves (**Chapter 4**). Transient expression of PiAP5 in *N. benthamiana* leaves resulted in slightly increased ion leakage, while *in planta* expression of PiAP10 and PiAP12 resulted in smaller lesion growth, suggesting that either PiAPs are active against plant proteins in the transformed cells or that they induce defense responses in the plant. Based on these results, we cannot distinguish between a role of PiAP5 in virulence and the effect due to the hampered growth and spore germination of the pathogen. In *Arabidopsis*, overexpression of an apoplastic AP, resulted in dwarfism and resistance to the plant pathogenic bacterium *Pseudomonas syringae* suggesting that the excess of AP in the cell activates the defense mechanism of the plant (Xia *et al*., 2004). Furthermore, other APs that function as virulence factors, like APs from the yeast *Candida* species, are secreted and contribute to the adherence of the pathogen to host cells (Monod & Zepelin, 2002, Naglik *et al*., 2003). Despite being homologous to PiAP10, PiAP11 did not have any effect on *P. infestans* growth and sporulation or virulence, indicating a different function (**Chapter 3**). Further studies with protease inhibitors would give more information on the role of PiAPs in virulence. Since targeted genome editing in *P. infestans* is not possible yet, an alternative strategy would be to study the homologous PiAPs in other *Phytophthora* species like *P. sojae*, or *P. palmivora,* species in which CRISPR/Cas9 has been proven to be applicable for genome editing.

The repertoire of **small PLD-likes** in *P. infestans*, includes one sPLD-like-A, three PLD-likes and nine sPLD-like-B's. Their high similarity leads to the expectation of genetic redundancy (Meijer & Govers, 2006). PLD-likes and sPLD-like-B's have alterations in one the HKD domains (Meijer & Govers, 2006). sPLD-like-A and –Bs are also predicted to have a signal peptide, hence are likely secreted, and extracellular PLD activity has been detected through the production of phosphatidic acid (PA) (Meijer *et al*., 2011). Therefore, studies based on gene silencing in the pathogen would not easily reveal their role, unless targeted genome editing would be feasible allowing to mutate multiple members of one gene family silmutaneously in one recipient strain. The most suitable approach was *in planta* expression to study their effect on the host cell and thereafter their effect on *P. infestans* virulence. Analysis of the three selected small PLD-likes (PLD-like-1, sPLD-like-1 and sPLD-like-12) showed that overexpression in the plant cell results in cell death while their presence in the host cells promotes virulence (**Chapter 5**). These results showed that small PLD-likes play a role in virulence, as has been shown for PLDs in several other pathogenic organisms (Lery *et al*., 2014, Dolan *et al*., 2004, Jacobs *et al*., 2010, Kay *et al*., 2011). The enhancement of the observed effects by calcium and the abolishment of the effect by mutating the HKD motifs, showed that the observed effects are related to PLD activity. Furthermore, the importance of the SP, supports the hypothesis that small PLD-

likes are secreted proteins. We hypothesize that small PLD-likes play a role in virulence by modifying the host cell membrane to facilitate pathogen entry and/or by deregulating downstream signaling through the production of phosphatidic acid (PA) (Fig. 1C).

Overall, from the functional analyses, it became apparent that several members of the selected enzyme groups are involved in *P. infestans* virulence. The different outcomes are indicative for distinct functions of the different enzymes during pathogen growth or during interaction with the host.

Unravelling the mechanisms of enzymatic action

After finding that several of the *P. infestans* enzymes play a role in virulence, our functional analysis focused on biochemical analysis for enzymatic activity. Since our inventory is the first study of the full repertoire of *P. infestans* MPs there is no direct indication for their functions or potential substrates making the choice for biochemical studies difficult. In mammalian systems, several MPs called sheddases, have been shown to modify transmembrane receptors leading to either activation or deactivation of the receptor and subsequent induction or inhibition of downstream signaling (Huovila *et al.*, 2005, Hayashida *et al.*, 2010). Although *P. infestans* has no MPs that are homologous to known sheddases, it is anticipated that other MPs have a similar function (Fig. 1A). Based on the functional analysis of *P. infestans* sMPs, there is an indication of the potential secretion and activity of these proteases on proteins in the host apoplast, since transient *in planta* expression of three astacin members and one sMP resembling leishmanolysins, led to increased *P. infestans* growth (**Box 2**). Leishmanolysin GP63 plays a role in *Leishmania* virulence by cleaving host tyrosine phosphatases leading to attenuation of innate inflammatory responses of the host (Hallé *et al.*, 2009, Gomez *et al.*, 2009). Mutation of the GP63 gene and its homologs in *L. major* resulted in reduction of virulence indicating GP63 as a virulence factor (Joshi *et al.*, 2002). Astacins from animal-parasitic nematodes have been shown to modify the extracellular matrix of the host by collagen hydrolysis (Joshi *et al*., 2002, Semenova & Rudenskaya, 2009). It is also possible that *P. infestans* possesses one or more MPs that show autoproteolytic activity. CLCAs, MPs from zincin family have been shown to be activated by cleaving themselves (Yurtsever *et al.*, 2012). Biochemical analysis of these proteins is essential, in order to show that they have proteolytic activity. In addition, identification of their substrates could lead to their connection with a particular molecular mechanism.

Commonalities between *P. infestans* APs and *Plasmodium* PMV

In the malaria parasite *Pl. falciparum*, effector export and translocation has been extensively studied. It has been found that PEXEL effectors are modified by PMV prior to their translocation into the host erythrocytes by the *Plasmodium* translocon complex (PTEX) (Elsworth *et al*., 2014, Russo *et al*., 2010, Boddey *et al*., 2010). Other studies have suggested a different mechanism where effector binding to PI(3)P by the PEXEL motif is necessary for export and works independently from PMV-mediated PEXEL modification (Bhattacharjee *et al*., 2012). However, Boddey *et al*. (2016), could not detect PI(3)P in *Plasmodium* ER. They also showed that PEXEL effectors are not able to bind PI(3)P and that the export of effectors is dependent on PMV-mediated modification (Boddey *et al*., 2016, Wawra *et al*., 2013).

Phytophthora RXLR and *Plasmodium* PEXEL effectors have similar motifs, i.e. RXLR and $RXLX^{E/Q/D}$ motifs, respectively. Furthermore, clade 5 PiAPs are similar to PMV. Due to these similarities, two mechanisms for effector delivery have been suggested; pathogen-dependent, RXLR motif-related delivery into the host cell (Petre *et al*., 2016, Wawra *et al*., 2017), or pathogen-independent endocytosis after motif-related binding to external PI(3)P (Kale & Tyler, 2011, Dou *et al*., 2008). However, Boddey *et al*. (2016) showed that RXLR effectors are not binding to PI(3)P, as also indicated for PEXEL effectors. Previous studies have reported that PEXEL and RXLR motifs were interchangeable (Bhattacharjee *et al*., 2006). However, later studies have shown that the RXLR effectors are not modified by PMV and the RXLR motif cannot functionally replace the PEXEL motif and therefore cannot mediate effector translocation to the erythrocyte (Boddey *et al*., 2016). Furthermore, no homologous genes encoding the proteins of PTEX are present in *P. infestans* genome (Wawra *et al*., 2017, Grouffaud *et al*., 2010, Elsworth *et al*., 2014). Recently, a study on *Toxoplasma gondii* effectors showed that a class of effectors translocate into the host nucleus. The modification of the RRL sequence of the TEXEL (*Toxoplasma* Export Element) motif of GRA effectors is essential for their translocation into the host (Coffey *et al*., 2015, Hammoudi *et al*., 2015, Marino *et al*., 2018). Mutation studies showed that MYR1 protein is essential for GRA translocation and the recently identified MYR2 and MYR3 proteins play a role in GRA transportation pointing to the translocon-mediated effector delivery in *Toxoplasma* (Marino *et al*., 2018). We could hypothesize that a not-yet-identified translocon complex is present in *Phytophthora*.

In our study, *in vitro* activity assays with the heterologously produced PiAPs showed that PiAP10 and PiAP12 are able to modify the *P. infestans* RXLR effector AVR4 (Fig. 1B). In contrast, a mutated RXLR motif was not modified by PiAP10 and PiAP12, suggesting that the RXLR motif is important either for the effector cleavage or for its recognition and/or binding to PiAPs (**Chapter 3**). This is in accordance with the study by Wawra *et al*. (2017), showing that the RXLR motif of the *P. infestans* RXLR effector AVR3 is cleaved before

secretion. But, in contrast to our results, they were not able to detect any proteolytic activity of PiAPs against AVR3 (Wawra *et al*., 2017). In their case, a different effector was used and a different experimental set-up. Moreover, Wawra *et al*. (2017) did not provide evidence that the PiAPs they produced were active proteases which might explain the differences with our findings. The proteolytic activity against AVR4 coupled with the induction of cell death upon inoculation with the PiAP overexpressing lines could be explained by an imbalance in protease activity, affecting the levels of the modified effectors secreted in the host cells, due to the alteration of *PiAPs* expression. These data, in combination with the reduced virulence of PiAP10 and PiAP12 transformants, indicate a redundancy in their function. The finding that PiAP11 it has general protease activity but was not able to modify AVR4, combined with the fact that PiAP11 is not involved in virulence, indicates a different function for this enzyme (**Chapter 3,** Fig. 1B). Based on their amino acid composition, PiAP10 and PiAP12 probably form homodimers, whereas PiAP11 lacks cysteine residues and likely acts as a monomer (Kay *et al*., 2011). This could potentially explain why PiAP11 behaves different compared to the other two clade 5 PiAPs.

These studies show that in different pathogenic organisms, APs have common functions and support the hypothesis that PiAPs may be involved in effector translocation and that the RXLR motif is essential for cleavage (Fig. 1B). Recently, it was demonstrated, for the first time, that an RXLR effector actually ends up in the host cells during natural infection (Wang *et al*., 2017). Cell uptake assays using suspension cultures to visualize effector translocation combined with infection assays and localization studies could be a follow-up study (**Box 1**). Moreover, biochemical analysis of the modified AVR4 with mass spectrometry to verify the cleavage site, or *in vitro* activity assays with mutated versions of PiAPs on several RXLR effectors or protease inhibition studies are the next steps to further unravel RXLR effector translocation mechanisms.

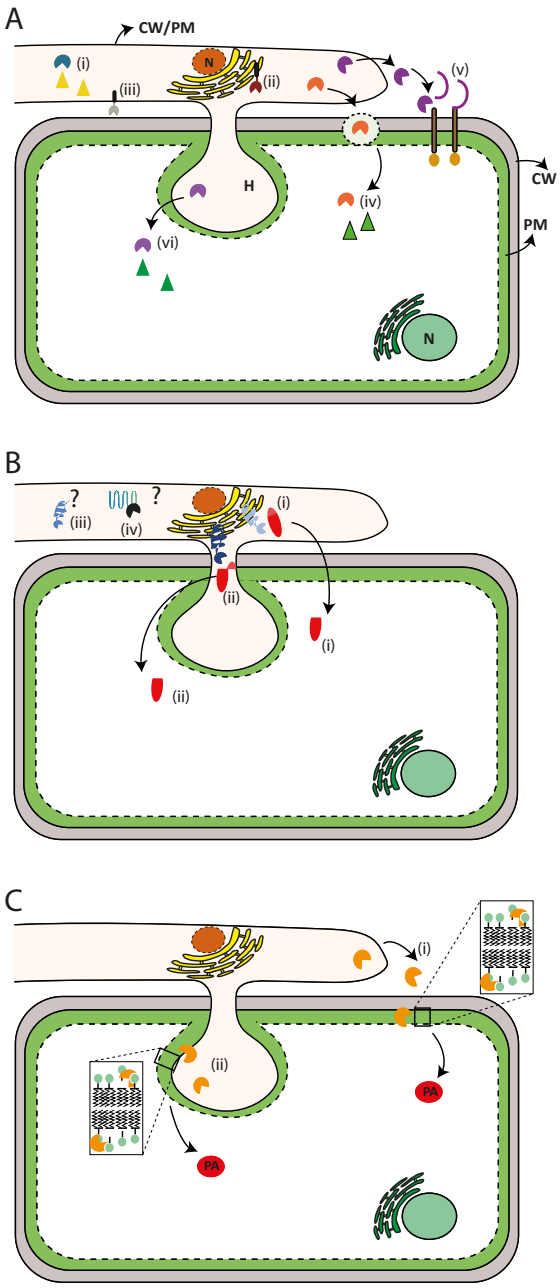


Figure 1. *Phytophthora infestans* enzymes participating in the host – pathogen interaction. Hypothetical models based on the main findings described in this thesis. A: *P. infestans* MPs can function inside the pathogen (i-iii), or are secreted (iv-vi). (i) MPs reside inside *P. infestans* and modify pathogen proteins. MPs with transmembrane domains are attached to (ii) inner membranes or, (iii) the outside membrane or cell wall. sMPs are secreted to the apoplast and (iv) taken up by the host cell via endocytosis. In the apoplast (v) sMPs may cleave receptor proteins in the plant membrane. (vi) sMPs secreted into the host cell through haustoria may modify intracellular host proteins (**Chapter 2**). B: *P. infestans*, PiAP10 (i) and PiAP12 (ii) cleave RXLR effectors in the ER, prior to their translocation into the host cell (ii). PiAP11 (iii) and PiAP5 (iv) may reside inside *P. infestans*, due to their predicted association with membranes (**Chapters 3 and 4**). C: small PLD-likes are (i) directly secreted to the apoplast or (ii) reside in the haustorium to hydrolyse phospholipids in the host membrane and/to activate downstream signaling through PA production (**Chapter 5**). Triangles represent substrates of either plant (green) or pathogen (yellow) origin. Red ovals represent RXLR effectors. N: nucleus; ER: endoplasmic reticulum; CW: cell wall; PM: plasma membrane; H: haustorium; PA: phosphatidic acid.

Are *P. infestans* enzymes functioning in synergy?

Sensing the environment is important for plant pathogens, as they have to receive signals from a nearby host to initiate spore germination. Knowing the molecular components governing signal perception and signal transduction is instrumental for unravelling pathogen-host interactions. The negative effect of overexpressing *PiAP5* on *P. infestans* sporulation (**Chapter 4**), is in accordance with the results obtained by Hua *et al*., (2014) on the GPCR-PIPK GK4, suggesting that *P. infestans* proteins containing GPCR domains could be involved in the fitness and asexual reproduction of the pathogen. A possible explanation could be the disruption of the signal transduction between the GPCR and the heterotrimeric G-protein, which in turn, affects downstream intracellular signaling. Silencing of G-protein subunits in *P. infestans* has been shown to negatively affect asexual spore formation and virulence (Latijnhouwers & Govers, 2003, Latijnhouwers *et al*., 2004). Based on the fact that we could not find protease activity of PiAP5, neither against general substrates nor the AVR4 effector, we can speculate that PiAP5 functions as a GPCR rather than an AP. However, it cannot be excluded that the produced protein fractions were not active due to the purification procedure, or that PiAP5 has protease activity against other substrates not tested in this study. It is also possible that PiAP5 has a dual function both as a GPRC and a protease, and that it is dependent on the conditions, like life stage or environment, when a particular function is active. Biochemical analysis and *P. infestans* transformations with mutated versions for either domain would shed more light on the function of this unique protein.

Besides the signaling mechanisms of the pathogen, membrane modification and downstream signaling in the host cells are also important for disease establishment. As all PLDs, small PLD-likes are expected to have phospholipase activity, thereby hydrolysing phospholipids residing in membranes. Alternatively, their mode of action in virulence could be the disturbance of downstream signaling through the formation of PA (Meijer & Munnik, 2003, Testerink & Munnik, 2005). In order to test the enzymatic activity of the small PLD-likes, we made attempts to produce them in *Escherichia coli* and in *Pichia pastoris* but all our efforts were unsuccessful. The presence of the small PLD-likes in bacterial or yeast cells, proved to be lethal, which may be related to their activity on the cell membranes and the inability of bacterial or fungal cells to control their expression (data not shown). Induction of cell death was also observed when small PLD-like genes were expressed *in planta,* and this was enhanced by the presence of calcium, adding to the premise that they have an effect on plants though membrane modification and cell death induction by catalyzing the production of phosphatidic acid (PA) (Fig. 1C). Loss of cell death inducing activity due to mutations in the catalytic motifs or removal of the SP, provided evidence that the observed effects are due to their function as an enzyme (**Chapter 5**).

Conclusions and future perspectives

For biologists, *P. infestans* and its relatives in the oomycete lineage are intriguing; there is still a lot to discover that can broaden our view of life on earth. For the farmers, *P. infestans* remains a nuisance that they want to get rid of. The challenge is to bridge the gap: deepen our knowledge on the biology and exploit this knowledge for developing durable and environmentally-friendly strategies to control late blight. There are still many things to discover when it comes to all the components that contribute to virulence. In depth understanding of the mechanisms underlying the infection process will provide us with new targets to combat *P. infestans*.

Throughout our studies it became apparent that several *P. infestans* enzymes are involved in the pathogen's lifestyle and virulence. Each enzyme group has different functions and likely acts at different stages of growth or infection. However, the exact mechanisms underlying the activity of these enzymes are still poorly understood. While we provide evidence for the importance of MPs, APs and small PLD-likes in *P. infestans* virulence, there are still many open questions; what are the roles of the different MP members? Are APs able to modify other RXLR effectors? Are there compounds that can block the activity of *P. infestans* enzymes? Is PiAP5 functioning as a protease or a GPCR? Altogether, the results presented in this thesis, provide a solid basis for further studies, which will give even more insight in the underlying mode of action of *P. infestans* enzymes. It is anticipated that finding ways to inhibit their activity or block their access to their targets inside the plant, would be useful for creating novel strategies to combat this devastating pathogen.

References

Ah-Fong, A.M.V., Kim, K.S. and Judelson, H.S. (2017a) RNA-seq of life stages of the oomycete *Phytophthora infestans* reveals dynamic changes in metabolic, signal transduction, and pathogenesis genes and a major role for calcium signaling in development. *BMC Genomics,* **18,** 198.

Ah-Fong, A.M.V., Shrivastava, J. and Judelson, H.S. (2017b) Lifestyle, gene gain and loss, and transcriptional remodeling cause divergence in the transcriptomes of *Phytophthora infestans* and *Pythium ultimum* during potato tuber colonization. *BMC Genomics,* **18,** 764.

Anderson, R.G., Deb, D., Fedkenheuer, K. and McDowell, J.M. (2015) Recent progress in RXLR effector research. *Mol Plant Microbe Interact,* **28,** 1063-1072.

Aoki, W., Kitahara, N., Miura, N., Morisaka, H., Yamamoto, Y., Kuroda, K. and Ueda, M. (2011) Comprehensive characterization of secreted aspartic proteases encoded by a virulence gene family in *Candida albicans*. *J Biochem,* **150,** 431-438.

Bhattacharjee, S., Hiller, N.L., Liolios, K., Win, J., Kanneganti, T.-D., Young, C., Kamoun, S. and Haldar, K. (2006) The malarial host-targeting signal is conserved in the Irish potato famine pathogen. *PLoS Pathog,* **2,** e50.

Bhattacharjee, S., Stahelin, Robert V., Speicher, Kaye D., Speicher, David W. and Haldar, K. (2012) Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell. *Cell,* **148,** 201-212.

Boddey, J.A. and Cowman, A.F. (2013) *Plasmodium* nesting: remaking the erythrocyte from the inside out. *Annu Rev Microbiol,* **67,** 243-269.

Boddey, J.A., Hodder, A.N., Gunther, S., Gilson, P.R., Patsiouras, H., Kapp, E.A., Pearce, J.A., de Koning-Ward, T.F., Simpson, R.J., Crabb, B.S. and Cowman, A.F. (2010) An aspartyl protease directs malaria effector proteins to the host cell. *Nature,* **463,** 627-631.

Boddey, J.A., O'Neill, M.T., Lopaticki, S., Carvalho, T.G., Hodder, A.N., Nebl, T., Wawra, S., van West, P., Ebrahimzadeh, Z., Richard, D., Flemming, S., Spielmann, T., Przyborski, J., Babon, J.J. and Cowman, A.F. (2016) Export of malaria proteins requires co-translational processing of the PEXEL motif independent of phosphatidylinositol-3-phosphate binding. *Nat Commun,* **7,** 10470.

Bozkurt, T.O., Schornack, S., Banfield, M.J. and Kamoun, S. (2012) Oomycetes, effectors, and all that jazz. *Curr Opin Plant Biol,* **15,** 483-492.

Cassone, A., Vecchiarelli, A. and Hube, B. (2016) Aspartyl proteinases of eukaryotic microbial pathogens: from eating to heating. *PLoS Pathog,* **12,** e1005992.

Coffey, M.J., Sleebs, B.E., Uboldi, A.D., Garnham, A., Franco, M., Marino, N.D., Panas, M.W., Ferguson, D.J.P., Enciso, M., O'Neill, M.T., Lopaticki, S., Stewart, R.J., Dewson, G., Smyth, G.K., Smith, B.J., Masters, S.L., Boothroyd, J.C., Boddey, J.A. and Tonkin, C.J. (2015) An aspartyl protease defines a novel pathway for export of *Toxoplasma* proteins into the host cell. *eLife,* **4,** e10809.

Dash, C., Ahmad, A., Nath, D. and Rao, M. (2001) Novel bifunctional inhibitor of xylanase and aspartic protease: implications for inhibition of fungal growth. *Antimicrob Agents Chemother,* **45,** 2008-2017.

Derevnina, L., Dagdas, Y.F., De la Concepcion, J.C., Bialas, A., Kellner, R., Petre, B., Domazakis, E., Du, J., Wu, C.-H., Lin, X., Aguilera-Galvez, C., Cruz-Mireles, N., Vleeshouwers, V.G.A.A. and Kamoun, S.C. (2016) Nine things to know about elicitins. *New Phytol,* **212,** 888–895.

Ding, Y., Wang, J., Wang, J., Stierhof, Y.-D., Robinson, D.G. and Jiang, L. (2012) Unconventional protein secretion. *Trends Plant Sci,* **17,** 606-615.

Dolan, J.W., Bell, A.C., Hube, B., Schaller, M., Warner, T.F. and Balish, E. (2004) *Candida albicans* PLD1 activity is required for full virulence. *Med Mycol,* **42,** 439-447.

Dou, D., Kale, S.D., Wang, X., Jiang, R.H.Y., Bruce, N.A., Arredondo, F.D., Zhang, X. and Tyler, B.M. (2008) RXLR-mediated entry of *Phytophthora sojae* effector AVR1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* , **20,** 1930-1947.

Du, Y., Mpina, M.H., Birch, P.R.J., Bouwmeester, K. and Govers, F. (2015) *Phytophthora infestans* RXLR effector AVR1 interacts with exocyst component Sec5 to manipulate plant immunity. *Plant Physiol,* **169,** 1975–1990.

Elsworth, B., Matthews, K., Nie, C.Q., Kalanon, M., Charnaud, S.C., Sanders, P.R., Chisholm, S.A., Counihan, N.A., Shaw, P.J., Pino, P., Chan, J.-A., Azevedo, M.F., Rogerson, S.J., Beeson, J.G., Crabb, B.S., Gilson, P.R. and de Koning-Ward, T.F. (2014) PTEX is an essential nexus for protein export in malaria parasites. *Nature Research letter,* **511,** 587-591.

Gomez, M.A., Contreras, I., Halle, M., Tremblay, M.L., McMaster, R.W. and Olivier, M. (2009) *Leishmania* GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. *Sci Signal,* **2,** 1-12.

Grouffaud, S., Whisson, S.C., Birch, P.R.J. and van West, P. (2010) Towards an understanding on how RxLR-effector proteins are translocated from oomycetes into host cells. *Fungal Biol Rev,* **24,** 27-36.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T. *et al*. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature,* **461,** 393 - 398.

Hallé, M., Gomez, M.A., Stuible, M., Shimizu, H., McMaster, W.R., Olivier, M. and Tremblay, M.L. (2009) The *Leishmania* surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. *J Biol Chem,* **284,** 6893-6908.

Hammoudi, P.-M., Jacot, D., Mueller, C., Di Cristina, M., Dogga, S.K., Marq, J.-B., Romano, J., Tosetti, N., Dubrot, J., Emre, Y., Lunghi, M., Coppens, I., Yamamoto, M., Sojka, D., Pino, P. and Soldati-Favre, D. (2015) Fundamental roles of the Golgi-associated *Toxoplasma* aspartyl protease, ASP5, at the host-parasite interface. *PLoS Pathog,* **11,** e1005211.

Hartmann, M., Herrlich, A. and Herrlich, P. (2013) Who decides when to cleave an ectodomain? *Trends Biochem Sci,* **38,** 111-120.

Hayashida, K., Bartlett, A.H., Chen, Y. and Park, P.W. (2010) Molecular and cellular mechanisms of ectodomain shedding. *Anat Rec,* **293,** 925-937.

Huovila, A.-P.J., Turner, A.J., Pelto-Huikko, M., Kärkkäinen, I. and Ortiz, R.M. (2005) Shedding light on ADAM metalloproteinases. *Trends Biochem Sci,* **30,** 413-422.

Jacobs, A.C., Hood, I., Boyd, K.L., Olson, P.D., Morrison, J.M., Carson, S., Sayood, K., Iwen, P.C., Skaar, E.P. and Dunman, P.M. (2010) Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infect Immun,* **78,** 1952-1962.

Jiang, R.H.Y., Tyler, B.M., Whisson, S.C., Hardham, A.R. and Govers, F. (2006) Ancient origin of elicitin gene clusters in *Phytophthora* genomes. *Mol Biol Evol,* **23,** 338 - 351.

Joshi, P.B., Kelly, B.L., Kamhawi, S., Sacks, D.L. and McMaster, W.R. (2002) Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. *Mol Biochem Parasitol,* **120,** 33-40.

Judelson, H. and Ah-Fong, A. (2010) The kinome of *Phytophthora infestans* reveals oomycete-specific innovations and links to other taxonomic groups. *BMC Genomics,* **11,** 700.

Judelson, H.S., Ah-Fong, A.M.V., Aux, G., Avrova, A.O., Bruce, C., Cakir, C., da Cunha, L., Grenville-Briggs, L., Latijnhouwers, M., Ligterink, W., Meijer, H.J.G., Roberts, S., Thurber, C.S., Whisson, S.C., Birch, P.R.J., Govers, F., Kamoun, S., van West, P. and Windass, J. (2008) Gene expression profiling during asexual development of the late blight pathogen *Phytophthora infestans* reveals a highly dynamic transcriptome. *Mol Plant Microbe Interact,* **21,** 433-447.

Kale, S.D. and Tyler, B.M. (2011) Entry of oomycete and fungal effectors into plant and animal host cells. *Cell microbiol,* **13,** 1839-1848.

Kay, J., Meijer, H.J.G., ten Have, A. and van Kan, J.A.L. (2011) The aspartic proteinase family of three *Phytophthora* species. *BMC Genomics,* **12,** 1-15.

Latijnhouwers, M. and Govers, F. (2003) A *Phytophthora infestans* G-protein β subunit is involved in sporangium formation. *Eukaryot Cell,* **2,** 971-977.

Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G.A.A., van West, P. and Govers, F. (2004) A Gα subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. *Mol Microbiol,* **51,** 925-936.

Lenarčič, T., Albert, I., Böhm, H., Hodnik, V., Pirc, K., Zavec, A.B., Podobnik, M., Pahovnik, D., Žagar, E., Pruitt, R., Greimel, P., Yamaji-Hasegawa, A., Kobayashi, T., Zienkiewicz, A., Gömann, J., Mortimer, J.C., Fang, L., Mamode-Cassim, A., Deleu, M., Lins, L., Oecking, C., Feussner, I., Mongrand, S., Anderluh, G. and Nürnberger, T. (2017) Eudicot plant-specific sphingolipids determine host selectivity of microbial NLP cytolysins. *Science,* **358,** 1431-1434.

Lery, L.M.S., Frangeul, L., Tomas, A., Passet, V., Almeida, A.S., Bialek-Davenet, S., Barbe, V., Bengoechea, J.A., Sansonetti, P., Brisse, S. and Tournebize, R. (2014) Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biology,* **12,** 41.

Marino, N.D., Panas, M.W., Franco, M., Theisen, T.C., Naor, A., Rastogi, S., Buchholz, K.R., Lorenzi, H.A. and Boothroyd, J.C. (2018) Identification of a novel protein complex essential for effector translocation across the parasitophorous vacuole membrane of *Toxoplasma gondii*. *PLoS Pathog,* **14,** e1006828.

Meijer and Munnik (2003) Phospholipid-based signaling in plants. *Annu Rev Plant Biol,* **54,** 265-306.

Meijer, H.J.G. and Govers, F. (2006) Genomewide analysis of phospholipid signaling genes in *Phytophthora* spp.: Novelties and a missing link. *Mol Plant Microbe Interact,* **19,** 1337-1347.

Meijer, H.J.G., Hassen, H.H. and Govers, F. (2011) *Phytophthora infestans* has a plethora of phospholipase D enzymes including a subclass that has extracellular activity. *PLoS ONE,* **6,** e17767.

Meijer, H.J.G., Mancuso, F.M., Espadas, G., Seidl, M.F., Chiva, C., Govers, F. and Sabido, E. (2014) Profiling the secretome and extracellular proteome of the potato late blight pathogen *Phytophthora infestans*. *Mol Cell Proteomics,* **13,** 2101-2113.

Mingora, C., Ewer, J. and Ospina-Giraldo, M. (2014) Comparative structural and functional analysis of genes encoding pectin methylesterases in *Phytophthora* spp. *Gene,* **538,** 74-83.

Monod, M. and Zepelin, M.B.-v. (2002) Secreted aspartic proteases as virulence factors of *Candida* species. *Biol Chem,* **383,** 1087-1093.

Naglik, J.R., Challacombe, S.J. and Hube, B. (2003) *Candida albicans* Secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews,* **67,** 400-428.

Oh, S.K., Kamoun, S. and Choi, D. (2010) Oomycetes RXLR effectors function as both activator and suppressor of plant immunity. *Plant Pathol J,* **26,** 209-215.

Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T.O., Chaparro-Garcia, A., Segretin, M.E., Stam, R., Schornack, S., Cano, L.M., van Damme, M., Huitema, E., Thines, M., Banfield, M.J. and Kamoun, S. (2010) Recent developments in effector biology of filamentous plant pathogens. *Cell microbiol,* **12,** 705-715.

Ospina-Giraldo, M.D., Griffith, J.G., Laird, E.W. and Mingora, C. (2010) The CAZyome of *Phytophthora* spp.: a comprehensive analysis of the gene complement coding for carbohydrate-active enzymes in species of the genus *Phytophthora*. *BMC Genomics,* **11,** 525.

Petre, B., Kopischke, M., Evrard, A., Robatzek, S. and Kamoun, S. (2016) Cell re-entry assays do not support models of pathogen-independent translocation of AvrM and AVR3a effectors into plant cells. *bioRxiv*, doi: https://doi.org/10.1101/038232.

Rabouille, C., Malhotra, V. and Nickel, W. (2012) Diversity in unconventional protein secretion. *J Cell Sci,* **125,** 5251-5255.

Rawlings, N.D., Barrett, A.J. and Finn, R. (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res,* **44,** D343-D350.

Rawlings, N.D. and Morton, F.R. (2008) The MEROPS batch BLAST: a tool to detect peptidases and their non-peptidase homologues in a genome. *Biochimie,* **90,** 243-259.

Russo, I., Babbitt, S., Muralidharan, V., Butler, T., Oksman, A. and Goldberg, D.E. (2010) Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature,* **463,** 632-636.

Schornack, S., Huitema, E., Cano, L.M., Bozkurt, T.O., Oliva, R., Van Damme, M., Schwizer, S., Raffaele, S., Chaparro-Garcia, A., Farrer, R., Segretin, M.E., Bos, J., Haas, B.J., Zody, M.C., Nusbaum, C., Win, J., Thines, M. and Kamoun, S. (2009) Ten things to know about oomycete effectors. *Mol Plant Pathol,* **10,** 795-803.

Seidl, M.F., Van den Ackerveken, G., Govers, F. and Snel, B. (2011) A domain-centric analysis of oomycete plant pathogen genomes reveals unique protein organization. *Plant Physiol,* **155,** 628-644.

Semenova, S.A. and Rudenskaya, G.N. (2009) The astacin family of metalloproteinases. *Biochem*, **3,** 17-32.

Sperschneider, J., Williams, A., Hane, J., Singh, K. and Taylor, J. (2015) Evaluation of secretion prediction highlights differing approaches needed for oomycete and fungal effectors. *Front Plant Sci,* **6**.

ten Have, A., Tenberge, K.B., Benen, J.A.E., Tudzynski, P., Visser, J. and van Kan, J.A.L. (2002) The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens. In: *Agricultural Applications.* (Kempken, F., ed.). Berlin, Heidelberg: Springer pp. 341-358.

Testerink, C. and Munnik, T. (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. *Trends Plant Sci,* **10,** 368-375.

Tyler, B.M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R.H.Y., Aerts, A., Arredondo, F.D., Baxter, L., Bensasson, D. and Beynon, J.L. (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science,* **313,** 1261 - 1266.

van den Hoogen, D.J., Meijer, H.J.G., Seidl, M.F. and Govers, F. (2018) The ancient link between G-protein-coupled receptors and C-terminal phospholipid kinase domains. *mBio,* **9,** e02119-02117.

Wang, S., Boevink, P.C., Welsh, L., Zhang, R., Whisson, S.C. and Birch, P.R.J. (2017) Delivery of cytoplasmic and apoplastic effectors from *Phytophthora infestans* haustoria by distinct secretion pathways. *New Phytol*, 1-11.

Wawra, S., Djamei, A., Albert, I., Nürnberger, T., Kahmann, R. and van West, P. (2013) *In vitro* translocation experiments with RxLR-reporter fusion proteins of Avr1b from *Phytophthora sojae* and AVR3a from *Phytophthora infestans* fail to demonstrate specific autonomous uptake in plant and animal cells. *Mol Plant Microbe Interact,* **26,** 528-536.

Wawra, S., Trusch, F., Matena, A., Apostolakis, K., Linne, U., Zhukov, I., Stanek, J., Koźmiński, W., Davidson, I., Secombes, C.J., Bayer, P. and van West, P. (2017) The RxLR motif of the host targeting effector AVR3a of *Phytophthora infestans* Is cleaved before secretion. *Plant Cell,* **29,** 1184-1195.

Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R.A. and Lamb, C. (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *EMBO J,* **23,** 980-988.

Yurtsever, Z., Sala-Rabanal, M., Randolph, D.T., Scheaffer, S.M., Roswit, W.T., Alevy, Y.G., Patel, A.C., Heier, R.F., Romero, A.G., Nichols, C.G., Holtzman, M.J. and Brett, T.J. (2012) Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation. *J Biol Chem,* **287,** 42138-42149.

Summary

Pathogens use a huge arsenal of proteins and metabolites in order to infect plants and establish disease. They function in facilitating pathogen entry or suppressing plant defence triggered upon pathogen attack. The proteins that pathogens employ to infect the host include enzymes such as cell wall-degrading enzymes, phospholipid modifying enzymes and proteases, but also effectors that target and disable the host cell machinery. To facilitate disease these proteins function at several levels. First of all, inside the pathogen, for example in processing or maturation of pathogenicity or virulence factors. Secondly in the apoplast, for example in cell wall degradation or receptor modification. And lastly, inside the host cell where they can have a role in a variety of processes such as disrupting vesicle transport, inhibiting transcription factors or deregulating the hormone balance.

The oomycete *Phytophthora infestans* is the causal agent of late blight, the most important disease in potato crops worldwide. Since its appearance in Europe in the mid-nineteenth century, when it destroyed the potato crop all over Western Europe and gave rise to the Irish potato famine, this pathogen has been studied extensively. Since the early 1990's, when the first molecular tools became available to study oomycete plant pathogens, attempts have been made to identify genes in *P. infestans* that play a role in pathogenicity or virulence and to unravel in depth the molecular and cellular mechanisms underlying pathogenicity. Despite rapid progress and the information provided by *Phytophthora* genome sequencing projects, these mechanisms are still poorly understood. The research described in this thesis deals with three different groups of enzymes in *P. infestans* that are anticipated to have a role in host-pathogen interactions and have not been studied previously.

In **Chapter 1** we first introduce potato and potato late blight and give a glimpse of the history of the disease and its societal impact. We then describe the life cycle of *P. infestans* and the molecular toolbox that is currently available to study this pathogen and the interaction with its host. Subsequently, we address the current practices of late blight control with emphasis on resistance genes and the ability of the pathogen to rapidly escape recognition. This is followed by an overview of enzymes and enzyme inhibitors that have been shown to play a role in virulence in a variety of pathogens and finally we describe the scope of this thesis.

In **Chapter 2** we present an inventory of metalloproteases (MPs) in *P. infestans*. MPs are a very diverse group of proteases that function by virtue of a divalent metal cation positioned at their catalytic site. Several MPs have been shown to be involved in the pathogenicity of mammalian and plant pathogens. Thorough genome mining and gene model corrections based on RNA-Seq data, revealed 99 MPs, divided over 20 families. Searches for homologs in other oomycetes and Stramenopiles species showed that some

MPs are expanded in *Phytophthora* species. Analyses of the domain compositions of MPs revealed a few MPs with a novel domain architecture exclusively found in *Phytophthora* species. Gene expression analyses showed that several MPs are highly expressed in infectious propagules. To our knowledge, this is the first systematic inventory of MPs in an oomycete. Based on these results MP genes can be selected as candidates for future functional studies in *P. infestans* and other oomycetes.

Chapters 3 and **4** deal with aspartic proteases (APs). In other pathogens APs were found to be involved in effector modification and virulence. The *Plasmodium falciparum* AP Plasmepsin V (PMV) has been shown to modify PEXEL effectors of *Plasmodium* prior to their translocation into the host cell. Of the twelve *P. infestans* APs, three are highly homologous to PMV. Due to this homology as well as the homology between the host translocation motifs in *Plasmodium* PEXEL effectors and *Phytophthora* RXLR effectors, it has been suggested that these three APs, PiAP10, PiAP11 and PiAP12, play a similar role in effector modification as PMV. In order to test their involvement in virulence we generated *P. infestans* transformants in which the PiAPs genes are silenced or overexpressed (**Chapter 3**). *PiAP11* transformants showed no obvious changes in virulence but transformants silenced for *PiAP10* and *PiAP12*, showed reduced infection efficiency and smaller lesions upon inoculation on potato leaves. Overexpression of these two genes also resulted in transformants with compromised virulence, while in several cases, they triggered cell death upon inoculation. A small reduction in colony growth and sporulation of the transformants was also observed. In-gel zymography assays with gelatin showed that all PiAPs have enzymatic activity that was inhibited by pepstatin. Moreover, PiAP10 and PiAP12 showed proteolytic activity against the RXLR effector AVR4 with its authentic RXLR motif, but not against AVR4 with a mutated RXLR motif showing that the modification only occurs when the RXLR motif is intact. PiAP11 had no proteolytic activity against AVR4 effector, suggesting a different function for the particular PiAP. These results show that PiAP10 and PiAP12 are involved in virulence and suggest their involvement in effector modification.

One of the *P. infestans* APs named PiAP5, is an AP with a unique domain architecture. It has a G-protein-coupled receptor (GPCR) domain adjacent to the AP domain and its topology suggests that PiAP5, with its seven transmembrane-spanning regions, is integrated in the membrane with the N-terminal AP domain as extracellular domain. Such an AP-GPCR is exclusively found in oomycetes. In **Chapter 4** we describe that alteration of *PiAP5* expression in *P. infestans* strongly reduced growth and sporulation and resulted in malformed germinating sporangia, especially in *P. infestans* transformants overexpressing the gene. Protease activity assays with general substrates and AVR4 did not show any activity of PiAP5 as a protease. These results show that PiAP5 is involved in fitness and sporulation of the pathogen, and consequently affects its virulence. Whether or not PiAP5 is active as a protease is unknown.

Chapter 5 focuses on the function of a subclass of phospholipase D's (PLDs). PLDs are enzymes that are involved in the hydrolysis of phospholipids, the main structural components of cell membranes, and in the production of the second messenger phosphatidic acid (PA). Three small PLD genes were selected for functional analysis by means of transient expression in *Nicotiana benthamiana* leaves. The presence of PLD-like-1, sPLD-like-1 or sPLD-like-12 in the leaves gave rise to calcium-dependent cell death and, when the leaves were inoculated with *P. infestans* it enhanced lesion growth. Mutations in the catalytic HKD motifs of the PLDs or removal of the signal peptide strongly reduced the cell death responses and abolished the virulence promotion demonstrating that the enzymatic activity of the PLDs is the major determinant and that the PLDs likely function outside the cell. These results show that that small PLD-likes play a role in virulence, either by modifying the host membranes or through PA signaling.

To gain more insight in the detailed mechanisms underlying *Phytophthora*-plant interactions, it is desirable to have experimental systems that enable high quality data generation. The establishment of model systems that fit that purpose, yet resembling the natural infection process are necessary. In **Chapter 6** we describe the development of a new infection system using the tomato cell line MsK8 as host for *Phytophthora* pathogens. The infection system was optimized by studying the interaction of MsK8 cells with several *Phytophthora* species over time. The experiments included infection assays, microscopy, gene expression profiling and ROS production measurements. The results show that the MsK8 infection system offers a versatile platform that can be used in studies ranging from analysing a single gene, testing chemical compounds, to large -omics studies.

Chapter 7 addresses the main findings of this thesis, and puts them in a broader perspective. The function and potential involvement of MPs, APs and PLDs in fitness or virulence of *P. infestans* are discussed, as well as the possible effects of the enzymatic activity in relation to virulence.

Overall, this thesis highlights the importance of enzymes in growth and virulence of *P. infestans* and gives insights in three different types of enzymes. The potential roles of these enzymes in *Phytophthora*-host interactions could serve as food for thought for further studies.

Samenvatting

Ziekteverwekkers gebruiken een enorm arsenaal aan eiwitten en metabolieten om planten te infecteren en ziektes te veroorzaken. Deze hebben een functie bij het binnendringen van ziekteverwekkers of in het onderdrukken van afweer van de plant die wordt geactiveerd bij een aanval door ziekteverwekkers. Voorbeelden zijn enzymen zoals celwand-afbrekende enzymen, fosfolipide-modificerende enzymen en proteasen, maar ook effectoren die het functioneren van de plantencel ondermijnen. Om infectie te bewerkstelligen, werken deze eiwitten op verschillende niveaus. Allereerst binnen de ziekteverwekker, bijvoorbeeld voor het modificeren virulentiefactoren die uitgescheiden worden. Daarnaast in de apoplast, bijvoorbeeld bij de afbraak van celwanden of het modificeren van receptoren. En tenslotte in de gastheercel waar ze een rol kunnen spelen in een verscheidenheid aan processen zoals het verstoren van vesikeltransport, het remmen van transcriptiefactoren of het dereguleren van de hormoonbalans.

De oomyceet *Phytophthora infestans* is de veroorzaker van de aardappelziekte, wereldwijd de meest beruchte ziekte in aardappelgewassen. Sinds de eerste uitbraken in Europa in het midden van de negentiende eeuw, toen de ziekte de aardappeloogst in heel West-Europa vernietigde en de Ierse hongersnood veroorzaakte, is deze ziekteverwekker uitgebreid bestudeerd. Vanaf het begin van de jaren negentig, toen de eerste moleculaire hulpmiddelen beschikbaar kwamen om deze ziekteverwekker te onderzoeken, zijn er pogingen gedaan om de moleculaire en cellulaire mechanismen die ten grondslag liggen aan pathogeniciteit te ontrafelen. Er is veel nieuwe kennis vergaard, mede dankzij grootschalige DNA genoom-sequencing projecten van verschillende *Phytophthora* soorten, maar desondanks is ons inzicht in de onerliggende mechanismen nog steeds beperkt. Het onderzoek beschreven in dit proefschrift is gericht op bepaalde enzymen in *P. infestans* die mogelijk een rol spelen in de interactie met de waardplant en die nog niet eerder zijn bestudeerd.

In **hoofdstuk 1** introduceren we de aardappelziekte en geven een kort overzicht van de geschiedenis van de ziekte en de maatschappelijke impact. Vervolgens beschrijven we de levenscyclus van *P. infestans* en de moleculaire toolbox die momenteel beschikbaar is om deze ziekteverwekker en de interactie met zijn gastheer te bestuderen. Vervolgens richten we ons op de huidige praktijken van bestrijding van de aardappelziekte met de nadruk op resistentiegenen en het vermogen van de ziekteverwekker om aan herkenning door de gastheer te ontsnappen. Dit wordt gevolgd door een overzicht van enzymen en enzymremmers waarvan is aangetoond dat ze een rol spelen bij virulentie in een verscheidenheid aan ziekteverwekkers en uiteindelijk presenteren we het doel van het onderzoek beschreven in dit proefschrift.

Hoofdstuk 2 geeft een overzicht van metalloproteases (MP's) in *P. infestans*. MP's vormen een zeer diverse groep van proteasen die voor hun functioneren afhankelijk zijn van een tweewaardig metaalkation dat op een katalytische plaats is gepositioneerd. Van verschillende MP's is aangetoond dat zij belangrijk zijn voor de virulentie van ziekteverwekkers op zoogdieren en planten. Potentiële MP genen in het *P. infestans* genoom werden geselecteerd op basis van annotaties en BLAST analyses en foutieve genmodellen werden gecorrigeerd met behulp van RNA-Seq data. Dit resulteerde in 99 MP's verdeeld over 20 families. Vergelijking met het MP repertoire in andere oomyceten en Stramenopila toonde aan dat sommige MP's in grotere aantallen voor komen in *Phytophthora* soorten. Uit analyse van de eiwitdomeinen bleken een aantal MP's een nieuwe domeinarchitectuur te hebben die uitsluitend wordt gevonden in *Phytophthora* soorten. Genexpressie-analyses toonden aan dat verschillende MP genen sterk tot expressie komen in sporangia en zoösporen. Voor zover bekend is dit de eerste systematische inventarisatie van MP's in een oömyceet. Op basis van deze resultaten kunnen MP-genen worden geselecteerd als kandidaten voor toekomstige functionele studies in *P. infestans* en andere oomyceten.

Hoofdstukken 3 en 4 hebben betrekking op asparagine proteasen (AP's). In andere ziekteverwekkers zijn AP's gevonden die betrokken zijn bij effectormodificatie en virulentie. Zo is bijvoorbeeld in de malaria parasiet *Plasmodium falciparum* aangetoond dat het AP PlasmepsinV (PMV), PEXEL-effectoren van *Plasmodium* modificeert voorafgaand aan hun translocatie in de gastheercel. Van de twaalf *P. infestans* AP's zijn er drie sterk homoloog aan PMV. Vanwege deze homologie en de homologie tussen de translocatiemotieven in *Plasmodium* PEXEL-effectoren en *Phytophthora* RXLR-effectoren, bestaat de mogelijkheid dat deze drie *P. infestans* AP's, PiAP10, PiAP11 en PiAP12, een vergelijkbare rol spelen bij effectormodificatie als PMV. Om hun betrokkenheid bij virulentie te testen, hebben we *P. infestans* transformanten gegenereerd waarin de expressie van PiAP genen is onderdrukt en transformanten waarin de PiAP genen tot overexpressie zijn gebracht **(hoofdstuk 3)**. PiAP11-transformanten vertoonden geen duidelijke veranderingen in virulentie, maar transformanten waarin *PiAP10* en *PiAP12* expressie werd onderdrukt, vertoonden verminderde infectie-efficiëntie en kleinere lesies na inoculatie op aardappelbladeren. Overexpressie van deze twee genen resulteerde ook in transformanten met verminderde virulentie, terwijl sommige celdood teweegbrachten. Ook vertoonden de transformanten een geringe afname in koloniegroei en sporulatie. Uit in-gel zymografie proeven met gelatine bleek dat de drie PiAP's enzymatische activiteit hebben die wordt geremd door pepstatine. Bovendien vertoonden PiAP10 en PiAP12 proteolytische activiteit in een assay waarin de RXLR-effector AVR4 met zijn authentieke RXLR-motief als substraat werd aangeboden, terwijl in dezelfde assay AVR4 met een gemuteerd RXLR-motief niet werd geknipt. Dit toont aan dat de proteolyse alleen optreedt wanneer het RXLR-motief intact is. PiAP11 vertoonde geen proteolytische activiteit en dit suggereert dat deze AP een andere functie heeft. Deze resultaten laten zien dat PiAP10 en PiAP12 belangrijk zijn voor virulentie en suggereren hun betrokkenheid bij effectormodificatie.

Een van de *P. infestans* AP's genaamd PiAP5, is een AP met een unieke domeinarchitectuur. Het heeft een G-eiwit gekoppelde receptor (GPCR) domein aansluitend aan het AP-domein en deze topologie suggereert dat PiAP5, met zijn zeven transmembraan domeinen, is geïntegreerd in het membraan met het N-terminale AP-domein als extracellulair domein. Zo'n AP-GPCR wordt uitsluitend aangetroffen in oomyceten. In **hoofdstuk 4** beschrijven we dat verandering van *PiAP5* expressie in *P. infestans* de groei en sporulatie sterk verminderen. Bovendien zijn de sporangia misvormd, vooral in *P. infestans*-transformanten die *PiAP5* tot overexpressie brengen. In assays met algemene substraten en AVR4 als substraat vertoonde PiAP5 geen enkele proteolytische activiteit. Deze resultaten tonen aan dat PiAP5 betrokken is bij de vitaliteit en sporulatie van *P. infestans* en als zodanig belangrijk is voor virulentie. Of PiAP5 al dan niet actief is als protease is onbekend.

Hoofdstuk 5 richt zich op de functie van een subklasse van fosfolipase D's (PLD's). PLD's zijn enzymen die betrokken zijn bij de hydrolyse van fosfolipiden, de belangrijkste structurele componenten van celmembranen, en bij de productie van de secundaire boodschapper fosfatidinezuur (PA). Drie genen coderend voor 'small' PLDs werden geselecteerd voor functionele analyse door middel van transiënte expressie in bladeren van *Nicotiana benthamiana*. De aanwezigheid van PLD-like-1, sPLD-like-1 of sPLD-like-12 in de bladeren leidde tot calciumafhankelijke celdood. Daarnaast had de aanwezigheid van deze PLD's een positieve invloed op de virulentie van *P. infestans*. Wanneer deze bladeren werden geïnoculeerd met *P. infestans* resulteerde dit in grotere lesies. Mutaties in de katalytische HKD-motieven in de PLD's of verwijdering van het signaalpeptide verminderden de celdoodresponsen en verminderden het vermogen om de virulentie te stimuleren, hetgeen aantoont dat de enzymatische activiteit van de PLD's essentieel is en dat de PLD's waarschijnlijk buiten de cel functioneren. Samengevat tonen deze resultaten aan dat deze 'small' PLD's een rol spelen bij virulentie, hetzij door het modificeren van de gastheermembranen of via PA-signalering.

Om meer inzicht te krijgen in de precieze mechanismen die ten grondslag liggen aan *Phytophthora*-plant interacties, is het van belang om over experimentele systemen te beschikken die het verzamelen van data van hoge kwaliteit mogelijk maken. Het opzetten van modelsystemen die aansluiten bij dat doel, en tegelijkertijd representatief zijn voor het natuurlijke infectieproces, is essentieel. In **hoofdstuk 6** beschrijven we de ontwikkeling van een nieuw infectiesysteem gebaseerd op de tomatencellijn MsK8 als gastheer voor *Phytophthora*. Het infectiesysteem werd geoptimaliseerd door de interactie van MsK8-cellen met verschillende *Phytophthora* soorten in de tijd te bestuderen. Het systeem werd geanalyseerd op basis van infectie-assays, microscopie, genexpressieprofilering en ROS-productiemetingen. De resultaten toonden aan dat het MsK8-infectiesysteem een veelzijdig platform is dat kan worden gebruikt in studies gaande van het analyseren van een enkel gen, het testen van chemische verbindingen, tot grootschalige studies.

Hoofdstuk 7 gaat in op de belangrijkste bevindingen van dit proefschrift en plaatst deze in een breder perspectief. De functie en potentiële betrokkenheid van MP's, AP's en PLD's in vitaliteit en virulentie van *P. infestans* worden besproken, evenals de mogelijke effecten van de enzymatische activiteit in relatie tot virulentie.

Alles samengenomen benadrukt dit proefschrift het belang van bepaalde enzymen in de groei en virulentie van *P. infestans* en geeft het inzicht in de activiteiten van drie verschillende groepen van enzymen. De potentiële rollen van deze enzymen in de interactie tussen *Phytophthora* en zijn waardplanten kunnen dienen als 'food for though' voor verdere studies.

Περίληψη

Τα παθογόνα χρησιμοποιούν ένα ευρύ 'οπλοστάσιο' ώστε να μολύνουν επιτυχημένα τα φυτά και να προκαλέσουν ασθένεια. Ως λειτουργίες έχουν την διευκόλυνση της εισόδου του παθογόνου ή την καταστολή της άμυνας του φυτού κατά τη διάρκεια της 'επίθεσης'. Οι πρωτεΐνες που χρησιμοποιούνται από τα παθογόνα περιλαμβάνουν ένζυμα που διασπούν τα κυτταρικά τοιχώματα, ένζυμα που τροποποιούν τα φοσφολιπίδια, πρωτεάσες, καθώς και τελεστές που απενεργοποιούν τις κυτταρικές λειτουργίες του ξενιστή. Για να προκαλέσουν ασθένεια, οι παραπάνω πρωτεΐνικές ομάδες λειτουργούν σε διαφορα επίπεδα. Αρχικά, στο εσωτερικό του παθογόνου, για παράδειγμα στην τροποποίηση ή ωρίμανση των παραγόντων παθογένειας. Δεύτερον, στον αποπλαστικό χώρο (αποπλάστη), για παράδειγμα στη διάσπαση του κυτταρικού τοιχώματος ή τη μορφοποίηση των υποδοχέων. Και τέλος, στο εσωτερικό του κυττάρου του ξενιστή, όπου μπορούν να παίξουν ρόλο σε διάφορες διεργασίες, όπως τη διακοπή της μεταφοράς των σωματιδίων του κυτάρου, την αναστολή των παραγόντων μεταγραφης ή την απορύθμιση της ορμονικής ισορροπίας.

Ο ωομύκητας Φυτόφθορα (*Phytophthora infestans*) είναι ο παθογόνος φορέας της ασθένειας 'περονόσπορος', μία από της συμαντικότερες ασθένειες στην καλλιέργεια πατάτας παγκοσμίως. Πρωτοεμφανίστηκε στην Ευρώπη στα μέσα του 19ου αιώνα, προκαλώντας καταστροφές στην καλλιέργεια πατάτας σε όλη τη δυτική Ευρώπη, με αποτέλεσμα τον μεγάλο λιμό της Ιρλανδίας. Από τις αρχές της δεκαετίας του '90, όταν οι μοριακές τεχνικές έγιναν διαθέσιμες για τη μελέτη των φυτοπαθογόνων ωομυκήτων, έχουν γίνει διάφορες προσπάθειες για την ταυτοποίηση των γονιδίων εκείνων που εμπλέκονται στην παθογένεια ή τη μολυσματικότητά του αλλά και για την ανάλυση των μοριακών και κυτταρικών μηχανισμών πίσω από αυτά. Παρά την ταχεία πρόοδο και τις πληροφορίες που παρέχονται από την αλληλούχιση του γονιδιώματος της Φυτόφθορας, οι μηχανισμοί αυτοί είναι ακόμα ελάχιστα κατανοητοί. Η έρευνα που περιγράφεται σε αυτή τη διατριβή, εστιάζει σε τρεις διαφορετικές ομάδες ενζύμων της Φυτόφθορας που πιθανώς παίζουν ρόλο στην αλληλεπίδραση του παθογόνου με τον ξενιστή και έως τώρα, δεν έχουν αναλυθεί.

Στο **κεφάλαιο 1** περιγράφεται η καλλιέργεια της πατάτας και η ασθένεια περονόσπορος και δίνεται μια σύντομη περιγραφή της ιστορίας της ασθένειας και της επιρροής της σε κοινωνικό επίπεδο. Ακολουθεί η περιγραφή του βιολογικού κύκλου της Φυτόφθορας και οι μοριακές τεχνικές που είναι διαθέσιμες για τη μελέτη του παθογόνου και της αλληλεπίδρασής του με τον ξενιστή. Στη συνέχεια, αναφέρονται οι σύγχρονες εφαρμογές για τον έλεγχο του περονόσπορου, δίνοντας έμφαση στα γονίδια αντοχής και τη δυνατότητα του παθογόνου να αποφύγει την αναγνώριση από το φυτό. Τέλος, δίνεται μία επισκόπιση των ενζύμων και των παρεμποδιστών τους, ο ρόλος των οποίων στην παθογένεια διαφόρων παθογόνων έχει περιγραφεί σε προηγούμενες μελέτες.

Το **κεφάλαιο 2** παρουσιάζει έναν πλήρη κατάλογο των μεταλλοπρωτεασών (MPs) της Φυτόφθορας. Οι μεταλλοπρωτεάσεις είναι μια μεγάλη ομάδα πρωτεασών, η λειτουργία των οποίων χαρακτηρίζεται από ένα κατιόν μετάλλου στο ενεργό κέντρο κατάλυσης του μορίου. Ένας αριθμός μεταλλοπρωτεασών εμπλέκεται στην μολυσματικότητα διαφόρων παθογόνων φυτών και ζώων. Μέσω της ανάλυσης του γονιδιώματος της Φυτόφθορας και διορθώσεων των γονιδιακών μοντέλων βασιζόμενα σε RNA-seq δεδομένα, εντοπίστηκαν 99 μεταλλοπρωτεάσες, διαχωρισμένες σε 20 οικογένειες. Έλεγχος για τον εντοπισμό ομόλογων γονιδίων σε άλλους ωομύκητες και ειδών που ανήκουν στα Stramenopiles, έδειξαν μια επέκταση του αριθμού των μεταλλοπρωτεασών στους ωομύκητες. Ανάλυση της δομής των πρωτεϊνών οδήγησε στην εύρεση μεταλλοπρωτεασών με δομή που βρέθηκε αποκλειστικά στους ωομύκητες. Η ανάλυση της έκφρασης των γονιδίων έδειξε ότι διάφορες μεταλλοπρωτεάσες εκφράζονται στα μολυσματικά στάδια του παθογόνου. Αυτή είναι η πρώτη συστηματική κατηγοριοποίηση των μεταλλοπρωτεασών ενός ωομύκητα. Σύμφωνα με αυτά τα αποτελέσματα, διάφορα γονίδια μπορούν να επιλεγούν για περαιτέρω αναλύσεις στη Φυτόφθορα και άλλους ωομύκητες.

Τα **κεφάλαια 3** και **4** ασχολούνται με τις ασπαρτικές πρωτεάσες (APs). Σε άλλα παθογόνα, οι ασπαρτικές πρωτεάσες έχουν βρεθεί να εμπλέκονται στην τροποποίηση των τελεστών και τη μολυσματικότητα. Η ασπαρτική πρωτεάση του πλασμώδιου της ελονοσίας *Plasmodium falciparum*, Plasmepsin V (PMV), τροποποιεί τους τελεστές PEXEL του πλασμώδιου πριν τη μεταφορά τους στο κύτταρο του ξενιστή. Από τις δώδεκα APs της φυτόφθορας, τρεις είναι ομόλογες της PMV σε μεγάλο βαθμό. Εξαιτίας αυτής της ομοιότητας καθώς και της ομοιότητας των τελεστών RXLR της Φυτόφθορας και των PEXEL του πλασμώδιου, μπορεί να υποτεθεί ότι αυτές οι τρεις πρωτεάσες, PiAP10, PiAP11 και PiAP12, παίζουν παρόμοιο ρόλο στην τροποποίηση των τελεστών της Φυτόφθορας. Για να αναλυθεί η εμπλοκή τους στη μολυσματικότητα, δημιουργήθηκαν τροποποιημένα στελεχη της Φυτόφθορας, όπου έγινε σίγαση ή υπερέκφραση αυτών των γονιδίων (**κεφάλαιο 3**). Η τροποποίηση της έκφρασης του PiAP11, δεν επέφερε εμφανείς αλλαγές στη μολυσματικότητα, ενώ η σίγαση των γονιδίων PiAP10 και PiAP12, οδήγησε σε μειωμένη μολυσματική ικανότητα στο παθογόνο και μικρότερες κηλίδες σε μολυσμένα φύλλα πατάτας. Η υπερέκφραση αυτών των γονιδίων είχε ως αποτέλεσμα τη μείωση της μολυσματικότητας, ενώ σε αρκετές περιπτώσεις τη νέκρωση των φυτικών ιστών. Επίσης, παρατηρήθηκε μια μικρή μείωση της ανάπτυξης της αποικίας του ωομύκητα σε τεχνητό θρεπτικό υλικό, όπως και μειωμένη παραγωγή σποριαγγείων. Η ανάλυση μέσω ζυμογραφίας (In-gel zymography) με ζελατίνη έδειξε ότι όλες οι ασπαρτικές πρωτεάσες είναι ενεργές και η δραστηριότητα τους παρεμποδίζεται από την πεπστατίνη (pepstatin). Επιπλέον, οι πρωτείνες PiAP10 και PiAP12 έδειξαν πρωτεολυτική δραστηριότητα εναντίον του RXLR τελεστή AVR4 της Φυτόφθορας με το αυθεντικό RXLR μοτίβο, αλλά όχι με την τροποποιημένη έκδοσή του. Το αποτέλεσμα αυτό έδειξε ότι η τροποποίηση του τελεστή γίνεται μόνο όταν η αλληλουχία RXLR είναι ακέραιη. Το PiAP11 δεν είχε πρωτεολυτική δραστηριότητα ενάντια

στον τελεστή AVR4, υποδεικνύοντας μία διαφορετική λειτουργία για τη συγκεκριμένη πρωτεάση. Τα αποτελέσματα δείχνουν ότι οι πρωτεάσες PiAP10 και PiAP12 εμπλέκονται στη μολυσματικότητα και υποδεικνύουν την εμπλοκή τους στην τροποποίηση των τελεστών.

Η PiAP5, μία ασπαρτική πρωτεάση της *Phytophthora infestans*, παρουσιάζει μια ξεχωριστή πρωτεϊνική δομή. Περιέχει έναν G-protein coupled receptor (GPCR) τομέα προσκείμενο στον ασπαρτικό τομέα της προτεάσης. Η τοπολογία της υποδεικνύει ότι η PiAP5, με 7 διαμεμβρανικές περιοχές, είναι ενσωματωμένη στη μεμβράνη με τον N-terminal ασπαρτικό τομέα ως εξωκυτταρικό. Ένα τέτοιο μόριο, που συνδιάζει τους τομείς AP-GPCR, έχει βρεθεί αποκλειστικά στους ωομύκητες. Στο **κεφάλαιο 4**, περιγράφεται ότι η αλλαγή της έκφρασης του PiAP5 γονιδίου μείωσε κατά πολύ την ανάπτυξη του παθογόνου και την παραγωγή σπορίων, καθώς και οδήγησε σε παραμόρφωση των βλαστημένων σποριαγγείων, ειδικά σε σειρές που υπερεκφράζουν το γονίδιο. Οι αναλύσεις της πρωτεολυτικής δραστηριότητας χρησιμοποιώντας γενικά υποκείμενα και τον τελεστή AVR4, δεν έδειξαν καμία δραστικότητα της PiAP5 ως πρωτεάση. Τα αποτελέσματα δείχνουν ότι η PiAP5 εμπλέκεται στην ανάπτυξη και τη σπορογένεση του παθογόνου και κατά συνέπεια επηρεάζει τη μολυσματικότητα. Το αν η PiAP5 είναι ενεργή πρωτεάση ή όχι, παραμένει άγνωστο.

Το **κεφάλαιο 5** εστιάζει στη λειτουργία μιας υποκατηγορίας των φωσφολιπασών D (PLDs). Οι φωσφολιπάσες D είναι ένζυμα που εμπλέκονται στην υδρόλυση των φωσφολιπιδίων, των κύριων δομικών υλικών των κυτταρικών μεμβρανών, αλλά και στην παραγωγή φωσφατιδικού οξέως (PA), ενός δευτερογενή αγγελιοφόρου των κυττάρων. Το γονίδια τριών φωσφολιπασών επιλέχτηκαν για ανάλυση, μέσω παροδικής έκφρασής τους σε φύλλα του φυτού *Nicotiana benthamiana*. Η παρουσία των PLD-like-1, sPLD-like-1 ή sPLD-like-12 στα φύλλα κατέληξε σε νέκρωση των κυττάρων, που σχετίζεται με την παρουσία ασβεστίου, ενώ μόλυνση των φύλλων με Φυτόφθορα είχε ως αποτέλεσμα την αύξηση των κηλίδων. Η μετάλλαξη του καταλυτικού κέντρου HKD των PLDs ή η αφαίρεση του οδηγού (σηματοδοτικού) πεπτιδίου, μείωσαν την κυτταρική νέκρωση και κατάργησαν την προώθηση της μολυσματικότητας, επιδεικνύοντας ότι η ενζυματική δραστηριότητα των PLDs είναι ο καθοριστικός παράγοντας για την αλλαγή της μολυσματικότητας και ότι πιθανόν τα PLDs λειτουργούν εξωτερικά των κυττάρων. Τα αποτελέσματα δείχνουν ότι τα PLDs παίζουν ρόλο στη μολυσματικότητα, είτε μέσω της τροποποίησης των μεμβρανών του ξενιστή, είτε μέσω των σημάτων του φωσφατιδικού οξέως.

Για να μελετηθεί σε βάθος η αλληλεπίδραση της Φυτόφθορας με τα φυτά και οι μηχανισμοί πίσω από αυτή την αλληλεπίδραση, είναι απαραίτητο να υπάρχουν πειραματικά συστήματα για την παραγωγή δεδομένων υψηλής ποιότητας. Η εγκαθίδρυση συστημάτων-μοντέλων γι'αυτό το σκοπό, που ταυτόχρονα προσομοιάζουν τις φυσικές συνθήκες της μόλυνσης είναι απαραίτητη. Στο **κεφάλαιο 6** περιγράφεται η ανάπτηξη ενός νέου συστήματος μόλυνσης

που χρησιμοποιεί τη σειρά κυτταροκαλλιέργειας τομάτας, MsK8 ως ξενιστή για διάφορα είδη φυτόφθορας. Το σύστημα βελτιστοποιήθηκε μελετώντας την αλληλεπίδραση των κυττάρων με διάφορα είδη Φυτόφθορας καθ'όλη τη διάρκεια της μόλυνσης. Τα πειράματα περιελάμβαναν μολύνσεις, μικροσκοπία, ανάλυση της έκφρασης διαφόρων γονιδίων των δύο οργανισμών και μετρήσεις παραγωγής των ελεύθερων ριζών οξυγόνου (ROS). Τα αποτελέσματα έδειξαν ότι το σύστημα προσφέρει μια ευέλικτη πλατφόρμα που μπορεί να χρησιμοποιηθεί σε ποικίλες μελέτες –από την ανάλυση ενός μόνο γονιδίου έως την μαζική συλλογή δεδομένων.

Το **κεφάλαιο 7** αναλύει τα κύρια ευρήματα αυτής της μελέτης και τα τοποθετεί σε ένα ευρύτερο πλαίσιο. Αναλύεται η λειτουργία και η δυνητική συμμετοχή των μετταλοπρωτεασών, των ασπαρτικών πρωτεασών και των φωσφολιπασών στην ανάπτυξη ή την μολυσματικότητα της Φυτόφθορας, όπως και τα πιθανά αποτελέσματα της ενζυματικής τους δραστηριότητας σε σχέση με την μολυσματικότητα.

Στο σύνολό της, αυτή η μελέτη τονίζει τη σημαντικότητα των ενζύμων στην ανάπτυζη και τη μολυσματικότητα του παθογόντου και παρέχονται γνώσεις πάνω στις τρεις διαφορετικές ενζυματικές ομάδες. Οι δυνητικοί ρόλοι αυτών των ενζύμων στην αλληλεπίδραση της Φυτόφθορας με τα φυτά προσφέρονται ως βάση για περαιτέρω μελέτες.

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Jasper, Jinbin, Jinling, Jordi, Luigi, Malaika, Mansoor, Martin, Nick, Ruth, Thomas, Viviane, Xiaoqian, Yan, Yin and Yu. Hanneke, It was great to work together at MusaRadix and it helped me a lot during the final stretch of my thesis.

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To my Greek friends Christos and Dimitris, Georgia, Maria and Alexandra, thank you for your friendship and for keeping my spirit up when I was homesick.

Στη 'νέα' οικογένειά μου, Μάρκο, Μαρία, Νικηφόρο, Αγάπη και Αρετή, σας ευχαριστώ που με καλωσορίσατε και σας εύχομαι ότι καλύτερο! Ευχαριστώ την αδερφή μου Μαρίνα αλλά και τους γονείς μου Παναγιώτη και Παρασκευή, που ήταν παντα διπλα μου και με υποστήριζαν, και με έμαθαν να είμαι ανεξάρτητη! Κυρίως τη μητέρα μου που παρόλο που αυτά τα χρόνια ήταν δύσκολα, και δεν μπορούσα να βοηθήσω από μακριά, δεν παραπονέθηκες ποτέ, αλλά πάντα νοιαζόσουν να είμαι εγώ καλά.

Manos there are no words enough to express my love and gratitude to you! You are the only person that understands the real me and can bring me real happiness. Thank you for you endless love and support (and incredible patience!) all these years. Doing this together was what made it possible!

Thank you, σας ευχαριστώ, bedankt!

About the author

Charikleia (Chara) Schoina was born in December 3rd, 1986 in Athens, Greece. Being always interested in biology, she studied Plant Sciences at the Agricultural University of Athens. Among the large variation of subjects she got fascinated by the interaction of plants and microbes, so she specialized on plant protection and environment. During her thesis, she worked on biological control of the fungus *Thielaviopsis basicola* in cotton. She performed her internship at the Benaki Phytopathological Institute where she got acquainted with disease trials and classical plant disease diagnosis. To gain more knowledge in the molecular aspects of plant-microbe interactions and experience living in a foreign country, she moved to Wageningen University for her MSc studies in Plant Sciences. Performing her major thesis in the Laboratory of Phytopathology, she focused on *Phytophthora infestans* PLD-likes and in her minor thesis, in a collaboration between Biochemistry and Phytopathology, she worked on biochemical studies on *Arabidopsis* LecRK-I.9. Fascinated by performing research, she started as a PhD candidate in the Laboratory of Phytopathology, under the supervision of Prof. Francine Govers and Dr. Klaas Bouwmeester. During her PhD, she focused on the devastating plant pathogenic oomycete *Phytophthora infestans*. After the completion of her PhD experiments, she joined the start-up company MusaRadix as trial coordinator, working on the major diseases threatening banana cultivation. Since January 2018, she works as Project Lead Phytopathology at Hobaho by Dümmen Orange, attempting to solve disease problems in the flower world.

Publications

Schoina, C., Rodenburg, S.Y.A., Meijer, H.J.G., Seidl, M.F., Bouwmeester, K., and Govers, F. (2018) *Phytophthora infestans* metalloproteases: an inventory of protein modifying enzymes. (submitted)

Meijer H.J.G., Schoina C., Wang S., Bouwmeester K., Hua C. and Govers F. (2018) *Phytophthora infestans* small phospholipase D-like proteins elicit plant cell death and promote virulence. (submitted)

Schoina, C., Bouwmeester, K. & Govers, F. (2017) Infection of a tomato cell culture by *Phytophthora infestans*; a versatile tool to study *Phytophthora*-host interactions. *Plant Methods,* **13,** 88.

Overdijk, E.J.R., de Keijzer, J., de Groot, D., Schoina, C., Bouwmeester, K., Ketelaar, T. and Govers, F. (2016) Interaction between the moss *Physcomitrella patens* and *Phytophthora*: a novel pathosystem for live-cell imaging of subcellular defence. *Journal of Microscopy*, **263**, 171-180.

Schoina, C. and Govers, F. (2015) The oomycete *Phytophthora infestans*, the Irish potato famine pathogen. In: *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture.* (Lugtenberg, B., ed.) Springer International Publishing. pp. 371-378.

Tzima, A.K., Paplomatas, E.J., Schoina, C., Domazakis, E., Kang, S. and Goodwin, P.H. (2014) Successful *Agrobacterium* mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. *Fungal Biology*, **118**, 675-682

Schoina, C., Stringlis I., Pantelides I., Tjamos S.E., and Paplomatas, E.J. (2011) Evaluation of application methods and biocontrol efficacy of *Paenibacillus alvei* strain K-165, against the cotton black root rot pathogen *Thielaviopsis basicola*. *Biological Control*, **58**, 68-73

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Charikleia Schoina**
Date: **1 June 2018**
Group: **Laboratory of Phytopathology**
University: **Wageningen University & Research**

1) Start-up phase

	date
▶ **First presentation of your project**	
Title: Towards innovative strategies for controlling late blight in potato	04 Sep 2013
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
The Oomycete Phytophthora infestans, the Irish Potato Famine Pathogen. IN: Principles of Plant-Microbe Interactions, Springer, Cham. DOI://doi.org/10.1007/978-3-319-08575-3_39	May, 2014
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure

	date
▶ **EPS PhD student days**	
EPS PhD student day, Amsterdam, NL	30 Nov 2012
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, NL	29-30 Jan 2015
EPS PhD student day 'Get2Gether', Soest, NL	28-29 Jan 2016
▶ **EPS theme symposia**	
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Utrecht, NL	24 Jan 2013
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Amsterdam, NL	25 Feb 2014
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Leiden, NL	22 Jan 2016
▶ **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015

	Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
►	**Seminars (series), workshops and symposia**	
	Seminars:	
	Phytopathology Seminar Series	Oct 2012 - Oct 2016
	Rob Goldbach Memorial Lecture by Prof. Dr. David Baulcombe	10 Oct 2012
	Ralph Panstruga: Comparative pathogenomics of powdery mildew fungi	04 Dec 2012
	Brian Staskawicz "Effector-Targeted Breeding for Durable Disease Control of Xanthomonas diseases in Tomato and Cassava	21 May 2013
	Pieter Dorrestein: A "GoogleMAP"-type molecular view of microbes - from culture to people	22 Aug 2013
	David Weller: Soilborne Pathogens and their Natural Biocontrol Agents in Cereal-Based Production Systems	25 Sep 2013
	Plant Sciences seminar; Robert Hall	08 Oct 2013
	Jos Raaijmakers:Back to the roots: exploring and exploiting the plant microbiome'	11 Mar 2014
	Jane Parker: Reprogramming cells for defense in plant innate immunity	09 Apr 2014
	Yuanchao Wang: Dissecting the interaction between Phytophthora sojae and soybean: making sense out of signaling and effectors	16 Jul 2014
	Frank van Breusegem: Metacaspases in plant cell death and development	09 Apr 2014
	Sophien Kamoun: Genome and effector evolution in the Irish potato famine pathogen lineage	28 May 2014
	Michael Freitag: Chromatin structure controls centromeres and secondary metabolism in filamentous fungi	21 Oct 2014
	Ortrun Mittelsten Scheid: Genetics and epigenetics: a complex relationship	19 Nov 2014
	Kevin Foster WEES seminar: The evolution of cooperation and competition in microbes	22 Jan 2015
	Monica Höfte: Towards understanding rice brown spot, a disease induced by physiological stress	06 Feb 2015
	Gero Steinberg: Long-distance endosome trafficking drives fungal effector production during plant infection	05 Jun 2015
	Jane Parker: Plant intracellular immunity: evolutionary and molecular underpinnings	21 Jan 2016
	Pierre-Marc Delaux: Evolution of symbiotic gene networks in land plants	08 Apr 2016
	Wenbo Ma: Effectors as molecular probes to understand pathogenesis	20 Jun 2016
	Edze Westra and Jennifer Doudna: Rewriting our genes? CRISPR-CAS systems as tools for genome editing	30 Sep 2016

	Workshops:	
	Advanced Light Microscopy Facilities, Wageningen University & Research	13 Jun 2013
	Applications of X-ray computed tomography (XRT)	18 Jun 2013
▶	**Seminar plus**	
▶	**International symposia and congresses**	
	Oomycete Molecular Genetics Network Meeting, Norwich, UK	02-04 Jul 2014
	2nd Annual Conference of the SUSTAIN Action, Zakopane, Poland	15-17 Oct 2014
	36th New Phytologist symposium, Munich, Germany	30 Nov-02 Dec 2015
	IS-MPMI XVII international congress, Portland, USA	17-21 Jul 2016
▶	**Presentations**	
	Talk: KNPV meeting: The process to progress	26 Oct 2013
	Talk: 2nd Annual Conference of the SUSTAIN Action, Zakopane, Poland	15 Oct 2014
	Poster: Oomycete Molecular Genetics Network Meeting, Norwich, UK	02-04 Jul 2014
	Talk: EPS theme 2 symposium and Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
	Poster: 36th New Phytologist symposium, Munich, Germany	30 Nov-02 Dec 2015
	Poster: IS-MPMI XVII international congress, Portland, USA	17-21 Jul 2016
	Talk: ALW meeting 'Experimental Plant Sciences', Lunteren	12 Apr 2016
▶	**IAB interview**	
▶	**Excursions**	
	Subtotal Scientific Exposure	*17.2 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
Advanced course 'Bioinformatics: A user's approach', Wageningen, NL	27-31 Aug 2012
Advanced Course 'The Power of RNA-seq', Wageningen, NL	16-18 Dec 2013
7th PhD Summerschool on Environmental Signaling, Utrecht, NL	26-28 Aug 2013
▶ **Journal club**	
Member of literature discussion P. infestans group	2012-2016
▶ **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**

4) Personal development	*date*
▶ **Skill training courses**	
Information literacy including EndNote introduction	11-12 Jun 2013
Interpersonal Communication for PhD candidates	19-20 Nov 2015
Career Perspectives	17 Mar-14 Apr 2016
Brain Training	20 Sep 2016

► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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