The biology and ecology of *Phytophthora infestans*: the role of cell wall proteins in development, pathogenicity and potato defence activation

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Doctoral thesis
Swedish University of Agricultural Sciences
Alnarp 2019

Acta Universitatis Agriculturae Sueciae
2019:81

Cover: *Phytophthora infestans* attacking potato plant. Illustration by Ida Szkalej.

ISSN 1652-6880
ISBN (print version) 978-91-7760-480-8
ISBN (electronic version) 978-91-7760-481-5

Print: SLU Repro, Alnarp 2019

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Abstract

Phytophthora infestans was one of the first eukaryotic microbes to be implicated as the causal agent of a plant disease. Despite a relentless research effort over the last 150 years, it remains one of the most economically important phytopathogens today. Late blight disease is the most devastating disease of potato and remains a global research focus. Contrary to its initial classification, *P. infestans* is not a fungus, but an oomycete, part of an entirely separate Kingdom of life and thus, broad spectrum fungicides are usually ineffective. Nonetheless, frequent spraying with synthetic pesticides remains the only efficient means of late blight control. The success of the pathogen stems from its remarkable adaptive ability and the high plasticity of the genome. *P. infestans* can overcome plant resistance, including resistance introduced through breeding, by rapid evolution of its effectors – molecules secreted to promote infection that can also be recognised by the plant immune system. Thus, non-chemical methods of control are not usually durable. Next-generation pesticides that retain current levels of crop protection whilst lowering their negative impact on the environment are needed. Therefore, the focus of our research was the biology and ecology of the pathogen and our aim was to identify new possible targets for future disease control measures. The cell wall is an essential component of microbial cells and unique components of the *P. infestans* cell wall are attractive targets for next generation pesticides. We identified two families of cell wall proteins necessary for growth, development and pathogenicity of *P. infestans*. Moreover, we analysed various commercially available chemicals for their effectiveness as *P. infestans* cell wall and growth inhibitors. The identification of a putative new *P. infestans* overwintering strategy in the rhizosphere of wild potatoes generated new hypotheses for the application of chemical fungicides in the future. Finally, in an attempt to increase late blight resistance in potato we expressed a *P. infestans* cell wall elicitor peptide *in planta*. This strategy was effective and resistance was increased under controlled conditions, but it was not durable in the field environment, indicating that caution needs to be taken when analysing transgenic plants and highlighting the importance of field trials. Overall, we have shown that several new targets in the oomycete cell wall could be promising leads for the development of next-generation pesticides and thus our data make a valuable addition to the understanding of the biology of *P. infestans* and the control of late blight disease.

Keywords: Phytophthora infestans, late blight, control strategy, pesticides, oomycete, cell wall

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Abstract

Phytophtora infestans är en av de första eukaryota mikroberna som kunde kopplas direkt till en växtsjukdom. Trots en intensiv forskningsinsats under de senaste 150 åren utgör den än idag en av de ekonomiskt viktigaste växtpatogenerna. Bland dessa finns potatisbladmögel som utgör den i synnerhet mest förödande potatissjukdomen med ett stort internationellt forskningsfokus. I motsats till sin ursprungliga klassifikation är *P. infestans* inte en svamp utan en oomycete, alltså tillhörande ett helt annat rike. Detta medför att bredspektrumsfungicider sällan når erforderlig verkan. Trots detta utgör regelbunden applicering av syntetiska pesticider det enda effektiva motmedlet mot potatisbladmögel. Patogenens framgång kommer till stor del från dess anmärkningsvärt höga anpassningsförmåga och genetiska plasticitet. *P. infestans* kan snabbt övervinna växtresistans, även sådan som åstadkommits genom resistensförädling, genom sin förmåga att hastigt anpassa sina effektorer. Effektorer är molekyler som utsöndras för att underlätta patogenens infektion men som även kan detekteras av växtens immunförsvar. Detta medför att icke-kemiska bekämpningsmetoder sällan är beständiga. Nästa generation av pesticider måste därför både erbjuda en hög nivå av grödoskydd samtidigt som den miljömässiga påverkan måste minska. Vårt forskningsfokus har därför varit att studera patogenens biologi och ekologi med syfte att identifiera potentiella måltavlor för framtida bekämpningsmetoder. Cellväggen utgör en essentiell del av mikrobiella celler och om en unik komponent hos *P. infestans* cellvägg kunde identifieras skulle den utgöra en attraktiv måltavla för nya pesticider. Vi har identifierat två cellväggsproteinfamiljer som är nödvändiga för tillväxt, utveckling och patogenicitet hos *P. infestans*. Dessutom analyserade vi olika kommersiellt tillgängliga kemikalier för deras effektivitet som cellväggs- och tillväxtinhiberare hos *P. infestans*. En hittills okänd förmodad övervintringsmekanism för *P. infestans* identifierades i rhizosfären från vild potatis viket ledde fram till nya hypoteser kring framtida appliceringen av kemiska fungicider. Slutligen, i ett försök att öka motståndskraften mot potatisbladmögel, uttrycktes en cellväggspecifik elicitor peptid från *P. infestans* i potatis. Denna strategi var effektiv och höjde potatisens motståndskraft när den odlades i en kontrollerad miljö men effekten var inte stabil under fältförsök. Detta indikerar att försiktighet måste iakttagas när resultat från transgena växter analyseras och lyfter fram vikten av fältförsök. Sammanlagt har vi visat att flertalet nya komponenter i oomyceternas cellväggar kan utgöra lovande måltavlor vid utvecklingen av nästa generation av pesticider. Vår data utgör därför ett värdefullt tillskott till vår förståelse av *P. infestans* biologi och bekämpningen av potatisbladmögel.

Keywords: Phytophthora infestans, potatisbladmögel, kontrollmetoder, pesticider, oomycete, cellvägg

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Dedication

To Ida

” Nikomu z nas życie, zdaje się, bardzo łatwo nie idzie, ale cóż robić, trzeba mieć odwagę i głównie wiarę w siebie, w to, że się jest do czegoś zdolnym i że do tego czegoś dojść potrzeba. A czasem wszystko się pokieruje dobrze, wtedy kiedy najmniej się człowiek tego spodziewa”.

“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained”.

Maria Skłodowska Curie

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Resjö, S.*, Brus, M., Ali, A., Meijer, H. J. G., Sandin, M., Govers, F., Levander, F., Grenville-Briggs, L. & Andreasson, E (2017). Proteomic Analysis of *Phytophthora infestans* Reveals the Importance of Cell Wall Proteins in Pathogenicity. *Molecular & Cellular Proteomics*, 16, pp. 1958-1971.

II Brus-Szkalej, M.*, Andersen, C. B., Mostafanezhad, H., Vetukuri, R. R., & Grenville-Briggs, L. J. A family of cell wall transglutaminases is essential for appressorium development and pathogenicity in *Phytophthora infestans*. (manuscript)

III Brus-Szkalej, M., Ibrahim Moushib, L., Lenman, M., Alexandersson, E., Hedley, P., Million, C., Andersson, M., Grenville-Briggs, L. J.* and Andreasson, E. Transgenic expression of the *Phytophthora infestans* elicitor peptide Pep13 induces potato defence responses and delays disease development under field conditions. (submitted)

IV Brus-Szkalej, M.*, Resjö, S., Vetukuri, R. R. & Grenville-Briggs, L. J. The role of cell wall polysaccharides in *Phytophthora infestans* development and as potential targets for anti-oomycete drugs. (manuscript)

V Vetukuri, R.R.*, Masini, L., McDougal, R., de Zinger, L., Brus,-Szkalej M, Williams, N., Lankinen, Å., & Grenville-Briggs, L. J. The presence of *Phytophthora infestans* in the rhizosphere of a wild Solanum species may contribute to off-season survival and pathogenicity. (submitted)

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* Corresponding author.

The contribution of Maja Brus-Szkalej to the papers included in this thesis was as follows:

I Planned and performed the molecular work. Participated in writing of the manuscript

II Developed research questions together with co-authors. Planned, performed and supervised laboratory work. Performed the *in silico* analysis. Wrote the manuscript with the input of the co-authors.

III Participated in the development of research questions. Planned and performed laboratory work and participated in the field work. Analysed the data. Wrote the manuscript together with co-authors.

IV Developed research questions together with co-authors. Planned and performed experimental work together with co-authors. Wrote the manuscript together with last author.

V Collected and analysed samples. Gave input on the manuscript.

Abbreviations

Avr	Avirulence
DCB	2,6-dichlorobenzonitrile
dpi	Days post inoculation
dsRNA	Double-stranded RNA
ETI	Effector- Triggered Immunity
ETS	Efector Tiggered Susceptibility
GM	Genetic Modification
hpi	Hours post inoculation
HR	Hypersensitive Response
IPM	Integrated Pest Managment
MPD	mandipropamid
PAMP	Pathogen-Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
R gene	Resistance gene
RISC	RNA-Induced Silencing Complex
RNAi	RNA interference
siRNA	Short interfering RNA

1 Introduction

1.1 *Phytophthora infestans* – the infamous plant destroyer

The *Phytophthora* genus contains over 120 species, all of which are plant pathogens (Martin et al., 2014). The damaging potential of these organisms is illustrated in their name, which literally means plant destroyer (from Greek: phyto – plant, phthora – destroy). The name was coined by Anton de Bary, who was first to formally classify a genus of fungi as plant pathogens and thus gave start to a new field of biology known as phytopathology. The debate on whether a microorganism could cause a plant disease started with the Great Irish Famine (1845-1849) - an epidemics of potato disease that cost Ireland a quarter of its population due to starvation and massive migrations (Ribeiro, 2013). The initial idea that the potato crops were lost due to excessive inbreeding was quickly debunked due to evidence that similar outbreaks were recorded in the North America between 1843 and 1845 (Ribeiro, 2013). Together with the new proposal that the blight was caused by a "fungus", later named by de Bary *Phytophthora infestans*, the facts led to a hypothesis that the pathogen originated in the South American Andes, precisely in current Bolivia and Peru, which were believed to be the origin of the cultivated potato (Gómez-Alpizar et al., 2007, Andrivon, 1996) and was brought to Europe in contaminated potato shipments. Based on historical documents Bourke and Lamb (1993) deduced that *P. infestans* arrived in Belgium in 1843 or 1844, when large shipments of potatoes were imported in order to restore the stocks lost to *Fusarium* dry rot outbreaks. The exact origin of the imported potato was, however, unclear.

An alternative theory arose in 1950s when it was discovered that *P. infestans* population in Mexico was very diverse, consisting of both A1 and

A2 mating types in equal proportions and that the *Solanum* plants in the region display a great variation in resistance (*R*) genes, suggesting co-evolution of the host plants and the pathogen (Gallegly and Galindo, 1958; cited in: Andrivon, 1996, Grünwald and Flier, 2005). Furthermore, it was shown that Mexico had been the source of the second *P. infestans* migration to Europe in the 1970s (Fry et al., 1993).

Based on both historical documents and biological investigations Andrivon (1996) proposed a three step process to explain *P. infestans* migrations: (1) limited migration of the pathogen from Mexico to South America, most likely of a single clonal lineage, and fixation of the isolate; (2) migration from South America to the US around 1841-1842, (3) migration of the same *P. infestans* isolate to Europe, either from South America or the US, or perhaps both places. The appeal of this theory is the fact that Mexican origin is acknowledged, but the migration through South America explains the lack of diversity in the pathogen populations in Europe before the second migration in the 1970s.

The second migration introduced the A2 mating type to Europe making sexual reproduction possible and hence introducing more diversity into *P. infestans* populations (Fry et al., 1993). The fact that the old clonal lineage had been completely replaced with the new populations throughout Europe suggests that the new isolates were more fit and thus most likely more aggressive than the old lineage, explaining the documented rise in occurrence and widespread nature of the disease in the 1980s (Fry et al., 1993). Sexual reproduction allows for formation of new genotypes and thus creates the possibility of accelerated evolutionary adaptation, particularly in new environments (Smith, 1971).

Independent of its exact origin and migration pattern *P. infestans* is a devastating pathogen of potato and tomato, and other *Solanaceae* plants that are of less or no economic importance (Becktell et al., 2006). Despite extensive research in the past 150 years, it remains the most serious pathogen of potato to this day (Fry et al., 2015). The annual global costs due to yield losses and management of late blight were recently estimated to be at least 7 billion euros (Haverkort et al., 2008, Haverkort et al., 2016).

1.2 "Bats are not birds, dolphins are not fish, oomycetes are not fungi"[1]

Up until the 1980s *Phytophthora* species were believed to be fungi making their systematic classification troublesome (Ribeiro, 2013). Initial studies in the 19th century were based purely on morphology and similarity to other known organisms and since oomycetes, including *P. infestans* form mycelia and spores and thus resemble the true Fungi they were classified as fungal organisms. The first indications that oomycetes might be different from true Fungi came already in the 19th century, shortly after the discovery of *P. infestans*, yet despite structural similarities to algae and being called oomycetes already in 1880 (Winter, 1880; cited in Lévesque, 2011), these organisms were still believed to share a close evolutionary relationship with Fungi. It was the differences in biochemical pathways (Vogel, 1960) and cell wall composition (Bartnicki-Garcia, 1968) that finally led to the creation of a new kingdom – Chromista that includes oomycetes and other fungal-like organisms (Cavalier-Smith, 1981). With the advance of technology and the development of molecular phylogenetics it became apparent that the relationship between Fungi and oomycetes is even more distant than predicted (Lévesque, 2011) and even the classification into Chromista might not be correct, as the name excludes organisms like colourless oomycetes and protists that are actually closely evolutionary related to organisms in that kingdom (Lévesque, 2011). The new proposed name stramenopiles (Patterson, 1989) was widely accepted, yet it is still unclear whether the name should replace Chromista as the kingdom name or function as an additional name of the group of organisms. A good solution to resolve the complex taxonomy issues was proposed by Burki et al. (2007) – division of the domain Eukaryota into supergroups and introduction of a new supergroup - SAR: Stramenopiles (including oomycetes) + Alveolata + Rhizaria. This new division not only orders diverse eukaryotes into clear groups, but also visibly separates oomycetes from Fungi, showing the strong relationship between oomycetes and algae, while linking true Fungi to animals (Metazoa) (Burki, 2014).

1. Sophien Kamoun

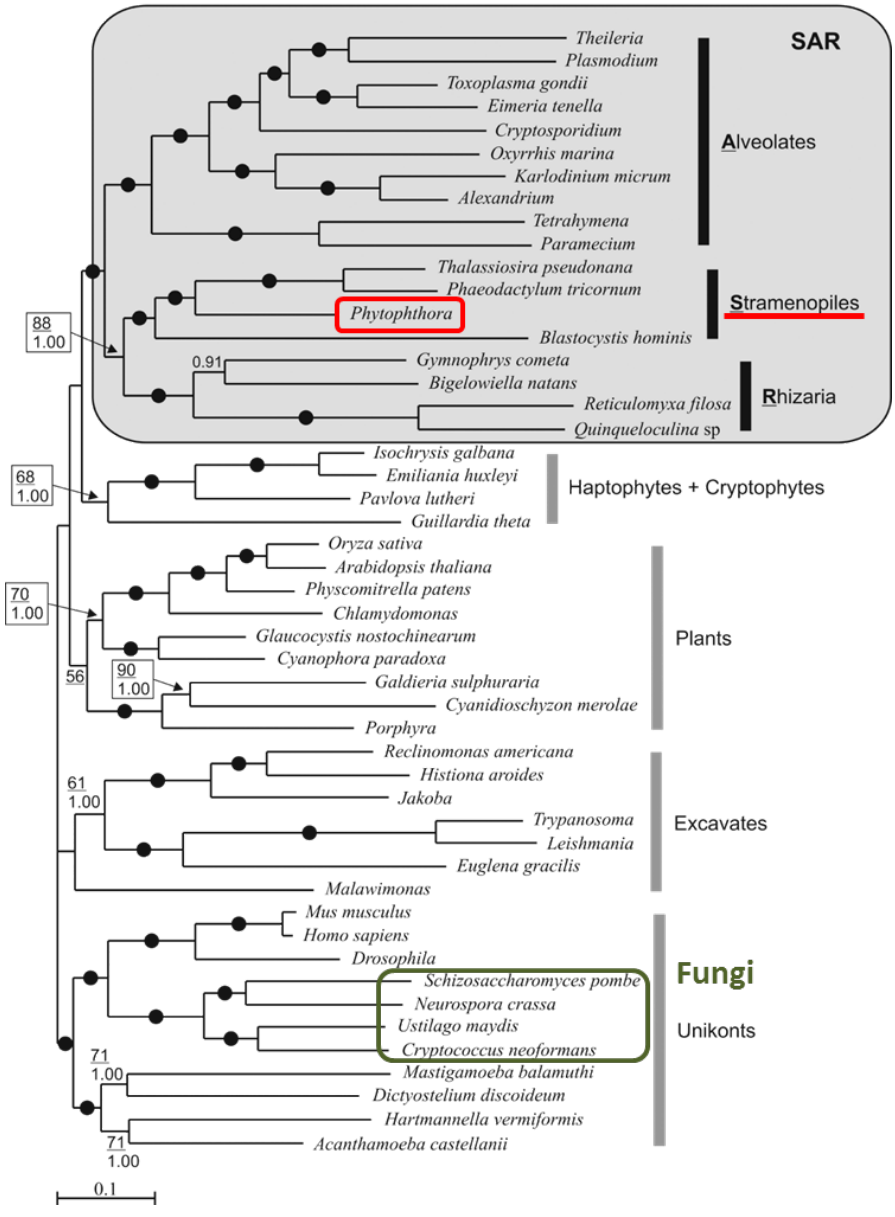

Figure 1. **The division of Eukaryota into super groups.** The tree was created using best maximum likelihood method and presents a few chosen representatives in each group. *Phytophthora infestans*, the only oomycete presented, and Stramenopiles are marked in red, while true Fungi are marked in green. Figure adapted from *Burki et al.* (2007).

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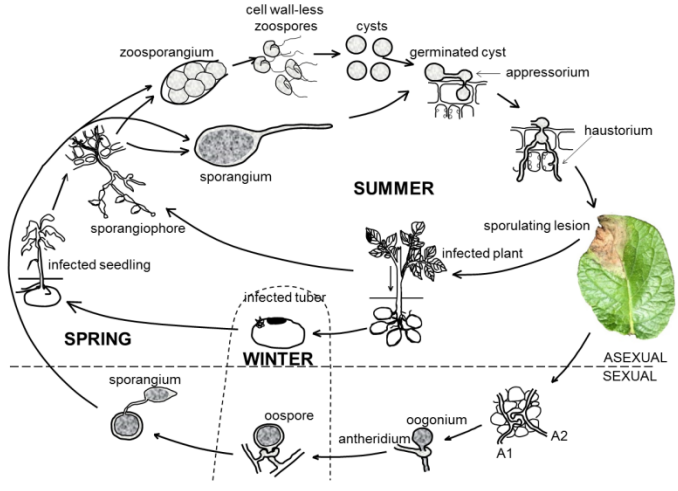
1.3 One day in life of a phytopathogenic oomycete

The recognition of oomycetes as distinct from Fungi has shifted the focus of many plant pathologists towards a better understanding of the biology, development and genetics of these organisms. *Phytophthora infestans*, as one of the most damaging pathogens but also one of the most recalcitrant organisms, has been given a particular attention (Kamoun et al., 2015). *P. infestans* is a hemibiotrophic organism, i.e. it requires a living host in the initial phases of the infection. The infection starts when a spore comes in contact with the plant surface and germinates. In the asexual life cycle (Figure 2, upper part) the spores are either sporangia which can germinate directly and infect the plant, or zoospores – small motile wall-less spores released from sporangia at lower temperatures. Each sporangium is able to release six to eight motile zoospores that are able to swim for several hours. Upon contact with the leaf surface, zoospores lose their flagella and build a cell wall, forming a cyst. The cysts, in turn, can germinate in the same manner as sporangia. In order to penetrate the surface of the leaf, the germ tube (from either sporangium or cyst) forms a specialised structure called an appressorium that, due to a thick cell wall and high turgor pressure, is able to break the plant cell wall and allows for the penetration peg to reach epidermal cells of the host. Germ tubes branch out into hyphae growing in the intercellular spaces and haustoria, the feeding structures, which invaginate host cells. After depletion of nutrients, *P. infestans* switches from a biotrophic to necrotrophic phase of growth. Plant cell death in this phase becomes visible as brownish lesions and when the pathogen reaches the exterior surface, sporangiophores are produced that release sporangia into the air and water. This whole cycle can be completed within just four days, thus producing billions of spores during one growing season. Moreover, the infection is not limited to the green parts of the plant, but affects stems and tubers as well (Judelson, 1997, Kamoun et al., 2015, Hardham, 2007).

Although not universally common in all of the countries where *P. infestans* is present, sexual reproduction is also possible (Figure 2, lower part). As mentioned before, *P. infestans* is heterothallic with both A1 and A2 mating types necessary for sexual reproduction. The two mating types differ in hormone production and sensing of the reciprocal hormone leads to production of sexual organs – antheridia (male) and oogonia (female). The gametangia produce haploid nuclei that fuse to form a diploid oospore. The offspring carries genetic material from the two parent isolates and hence can exhibit properties of either or both of them, resulting in a potentially large population diversity. Even if sexual reproduction does not necessarily contribute to the aggressiveness of the strains, it increases the availability of inoculum in the

field through overwintering of the pathogen in the soil (Medina and Platt, 1999). While sporangia are only able to survive a few weeks in the soil and their infectivity ranges from 15 to 45 days, depending on the pathogen isolate and the soil type (Andrivon, 1994); oospores were shown to persist for years and their infectivity declined only slightly after 18 months storage in various environments including soil where the pathogen was subjected to temperatures below zero. What is more, the majority of the investigated oospores were able to infect potato and tomato plants after subjection to harsh weather conditions (Mayton et al., 2000). The increased inoculum means not only greater risk of epidemics, but also a potentially faster rate of disease progression (Hannukkala et al., 2007).



.Figure 2. **Life cycle stages of *Phytophthora infestans*.** Adapted from Van West and Vleeshouwers (2004).

1.4 Enemy at the gates

The first layer of plant immunity, the identification of pathogen as non-self, is achieved by the recognition of pathogen associated molecular patterns (PAMPs) by cell surface recognition receptors (PRRs) and subsequent activation of defence mechanisms (Jones and Dangl, 2006, Zipfel, 2014). To evade PAMP-triggered immunity (PTI) which prevents spreading of the infection, pathogens rely on effector molecules. The role of these proteins is

suppression of plant defence mechanisms and alteration of host cell processes resulting in Effector-Triggered Susceptibility (ETS). The second layer of plant defences is the recognition of effectors by proteins encoded by resistance (*R*) genes. Particular effectors, encoded by avirulence (*Avr*) genes, are recognised by particular resistance (R) proteins in a gene-for-gene manner, thus the recognition at this level is specific to an individual pathosystem. The recognition of an effector leads to activation of effector triggered immunity (ETI) (Jones and Dangl, 2006). Activation of ETI in the host plant often results in killing off the infected cells, a mechanisms termed Hypersensitive Response (HR). There are two types of effectors – apoplastic effectors that act outside plant cells in the apoplastic space, and cytoplasmic effectors that are delivered into host cells. Secretion of both types of effectors is believed to occur through haustoria, but using distinct pathways (Wang et al., 2017). The majority of apoplastic effectors act to neutralise plant enzymes that could be harmful to the microorganism, while cytoplasmic effectors typically have a role in altering host processes (Kamoun, 2006). Thus, cytoplasmic effectors are of particular interest in studies into what determines disease resistance or susceptibility. All of the oomycete *Avr* genes identified to-date encode effectors with an RXLR motif (Birch et al., 2008, Birch et al., 2009). The motif consisting of arginine, any amino acid, leucine and arginine was shown to be necessary for the delivery of these types of effectors into plant cells (Whisson et al., 2007).

1.5 Always on top

The seemingly most sustainable method of disease control is breeding for resistant varieties of host plants. Late blight resistance breeding in potato has a long history worldwide and started already at the end of 19th century (Kamoun et al., 2015). Many of the wild relatives of cultivated potato (*Solanum tuberosum*) display late blight resistance and thus continue to be the focus of the search for resistance determinants. In the mid-twentieth century there were already eleven *R* genes, called *R1-R11*, identified from *Solanum demissum* (Black et al., 1953). Today there are at least 21 identified *R* genes conferring either full or partial late blight resistance (Vleeshouwers et al., 2011). The subsequent use of these *R* genes in various breeding programs has largely proved unsuccessful, as the pathogen was able to overcome the newly introduced resistance within a very short time, in the worst cases after a few growing seasons (Fry, 2008, Haverkort et al., 2016). The ability of *P. infestans* to overcome R proteins stems from its large population and effector diversity – R proteins are only effective against isolates that carry and express the gene for the corresponding effector protein. *P. infestans* has a highly plastic genome

with large repetitive regions that are likely to contribute to its remarkable adaptive ability. The effector genes are localised in the highly dynamic (repetitive) and expanded regions of the genome, thus allowing for their fast evolution (Haas et al., 2009). The pathogen can mutate, pseudogenise or silence avirulence (*Avr*) genes thus changing the repertoire of the RXLR effectors that they encode and evading recognition by the plant (Gilroy et al., 2011, Vetukuri et al., 2012, Haas et al., 2009). Therefore, for *R* gene breeding to be successful it is necessary to incorporate the knowledge of effectors and the *Avr* genes in order to achieve durability. Moreover, recent studies show that the best way of identifying and characterising new *R* genes is through the use of effectors (Vleeshouwers et al., 2011). Introduction of resistance through traditional breeding is a very long and tedious process, especially when multiple *R* genes are to be introduced, so called *R* gene stacking. Given the fast evolution of *P. infestans* and its adaptive abilities and quick population changes, this process is simply too long to be an efficient way of introducing resistance (Haverkort et al., 2016). Thus the possibility of introducing several *R* genes at once through genetic modification (GM) certainly seems appealing. The relatively new strategy to do so through cisgenesis, i.e. to only introduce genes from closely related species that are crossable with the cultivated potato, eliminates the introduction of selectable marker genes (e.g. antibiotic resistance genes) which are used in transgenic plants, and in principle generates plants that could have been produced by traditional breeding. What is more, only the selected gene(s), with the native introns and promoters, are being introduced into the well-established and commercially valued cultivars, without the risk of linkage drag, i.e. introduction of genes that might carry unfavourable qualities that significantly slows down traditional breeding (Schouten et al., 2006).

Since potato is one of the most important staple crops in the world and its consumption is based largely on regional and local products (Eriksson et al., 2016), new cultivars to be introduced should not only carry resistance genes, but also the characteristics the consumers and industry require. These cultivars also need to be adapted to the growing conditions in the region where they are to be introduced.

Another method of disease control is activation of plant defence responses by treatment with inducers. There are three types of inducers studied: microorganisms other than the causal agent (biocontrol), chemicals with low-risk active substances and attenuated pathogens (equivalent to vaccines used in animals) (Deverall, 1995). There are four main ways in which biocontrol agents can be used to limit or prevent plant diseases: the applied microorganism might compete with the pathogen for the colonisation of the rhizosphere, prime plant defence reactions, produce toxins or antibiotics that

kill the pathogen, or act directly as a mycoparasite, i.e. feed on and destroy the pathogenic organisms (Syed Ab Rahman et al., 2018). Organisms with all of these modes of action have been identified that could be used as a mean of late blight control. Unfortunately, none of the tested organisms or commercial formulations have so far proved efficient enough to fight late blight in the field conditions.

Studies focusing on chemical inducers of plant defences in potato have yielded some promising results, the most interesting examples including β-aminobutyric acid (BABA) (Olivieri et al., 2009, Liljeroth et al., 2010), sugar beet extract (Moushib et al., 2013) and potassium phosphite (Liljeroth et al., 2016) that not only induces defence mechanisms in potato, but can also have an anti-oomycete effect as well.

As of today, there are no reports on the use of attenuated fungal and oomycete pathogens as inducers of plant defences. It has, however, been shown that treatment with a single elicitor molecule derived from a pathogen can have such an effect. The best known examples of such elicitors include flg22 peptide derived from bacterial flagellin (Zipfel et al., 2004, Felix et al., 1999), and Pep13 (Schwessinger and Zipfel, 2008) - a thirteen amino acid-long peptide found in *Phytophthora spp.* including *P. infestans* (Brunner et al., 2002). Infiltration of such elicitors induces activation of defence systems in plants and confers resistance to pathogens, either specifically to the pathogen that was the source of the elicitor or broad spectrum resistance to a variety of pathogens. Expression of genes encoding elicitors *in planta* has yielded similar results in several pathosystems, including the oomycete elicitor - cryptogein expression in tobacco plants (Keller et al., 1999). However, before the work reported in Paper II, there were no other reports of the transgenic expression of *P. infestans* elicitors in potato as a defence elicitation strategy.

It is nonetheless unlikely that, with current agricultural practices where most crops are cultivated as monocultures, either resistance breeding or induction of plant defences will provide sustainable control of diseases such as potato late blight (Zhan et al., 2015). The use of a single cultivar and lack of genetic diversity in most crops allows for epidemics to occur – a pathogen only has to overcome defences in one plant genotype, so a single infection can spread in a very short time. *P. infestans* has very diverse population genetics and due to a short generation time is able to evolve very quickly. The short generation time of the pathogen as compared to the host plant is, however, counterbalanced by the trade-offs between pathogenicity and fitness of the pathogen and diversity of the plant population in natural environments (Zhan et al., 2014). It is the practice of monoculture, and especially introduction of a single cultivar carrying new resistance genes, that creates the genetic

homogeneity of the plants and thus causes strong directional selection on the pathogen and unsurprisingly makes the crop vulnerable to the disease (Zhan et al., 2015). Furthermore, the introduction of quantitative resistance that aims at a reduction of disease severity rather than complete resistance is problematic, as it can lead to increases in pathogen aggressiveness through intra-species competition (Zhan et al., 2015).

Despite ongoing efforts to decrease the use of chemical pesticides in agriculture, spraying with synthetic fungicides remains the only efficient way of preventing and managing late blight. Currently there are a wide variety of chemical control agents available and proper applications according to manufacturers' guidelines can significantly limit the scope of damage caused by the disease (Damalas and Eleftherohorinos, 2011). Nonetheless, to achieve a satisfactory level of crop protection, very large quantities of pesticides are required. In Sweden, specifically, potato occupies less than 1% of the cultivated land, but consumes as much as 21% of all fungicides used in Swedish agriculture (Eriksson et al., 2016). Eriksson *at al.* (2016) propose that climate change will increase the severity of late blight in the Nordic countries resulting in further increases in pesticide use. Statistical analyses of late blight epidemics in Finland and late blight modelling in Scotland, two countries with very similar climates to Sweden, showed that indeed the fungicide use is predicted to become higher due to climate change, but the reason for that is going to be earlier incidence of late blight disease and hence higher spread of inoculum (estimated as crop-connectivity) rather than severity of the disease (Hannukkala et al., 2007, Skelsey et al., 2016). Climate change is in fact predicted to only slightly influence the growth of *P. infestans* (Yang et al., 2016) and the risk for higher severity of late blight might be compensated for by a potential temperature-induced shift in potato growing season (Sparks et al., 2014).

Use of high doses of pesticides poses a huge burden on the environment, residues from such chemicals contaminate soil and water, and besides being toxic to humans and other animals introduce substantial changes to (agro)ecosystems. The majority of current pesticides used in Europe, are not specific to one species or even group of organisms and in addition to acting on the intended pathogen targets, they can also influence populations of beneficial microbes in the vicinity of the sprayed crops (Aktar et al., 2009, Pacilly et al., 2016). In addition to the harmful effect on the environment, such frequent sprayings are very expensive and thus alternative solutions are necessary. Furthermore, since mandatory implementation of the EU directive 2009/128/EU came into force in 2014 requiring member states to reduce their overall use of synthetic pesticides and increased the use of integrated pest

management (IPM) strategies, many previous plant protection products have been withdrawn from use in the EU and specifically also in Sweden.

The current state of resistance breeding and studies on plant resistance inducers (PRIs) do not allow regulators to treat them as alternatives to synthetic chemical control methods, but rather as a mean of decreasing the amount of pesticides used. The reduction of fungicide dosage can also be achieved through adjustment of the spraying schedule according to disease forecasting tools such as Decision Support Systems (DSS) which are tools used to predict particular risks of disease development and to time pesticide applications to only those times when a significant risk of infection is posed (Gu et al., 2016, Henderson et al., 2007, Johnson et al., 1998). However, reduction of fungicide doses can lead to selection for isolates with higher fitness and aggressiveness in a similar way to quantitative resistance (Zhan et al., 2015). This is especially true since the majority of synthetic chemical controls used nowadays do not eradicate the pathogen but rather slow down disease progression (Amaradasa and Everhart, 2016). Additionally, it has been proposed that application of lower doses of pesticides might also increase the risk of pesticide resistance, however, there is very little data supporting that hypothesis (van den Bosch et al., 2011). In fact, in clinical trials on humans, it was shown that the opposite scenario might be likely and that excessively high doses of antimicrobial drugs could potentially lead to development of resistance (Day and Read, 2016). Other studies of agrochemicals show that the dose is not the only determinant of the resistance build-up. The number of applications and combinations with other chemical agents with different modes of action can also play a role in the development of fungicide resistance in pathogen populations (van den Berg et al., 2016, Dooley et al., 2016).

The best example of pesticide resistance in *P. infestans* is resistance to metalaxyl, which has been reported in many countries around the world (Matson et al., 2015, Saville et al., 2014, Davidse et al., 1981, Gisi et al., 2011, Mazáková et al., 2018). Although the risk of resistance development is calculated to be very low for the modern late blight control agents, such as iprovalicarb (Chen et al., 2018) and azoxystrobin (Qin et al., 2016), extensive and prolonged use of one substance can result in resistance development even to such low risk agents, as was the case with fluazinam (Schepers et al., 2018).

Moreover, it has been shown that application of copious amounts of fungicides, as is practiced in modern agriculture, can lead to an increased rate of sexual reproduction of *P. infestans*. The production of oospores has been observed in single mating type isolates without the presence of the second mating type and between normally incompatible mating type isolates after treatment with commercially available pesticides (Groves and Ristaino, 2000).

All together, these data clearly show that synthetic chemical control agents need to be more effective and species specific in order to decrease their use without imposing further risks for development of more aggressive or resistant isolates. And thus, more detailed studies on the biology and genetics of oomycetes, such as *P. infestans,* are necessary. These should also be combined with detailed knowledge of the biological processes occurring in host plants during plant-microbe interactions.

1.6 Hit it where it hurts

Cell walls are necessary for the survival of oomycetes. They provide a barrier, protecting the cell from the surrounding environment, and contribute to cell shape. They also facilitate maintenance of osmotic balance and function as a biointerface between a pathogen and its host (Grenville-Briggs et al., 2010). The difference in the cell wall composition was one of the reasons the oomycetes were suspected not to be true Fungi. The oomycete cell wall is composed mainly of (1→3) and (1→6) β-D-glucans with a relatively small portion of cellulose and reportedly lacks chitin, the main component of fungal cell wall (Bartnicki-Garcia, 1968, Aronson et al., 1967). However, it was recently proposed that *P. infestans* most likely contains more cellulose than previously predicted and that this is higher than several other oomycetes (Mélida et al., 2013). Additionally, even though the presence of chitin has never been demonstrated in any bioassays, a putative chitin synthase gene has been identified in *P. infestans*. The expression of this gene has been demonstrated at the mRNA level (Ospina-Giraldo et al., 2010, Hinkel and Ospina-Giraldo, 2017). The fact that oomycete cell walls differ significantly from plant and other organisms' cell walls makes them an excellent target for disease control, and especially for new improved chemical agents.

There are four cellulose synthase genes identified in *P. infestans* and their expression has been shown to be upregulated in cysts and appressoria, the structures important for infection, and at early infection time points (Grenville-Briggs et al., 2008). Both silencing of this family of proteins by RNAi and chemical inhibition with 2,6-dichlorobenzonitrile (DCB) resulted in severe deformations of the appressoria and germ tubes, lowered appressoria production and caused loss of pathogenicity, clearly indicating the importance of cellulose in correct cell wall formation and pathogenicity in *P. infestans*. The same inhibitory effects were later obtained with mandipropamid (MPD), commercially available as Revus (Syngenta) that also targets cellulose synthesis, specifically the PiCesA3 gene (Blum et al., 2010). The use of Revus in the control regime of late blight proved to be efficient and good way of the

disease control and so far there are no reports on the pathogen resistance. This is a good example of the application of molecular biology and new research findings to the development of chemical control agents, since after Grenville-Briggs *et al.* (2008) determined the function of the cellulose synthase genes in *P. infestans,* Syngenta were able to determine the mode of action of their newly developed drug - Mandipropamid (Blum et al 2010).

The most studied inhibitor of β-D-glucan synthases is caspofungin, a member of the class of drugs called echinocandins and the first one to be approved by FDA (Douglas et al., 1997, Johnson and Perfect, 2003). The primary use of the drug is treatment of fungal and oomycete infections in humans and other animals (Deresinski and Stevens, 2003, Zhang et al., 2014, Pereira et al., 2007). The efficacy of the drug in treatment of *Pythium* infections indicates that it might have a similar effect on *P. infestans*, a hypothesis that we have tested in our study on chemical inhibitors of cell wall synthesis (Paper IV).

Finally, even though chitin has not been found in *P. infestans*, the presence of the putative chitin synthase gene and the high importance of chitin in other *Phytophthora* species (Cheng et al., 2019) led us and another research group (Klinter et al., 2019) to test the effect of the chitin synthase inhibitor nikkomycin Z on *P. infestans*. Similar to caspofungin, nikkomycin Z is used as a human antifungal therapy drug and so far has no reported uses in agriculture (Li and Rinaldi, 1999). Klinter *et al.* (2019) have demonstrated that treatment with nikkomycin Z causes rupture of the hyphal tips of *P. infestans*. In our study we have also tested the effect of the drug on the cyst germination and formation of appressoria, and on the protein composition of the cell wall (Paper IV).

2 Thesis Aims

The aim of this project was to learn more about the biology and ecology of *Phytophthora infestans*, with the main focus on the cell wall and its function in the development of infectious structures and pathogenicity. The rationale behind this approach was that by learning more about the pathogen itself we can discover new targets and strategies for the control of late blight. While potentially severe future climate changes direct agriculture towards abolishment of chemical control of plant diseases, the current state of our knowledge and biotechnology does not allow for such drastic steps yet. A deeper understanding of *P. infestans* cell wall composition and formation processes can potentially be used in the development of new and improved chemical control agents. Such new generation pesticides would reduce the harmful effect on the environment exerted by agriculture while maintaining the level of crop protection from pathogens that we are accustomed to today.

Furthermore, components of the cell wall are often recognised by the plant immune system, (e.g. acting as PAMPs) and thus can potentially be used as inducers of defence mechanisms.

The specific aims of the research articles included in this thesis were:

1. To analyse the *P. infestans* proteome at different asexual life cycle stages to reveal changes occurring during the development of infectious structures (Paper I); and to subsequently specifically study the interplay between the cell wall proteome and the synthesis of cell wall carbohydrates, by assessing the effects of chemical inhibitors on cell wall development and protein expression (Paper IV).

2. To identify cell wall proteins necessary for cyst germination and formation of appressoria and hence for pathogenicity of *P. infestans* (Papers I and II).

3. To induce potato defence responses through transgenic expression of the *P. infestans* cell wall elicitor PAMP peptide Pep13 and evaluate the disease resistance in the subsequent transgenic plants (Paper III).

4. To evaluate the potential of wild Solanum species as alternate hosts and contributors to both off-season survival and variations in pathogenicity or aggressiveness of *P. infestans* in the agroecosystem (Paper V).

3 Methods

3.1 RNAi-based transient silencing as a screening method

Stable transformation of *P. infestans* is troublesome. The promoters used in fungal transformations were shown to be non-functional in *Phytophthora* species, suggesting unique transcription machinery and complicating establishment of transformation protocols (Judelson et al., 1991, Judelson and Michelmore, 1991). New and unique transformation vectors had to be constructed. The development of transformation protocols was further complicated by the lack of homologous recombination, limiting transformation rates and making it very difficult to delete or knockout specific genes. Furthermore, gene deletions are impossible due to *P. infestans* diploidy (Birch and Whisson, 2001).

The discovery of RNA interference (RNAi) and the subsequent use of this mechanism in transient gene silencing allowed for determination of gene function based on the knock-down phenotype (Whisson et al., 2005). RNAi is a natural process occurring in most eukaryotic organisms that protects cells from transposons and viruses and regulates gene expression (Mascia et al., 2019). Shortly, the presence of double stranded RNA (dsRNA) activates a protein called Dicer that cuts the molecule into short 21-25nt fragments, called small interfering RNA (siRNA). SiRNAs are then bound by Argonaute proteins, forming the RNA-induced silencing complex (RISC). The two strands of siRNA are separated and the passenger (sense) strand gets degraded directly, while the guide (antisense) strand is used by the complex as a template to recognise complementary mRNA sequences and to subsequently degrade them. Thus, introduction to protoplasts of synthetic dsRNA complementary to the gene of interest allows for its silencing at the post-transcriptional stage

(Whisson et al., 2005). Since the dsRNA is introduced once and its amount depletes with time, the effects of the silencing are transient and last around 14 days. The main use of the RNAi-based silencing is, therefore, to screen for changes in the phenotype and thus to preliminarily determine the function of the gene of interest.

This approach has been used in this thesis as a screening method, allowing us to identify the components of the cell wall that are necessary for the development of infectious structures and hence for the pathogenicity of *P. infestans.* The advantages of RNAi-based gene silencing are the relative simplicity of the protocol, its speed and the fact that it has been developed to allow multiple genes or whole gene families to be silenced at once (Grenville-Briggs et al 2008), which is not easily achievable with stable transformation. Although in same cases this can be seen as a disadvantage, the varying level of silencing observed between different lines can also be advantageous, as it allows for the assessment of intermediate phenotypes and to assess the functions of essential genes, where complete knock-down would be lethal (see Paper II).

3.2 Expression of pathogen elicitor peptide in planta

A promising alternative to the introduction of major *R* genes into cultivated plants, in an attempt to confer full resistance to the pathogen, is induction of plant defence mechanisms. This approach is very similar to vaccinations used in animals as its purpose is to "pre-activate" the immune system and thus prepare the plant for incoming pathogens. Such activation can be achieved by *in planta* expression of elicitor molecules from the pathogen. Elicitors are normally recognised by the plant defence mechanisms during infection and thus their constitutive expression *in planta* may result in constitutive activation of the innate immune responses (see above). The main advantage of this approach is its potentially longer durability than the resistance conferred by the newly introduced *R* genes. The biggest disadvantage is that the exact effect of such induction cannot be predicted with high certainty and it might lead to only partial reduction of disease symptoms rather than full resistance. Further there may be fitness costs to the expression of non-endogenous genes in host plants (see Paper III).

3.3 Analysis of the proteome

Proteomic analysis yields data on the identity and quantity of all the proteins found in a particular sample. We decided to use it to compare differences in protein composition in different life cycle stages of *P. infestans* and then again to look specifically at the cell wall. Initial investigations of plant pathogens and their interactions with their hosts were focused on single genes or proteins and the first large scale analyses were sequencing of the genomes of model organisms (González-Fernández et al., 2010). The subsequent development of transcriptomics, proteomics and metabolomics had a significant impact on the scope of our knowledge of plant-pathogen interactions. Combining the different "omics" can give a comprehensive view of the studied organism or system (Tan et al., 2009). We have chosen proteomics for our studies, since our main interest lies in the role of proteins in the cell wall formation and the changes in protein composition during transition from dormant to infectious life cycle stages.

4 Results and Discussion

The proteomic analysis of asexual life cycle stages of *P. infestans* (Paper I) showed that there were 59 proteins that were specific to germinating cysts and appressoria, thus the two stages that are important for the establishment of infection. Out of these six were proteins involved in cell wall formation and maintenance, and as the cell wall was the main focus of this thesis, we concentrated on these proteins specifically. The expression of the genes encoding several of the identified proteins confirmed that they are highly expressed at 6hpi of potato leaves, the time point when germinated cysts start forming appressoria and penetrate the leaf surface, validating the hypothesis that these proteins play a role in appressorium development and the initiation of infection. To further investigate the importance of the selected proteins in the pathogenicity of *P. infestans*, we have used our RNAi protocol to transiently silence a family of three previously uncharacterised proteins. These three proteins were also identified in a previous study where the cell wall proteome of *P. infestans* was analysed specifically (Grenville-Briggs et al., 2010), further confirming their importance in cell wall formation. This family of three genes did not have significant similarity to known proteins in the NCBI non-redundant (nr) database and the protein sequences were lacking similarity to previously characterised protein domains. The phenotype we observed in the silenced lines was also consistent with the hypothesis, a significant portion of the appressoria displayed abnormalities and many of the germ tubes were twisted and unnaturally long and did not form appressoria at all (see Figure 3 for example picture). The lines where the altered phenotype was observed were also either completely non-pathogenic or were severely attenuated in pathogenicity, as tested in detached leaf infection assays. We have thus identified a family of three proteins necessary for the formation of healthy appressoria and the subsequent infection of host plants.

Both the Grenville-Briggs study (2010) and our proteomic analysis (Resjö et al., 2017) identified a transglutaminase as highly abundant in the cell wall.

The two transglutaminases were encoded by different genes, but *in silico* analysis revealed that they share very high sequence similarity and were grouped together with six other genes encoding transglutaminases in *P. infestans* (Paper II). Silencing of all members of this group of transglutaminases at once using the RNAi protocol resulted in a lethal phenotype. We were only able to find debris and a few bursting cysts on the plates. However, we were able to obtain an intermediate phenotype by introducing a few mismatches into the primers used for synthesis of dsRNA. The imperfect match of dsRNA to the sequences of the target mRNA resulted in a large range of phenotypes observed, most likely reflecting the different degree of silencing of individual genes. The most severe phenotype observed was still lethal, but more bursting cysts were observed and in some cases even a few bursting appressoria were found (Figure 3B: I and IX). Several of the transformed lines displayed the wild type phenotype, which could be the effect of both lack of introduction of the dsRNA into the protoplasts that gave rise to these lines or very low level of silencing with no effect on the morphology of the germ tubes and appressoria, e.g. only one of the genes was silenced. In the intermediate phenotypes the cysts germinated, but the germ tubes were swollen or twisted (Figure 3B: II, IV, VI and VIII). The appressoria count was lower in these lines and we observed higher count of appressoria-like structures (Figure 3B: III, V and VII) Moreover, the lines with both the extreme and the intermediate phenotypes showed reduced or no pathogenicity in detached leaf infection assays. We conclude that the immediate determinant of pathogenicity is the number of healthy appressoria produced rather than the rate of cyst germination. These findings are even more interesting given the fact that previous attempts at silencing transglutaminase genes failed (Fabritius and Judelson, 2003).

Furthermore, we have tested the effect of a chemical inhibitor of transglutaminases - cystamine on *P. infestans* growth and development. The concentration of cystamine that resulted in growth inhibition was about ten-fold lower than the ones reported for fungi, indicating that transglutaminases may play a more important role in the development of cell wall of oomycetes than they do in fungi (Iranzo et al., 2002, Ruiz-Herrera et al., 1995). The germination rate of sporangia and cysts and their ability to form appressoria were influenced by the chemical. The phenotype of the cultures treated with cystamine was the same as the phenotypes of the silenced lines, with swollen and deformed germ tubes, lower number of appressoria and bursting at higher concentrations. These changes to the morphology of the cysts, germ tubes and appressoria are, however, not specific to cystamine treatments and similar observations were also made when other components of the cell wall were

targeted with chemicals that affect their synthesis. In another study we have focused on the main carbohydrate components of *P. infestans* cell wall – β-glucans, cellulose and potentially chitin or the product of the putative chitin synthase gene, and showed that chemical inhibition of their synthesis also leads to similar disruptions in cell wall (Paper IV). The full range of morphological changes observed after various chemical treatments are shown in Figure 4, together with pictures of the different life cycle stages in the untreated (control) cultures.

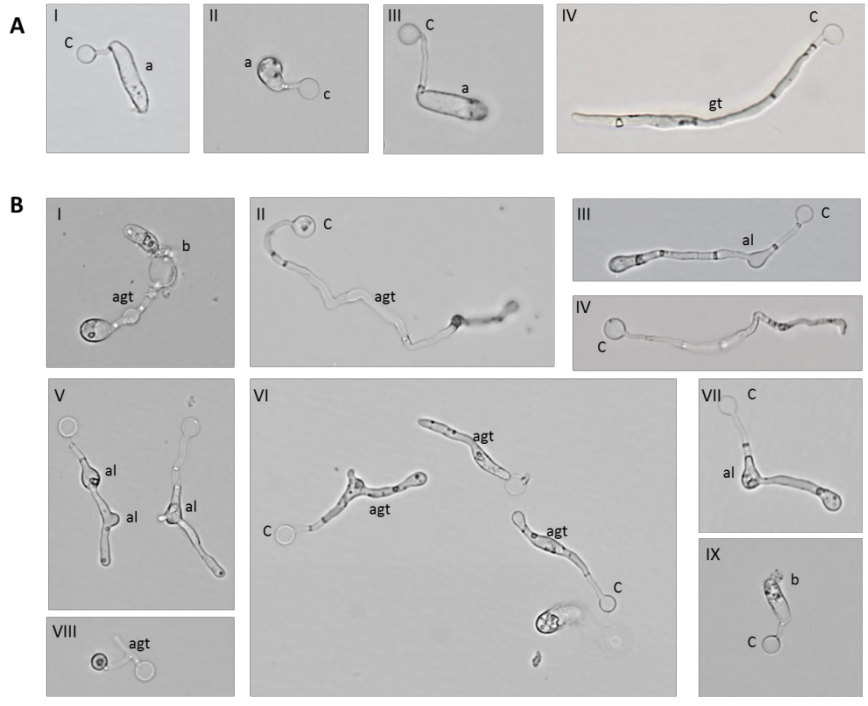


Figure 3. **Images of structures observed in *P. infestans* after RNAi-basesd silencing of a family of cell wall transglutaminases**. **A**. GFP non-endogenous positive control; **B**. silenced lines. c – cyst, a – appressorium, gt – germ tube, b- burst, agt – abnormal germ tube, al-apressoria-like structure.

Since the chemicals used in this study were all commercially available products, it indicates that our new possible targets for late blight control – the appressorial proteins (PiACWPs) and transglutaminases could also be used in the development of new control agents. Moreover, since the dose of cystamine required to inhibit growth and development of *P. infestans* is lower than for fungi, smaller doses could be used. The biggest limitation in the use of transglutaminases as target for chemical control is their high conservation

among both eukaryotic and prokaryotic organisms. Although transglutaminases from animals, plants and microbes share very little sequence similarity, their modes of action seem to be similar and depend on cysteine residue at the active site (Kashiwagi et al., 2002, Pedersen et al., 1994). The exact mode of action of cystamine is not known yet, but it has been proposed that it introduces structural changes to the transglutaminases thus rendering them inactive (Palanski and Khosla, 2018). The primary use of cystamine is treatment of human diseases, including nephrophatic cystinosis, suggesting that it might be effective against transglutaminases from a wide range of organisms, including plants. This poses a significant limitation to the use of cystamine as a control agent, as spraying with the chemical would potentially damage the plants as well. However, a possible solution to that would be the use of cystamine based pesticide for pre-treatment of the field before potato plants are sown. Since *P. infestans* spores are often present in the soil, mostly as oospores as described above, the application of the chemical would possibly limit or even eradicate the inoculum before the plants are introduced. Expression of the cell wall transglutaminase genes studied in this thesis was also demonstrated in *P. infestans* oospores (Ramesh Vetukuri, personal communication).

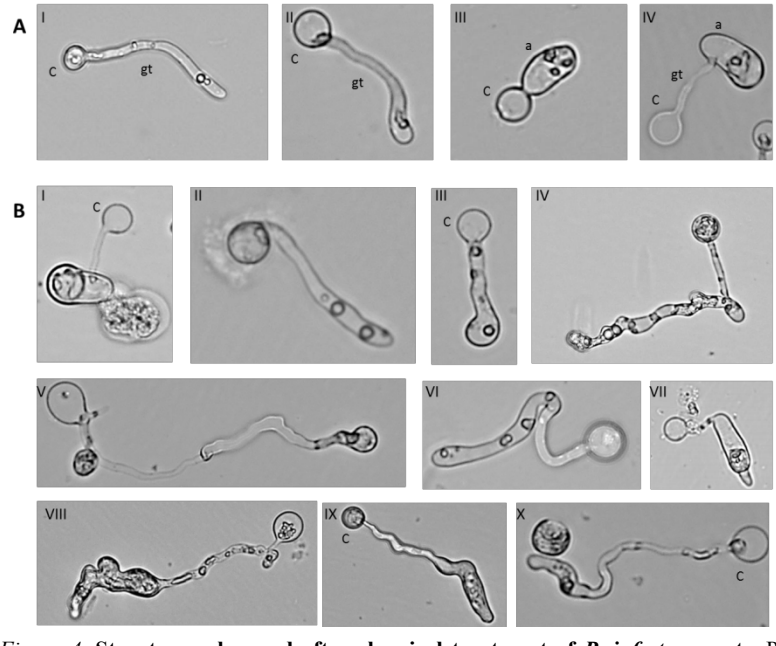


Figure 4. **Structures observed after chemical treatment of *P. infestans* cysts**. Pictures from different chemical treatments are included here as example of observed deformations. **A**. wt; **B**. treated samples. c – cyst, a – appressorium, gt – germ tube, b- burst, agt – abnormal germ tube, al-apressoria-like structure.

Such a method of late blight control is particularly interesting in the light of our discovery of a new putative overwintering strategy of the pathogen (Paper V). We have found *P. infestans* isolates in the rhizosphere of the wild relative of the cultivated potato – *Solanum dulcamara*. The isolates were found in samples collected in Sweden in three seasons: summer, spring and winter. The lack of negative effects on the growth rate of the roots of *S. dulcamara* and no symptoms of disease observed in these plants indicate that the plants serve just as overwintering host or associated refuge plant for the pathogen. Importantly, isolates collected from the *S. dulcamara* rhizosphere displayed a higher aggressiveness on potato plants than a laboratory strain passaged through potato (Paper V). This finding is in line with another recent study that showed that *P. infestans* isolates collected from *S. physalifolium,* were more aggressive on cultivated potato (*S. tuberosum*) compared to *P. infestans* isolates that were isolated directly from potato (Grönberg et al., 2012). Furthermore, the strains that we identified in the *S. dulcamara* rhizosphere were of the A2 mating type and all formed oospores when cultured with A1 mating type isolates *in vitro*. Presence of these isolates in the field increases the genetic population of *P. infestans* and provides a possibility for sexual reproduction that can further influence the gene pool and result in production of oospores able of withstanding harsh winter weather conditions.

This study highlights the importance of the incorporation of ecological studies into late blight research and management strategies. The possibility of *P. infestans* overwintering in the field could, for example, be reduced by removal of the alternate hosts; while knowledge on its distribution should be taken into consideration in the process of creating new pesticide application regimes.

The significance of field studies was also shown in Paper III – a study in which we expressed *P. infestans* elicitor molecule Pep13 in potato plants. Pep13 is a thirteen amino acid-long peptide found in many of the *P. infestans* transglutaminases (also in the ones mentioned above and described in detail in Paper II). It was previously shown to act as Pathogen Associated Molecular Pattern (PAMP) and to induce potato defence responses when infiltrated into plants (Brunner et al., 2002). We have expressed the peptide in potato, in an attempt to increase potato resistance to late blight. The transgenic plants did, as expected, display reduced disease severity symptoms when inoculated with *P. infestans* in controlled laboratory conditions. However, when the Pep13-expressing plants were introduced to the field the initial reduction of disease symptoms was quickly overcome by the pathogen, meaning that at the end of the season under high infection pressure the Pep13-expressing plants were infected to the same or greater degree as the wild-type control plants.

Moreover, early senescence was observed as a fitness cost associated with the expression of the transgene, which was not apparent in the controlled environment. We have investigated the phenotype further by microarray analysis of the field samples and were able to link the upregulation of various stress and metabolism related genes to the senescence and partial resistance phenotypes. The important conclusion from this study was the necessity of field analysis of new transgenic lines before their disease resistance potential could be fully assessed. It also shows the overall importance of applied biology in plant pathology research and in research in general. It is often believed that so called "basic biology" plays a more important role in elucidating the mechanisms and modes of action of various organisms and their interactions, while applied biology focuses on solutions. In fact, both types of research have exactly the same goals – to learn more, understand better and be able to use that knowledge in practice. Therefore, I strongly believe that the best approach is to compile as much knowledge as possible, both basic and translational independent of whether it comes from a big project or just a small study, and try to use it all in the fight with late blight, the fight we are still losing.

5 Future perspectives

Independent of the type of research done and the amount of knowledge obtained as a result, the goal for the future is always the same - to learn more. And while it is of course true, any research project is just a piece of an infinitely large puzzle, it is the time we start putting our pieces of the puzzle together. To truly apply our current knowledge of late blight and the interactions of *P. infestans* with potato, we need to start thinking in broader terms and incorporate the findings of molecular biology, genetics, chemistry, ecology and evolutionary biology into new common solutions. A view that is very close to my options was presented by Zhan *et al.* (2015). They argue that the only truly sustainable method of late blight control is the adjustment of current techniques and practices in order to minimise pathogen evolution. The revolutionary new solutions to the late blight problem, no matter how much better than the previously used ones, will still have time limitation unless they incorporate long-term predictions of pathogen evolutionary patterns. Furthermore, Zhan and colleagues (2015) suggest that sometimes the less efficient, but longer lasting solutions might be the better choice, and to be able to make such decisions evolutionary biologists need to play a larger role in the design of new management strategies.

Applying this way of thinking is particularly relevant to our studies on new targets for chemical control of late blight. Whereas to achieve our goal of identifying the potential targets we studied the biology of the cell wall in detail and focused on a narrow view, to be able to use that knowledge in practice, we need to consider the big picture. The best means and time of application of the potential new chemical control agent should be considered in order to assure the highest efficiency of late blight control and the lowest impact on the environment. Besides considering direct toxicity to non-target organisms, the small changes, even non-lethal ones, should be considered. Changes to the populations of non-target microorganisms might have an influence on plant growth, on the growth, spread and evolution of the pathogen of interest or other

pathogens, and might possibly have long-term effects on the ecosystem (Staley et al., 2015, Wainwright and Pugh, 1975, Yang et al., 2011). To increase the specificity of a fungicide it is also important to know the diversity, exact location and form in which the pathogen is present. The discovery of a new putative overwintering strategy of *P. infestans* and identification of the collected isolates (Paper V) provides us with more information on the diversity of the pathogen in the fields in Sweden, but also provides new opportunities. Application of a chemical control agent as a pre-treatment of the fields, possibly in winter or early spring, long before the potato growing season, might be an effective mean of decreasing the amount of inoculum present and a good way to decrease the uptake of the chemical by the plants, as the fungicide could decompose before the potato is planted (Thom et al., 1997). The fact that the collected isolates were of the A2 mating type indicates also the possibility of sexual reproduction and formation of highly persistent oospores, and thus suggests that oospores might play an important role as the disease inoculum in the soil in that region. This hypothesis is consistent with the observations made by Hannukkala *et al*. (2007) in Finland, where they showed oospores to be the primary inoculum in the soil and the reason for earlier onset of late blight epidemics. Thus, it is crucial to ensure that newly developed fungicides to be used in the Nordic countries are able to affect oospores, which are usually much harder to eradicate than asexual spores.

An awareness and acknowledgment of the changing view of plant immunity is extremely important for molecular studies of plant-pathogen interactions and the use of that knowledge in plant breeding. Traditionally plant-pathogen interactions were depicted as the zig-zag model (see section *Enemy at the gates* above) proposed by Jones and Dangl (2006) and widely accepted by the community. Nowadays, there is a new trend to think of the PTI and ETI immune responses as a continuous reaction rather than a dichotomy. In their comprehensive review Thomma *et al.* (2011) list multiple examples of PAMPs and effectors that do not follow the classical distinction. They give examples of effectors that are highly conserved among different groups of organisms and thus could be classified as PAMPs if they did not also have the potential to suppress plant immune responses (PTI), and PAMPs that are only conserved in one species. An example of such an elicitor is the Pep13 peptide from *Phytophthora* genus studied in this thesis. Furthermore, Pep13 was suggested to be necessary for pathogenicity – typically a characteristic of an effector and not a PAMP, since it contains the active site for transglutaminase activity of the protein it is a part of.

The authors propose that the models built on a few examples might give oversimplified view of the plant-pathogen interactions and that both PTI and

ETI might be either strong or weak and the prevalent response is determined by a particular interaction (Thomma et al., 2011).

Jonathan Jones, the author of the original zig-zag model, went a step further, proposing that the resistance is primarily, if not exclusively, based on PTI – the initial recognition of the pathogen, and the role of ETI is to attenuate the level of response (personal communication).

Exceptions to the gene-for-gene hypothesis are well known, as in addition to the direct recognition of the effector by a specific R protein, the interaction can also occur through a variety of different models, known as "guard, "decoy", and "integrated decoy". Kamoun and co-workers (Wu et al., 2018, Adachi et al., 2019) suggest that the interaction is even more complex - the receptors form large and heavily interconnected networks. They present an evolutionary scenario in which receptor networks evolved from a single molecule, referred to as singleton, that carried both the sensor (pathogen recognition) and helper (initiation of immune response) function, into pairs of receptors where the two functions were carried by separate molecules. The pair model may have further evolved through small networks where several sensors were associated with one helper into the current complicated networks with multiple sensors being necessary for the function of multiple helpers. They propose that singleton, pair and network receptors all coexist in nature.

These new data and ideas all show that plant-pathogen interactions are much more complicated than initially anticipated and cannot be thought of in terms of and precise and confined models, but rather each particular system needs to be treated as an individual case.

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Popular science summary

The global sustainable development goals set by the United Nations in 2015[2] list No Poverty and Zero Hunger as the two most important goals for the humanity in the upcoming years. With the growing world population the demand for food is also growing, and since crops are our main source of nutrition the focus of this goal is improvement of agriculture. By increasing the efficiency of crop production we could produce more food without the need for more arable lands. Making agriculture more sustainable is going to directly affect the climate action (goal 13) as well. One of the means of improving agricultural efficiency is to decrease crop losses. And the major reason for high yield loss in crop production are plant pathogens and diseases they cause. In line with the rationale of the UN goals, we believe food security to be one of the most important challenges nowadays and thus the research goal in this project was improvement of the management strategies for the control of late bight, a devastating disease of potato. Potato is one of the most important staple crops in the world and the one with the highest yield potential in Sweden. Late blight caused by the oomycete pathogen *Phytophthora infestans* is a disease that affects both the aboveground green parts of the plant and the tubers and can cause 100% yield losses if no control measures are taken. Over the 150 years, since the pathogen was discovered and identified, extensive research efforts have been directed towards better understanding of the pathogen and development of control measures. Unfortunately, breeding for resistance in potato is still not effective enough to be used as the sole method of prevention of late blight and potato growers depend heavily on the use of chemical pesticides. The frequent spraying regime required to provide appropriate level of protection from late blight is a huge burden on the environment and economy. Since abolishment of chemical control does not seem likely in the upcoming future, a promising alternative is improvement of the pesticides. So called, next-generation pesticides are much more target specific, i.e. they act on a specific organism or group of organisms only, and thus have less of the negative effect on the environment. In order to develop

[2] http://www.undp.org/content/undp/en/home/sustainable-development-goals.html

such pesticides, better understanding of the pathogen biology, genetics, ecology, evolution and mode of action is required. We have focused our studies largely on the cell wall of *P. infestans* searching for molecules necessary for its growth, development and pathogenicity on potato. We were able to identify two families of cell wall proteins indispensable for the survival and growth of the pathogen and analysed in detail the effect of several commercially available chemicals. Our conclusions are that there are multiple molecules in the cell wall of *P. infestans* that are necessary for the pathogen and that could, therefore, be analysed further as potential new targets for the next-generation pesticides.

The study on the population of oomycetes in the soil surrounding roots of wild potato relatives revealed that *P. infestans* is able to survive on the roots of these plants even in winter, suggesting that pre-treatment of fields with pesticides might be a good disease preventive measure.

Finally, we have also attempted to improve potato resistance to late blight by introducing a *P. infestans* gene into the plant. The introduced gene encodes an elicitor peptide – a molecule that triggers plant immune responses. Therefore, by introduction of that molecule to the plant we have activated its immune system in manner similar to the mode of action of vaccines in animals. The transgenic plants were indeed more resistant to late blight in controlled laboratory conditions, however the resistance was lost in the field where they were subjected to a multitude of stresses, such as adverse weather conditions and their rapid changes, and a variety of different pathogens and pests. The lesson from this project was that field studies are immensely important in evaluation of new resistant plants, which highlights the significance of applied biology.

Populärvetenskaplig sammanfattning

I FNs globala mål för hållbar utveckling[3] anges avskaffandet av fattigdom och hunger som de två viktigaste målen för mänskligheten under de kommande åren. Med en växande världsbefolkning ökar även efterfrågan på mat och då grödor utgör den huvudsakliga näringskällan syftar dessa mål till att förbättra jordbruket. Genom att öka jordbrukets effektivitet skulle vi kunna producera mer mat utan behov av mer åkermark. Ett mer hållbart jordbruk skulle även ge positiva effekter på klimatet (mål 13). En av metoderna för att förbättra jordbrukets effektivitet är att minska skördeförlusterna där en av de främsta orsakerna är växtpatogener och de sjukdomar de orsakar. I linje med FNs mål anser vi att livsmedelssäkerhet utgör en av de viktigaste utmaningarna idag och forskningsmålet i detta projekt var att förbättra förvaltningsstrategierna kring bekämpningen av potatisbladmögel, en förödande sjukdom som angriper potatis. Potatis utgör en av världens viktigaste stapelgrödor och är den gröda med högst avkastningspotential i Sverige. Potatisbladmögel orsakas av oomyceten *Phytophthora infestans* och angriper både den ovanjordiska delen av växten såväl som de underjordiska knölarna och kan orsaka total förlust av skörden om inte motåtgärder sätts in. Allt sedan patogenen identifierades för 150 år sedan har den varit målet för stora forskningsinsatser med för att förstå patogenen och utveckla nya bekämpningstekniker. Tyvärr är resistensförädlingen av potatis inte tillräckligt utvecklad för att effektivt kunna hindra potatisbladmögel och potatisodlare är starkt beroende av kemiska bekämpningsmedel för att minska skördeförluster. Behovet av regelbunden besprutning för att åstadkomma en acceptabel skyddsnivå är inte enbart kostsamt för miljön utan även en stor ekonomisk börda för potatisodlarna. Eftersom att det troligen kommer dröja länge innan besprutning kan avskaffas är det istället attraktivt att försöka förbättra de pesticider som används. Målet är att nästa generation av pesticider skall vara mer specifika, alltså enbart påverka en eller ett fåtal organismer, och därmed ha mindre negativ påverkan på miljön. För att kunna utveckla framtidens bekämpningsmedel krävs en bättre

3. http://www.undp.org/content/undp/en/home/sustainable-development-goals.html

förståelse för patogenens biologi, genetik, ekologi, evolution samt de mekanismer den använder för att angripa växten. Vår forskning har primärt varit fokuserad på *P. infestans* cellvägg i jakten på molekyler som krävs för dess tillväxt och för att kunna angripa potatisplantan. Vi har lyckats identifiera två familjer av cellväggsprotein som är nödvändiga för organismens överlevnad och tillväxt och analyserat hur dessa protein påverkas av kommersiellt tillgängliga kemikalier. Vår slutsats är att det finns flertalet molekyler i cellväggarna hos *P. infestans* som är livsviktig för dess överlevnad och därför utgör potentiellt lämpliga mål för nästa generation av pesticider.

Vår studie av de oomycete-populationer som återfinns i jorden kring vila potatissläktingar visade på att *P. infestans* kan övervintra omkring dessa växters rotsystem vilket tyder på att besprutning av fält innan plantering kan vara en effektiv förebyggande bekämpningsmetod.

Slutligen försökte vi även öka potatisens motståndskraft mot potatisbladmögel genom att introducera en gen från *P. infestans* in i växten. Denna gen kodar för en kort aminosyrasekvens som aktiverar växtens immunförsvar på ett liknande sett som ett vaccin fungerar hos djur. Dessa modifierade växter visade sig vara mer resistenta mot potatisbladmögel i laboratoriemiljö men denna resistens försvann under fältförsök när växten blev utsatt för många olika faktorer såsom förändringar i väderlek, växtpatogener och andra skadegörare. Slutsatsen från dessa försök var att fältförsök är oerhört viktiga för att utvärdera resistansen hos nya växtlinjer vilket lyfter fram betydelsen för applicerad biologisk forskning.

Acknowledgements

My PhD education has been a wonderful, exciting, and at times difficult process that allowed me to grow as a researcher and as a person. A large part of that growth I owe to the fantastic people that I worked and interacted with. You have all been an important part of this journey and I would like to thank you all. There a few people that deserve a special thank you:

Laura, thank you for giving me this opportunity, for being my supervisor, mentor and a great friend. Thank you for always being there for me, supporting and encouraging me every step of the way. For making me believe in myself and being my role model.

Ramesh, from the first moment we have met you have always believed in me and treated me as your equal. You have been a kind and patient supervisor and I am grateful for all your help.

Mia, you have been the person to turn to with all scientific (or not) questions. Thank you for always finding the time to help me. I was truly lucky to share the office with you!

Per, **Sophie** and **Fredrik**, you are my support network. Thank you for always being there to share my small victories, to laugh at my mistakes and for listening when I really needed it. It is a great feeling to know that you have friends you can always count on.

Christian, thank you for you contagious positivity and all the hard work to help me finish my thesis on time!

I would like to thank all of the **Integrated Plant Protection Unit** for creating an environment of open scientific discussion and **Erik Andreasson** and the whole **Resistance Biology Unit** for all our collaborations. I am especially grateful to **Svante**, for your tireless and detailed work and help analysing all of our proteomics data.

I could not achieve this without the love and support of the most important people in my life – my family. Kacper, thank you for not letting me give up when I didn't believe I can do it. For always being there to catch me and give me the strength to go on. Ida, thank you for being the perfect counterbalance to all the craziness. For showing me what is really important in life and being my constant source of happiness. Thank you, mum and dad for giving me the freedom to follow my own path and the courage to do it. Grażyna and Tomek for all your help and support through the years. Alicja and Marta for being the best sisters in the world!