Stress biology and interactions between *Solanum* species and *Phytophthora infestans*

Studies in laboratory and field conditions

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Cover: Resistance screening in wild *Solanum* species, quality control (QC) of RNA-seq data (credit: Itziar Frades), quantitative proteomics analysis, and SDS-PAGE analysis of proteins labelled in Activity based protein profiling (ABPP).

(Figure: Kibrom Berhe Abreha)

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Abstract

Phytophthora infestans is a highly destructive pathogen in potato production. Developing potato cultivars with resistance against the pathogen is considered the most sustainable solution to address the problem. Wild *Solanum* species are a source of genes conferring resistance to *P. infestans* (*Rpi*-genes); however, the *Solanum* species genepool from Europe remains untapped. Since the pathogen can overcome host resistance, it is crucial to also consider alternative ways to enhance basal defence using resistance-inducers. Improving the knowledge base of *Solanum* - *Phytophthora* interactions, as well as unravelling possible effects of the interactions on other microbes and herbivores, can facilitate the use of host resistance to reduce yield losses. Furthermore, understanding plant innate immunity activation and the response to stress of host plants growing in field conditions can increase the efficiency of future disease control efforts.

Characterization of the resistance against *P. infestans* in the three wild *Solanum* species growing in Sweden showed that *S. nigrum* is resistant and *S. physalifolium* is susceptible whereas there was large resistance variation among *S. dulcamara* accessions. A study of *S. physalifoilum* showed direct and transgenerational BABA-induced resistance against *P. infestans*. To further understand the molecular basis of these interactions, a transcriptome comparison based on RNA-seq data was performed in the three wild *Solanum* species and three potato clones with varying resistance level to the pathogen, after inoculation with *P. infestans*. The transcriptome analysis identified expanded or depleted transcript families which are associated with resistance. It also retrieved host *R*-gene like sequences and possible pathogenicity factors produced by the pathogen during the infection process. Moreover, a tritrophic interaction study showed that a generalist moth (*Spodoptera littoralis*) prefers to oviposit on a susceptible potato clone inoculated with *P. infestans* compared to uninoculated control plants as well as inoculated resistant clone. Introduction of a resistance gene from a wild *Solanum* species into potato can reduce the effects of *P. infestans* as well as *S. littoralis*.

To understand the prevalence of innate immunity activation in agriculture and nature, more than 500 apoplastic leaf samples isolated from *S. nigrum* and *S. dulcamara* growing in natural populations as well as from five potato cultivars with varying levels of resistance to *P. infestans* were analysed for the presence of PR proteins. The results showed that only one third of the plants have the innate immunity activated. Presence of PR proteins increases towards the end of the growing season, which may be is linked to an increased presence of natural enemies. Moreover, we performed apoplastic

proteome analysis, using label free quantitative proteomics and activity based protein profiling (ABPP) in order to get overview of involved processes. We found that most of the proteins with increased abundance in the field compared to in greenhouse condition were related to biotic stress response. ABPP also showed differential activity statuses of serine hydrolases and β-glucosidases in field and greenhouse growing conditions. Furthermore, the activity of serine hydrolases and β-glucosidases varies across the growing season within the same field. Non-plastic peptide biomarkers for potato stress response were suggested.

Keywords: Solanum, *P. infestans*, *Rpi*-genes, BABA, tritrophic, apoplast, quantitative proteomics, ABPP, Biomarkers

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Dedication

To my family

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 **Kibrom B. Abreha**, Åsa Lankinen, Laura Masini, Sofia Hydbom, Erik Andreasson. Investigation of late blight resistance of all three putative natural *Solanum* hosts in Sweden reveals large variation in *S. dulcamara*. Manuscript

II Åsa Lankinen, **Kibrom B. Abreha**, Erik Alexandersson, Stefan Andersson, and Erik Andreasson (2016). Nongenetic inheritance of induced resistance in a wild annual plant. *Phytopathology* 106:877-883.

III Itziar Frades***, Kibrom B. Abreha***, Estelle Proux-Wéra, Åsa Lankinen, Erik Andreasson and Erik Alexandersson (2015). A novel workflow correlating RNA-seq data to *Phythophthora infestans* resistance levels in wild *Solanum* species and potato clones. *Front.PlantSci*.6:718. doi: 10.3389/fpls.2015.00718. *co-first author

IV **Kibrom B. Abreha**, Erik Alexandersson, Jack H. Vossen, Peter Anderson, Erik Andreasson (2015). Inoculation of transgenic resistant potato by *Phytophthora infestans* affects host plant choice of a generalist moth. PLoS ONE 10(6): e0129815. doi:10.1371/journal.pone.0129815.

V Åsa Lankinen, **Kibrom B. Abreha**, Laura Masini, Ashfaq Ali, Erik Andreasson. Plant immunity is seldom activated in natural populations and agricultural fields. Submitted manuscript

VI **Kibrom B. Abreha**, Erik Alexandersson, Åsa Lankinen, Daniela Sueldo, Kaschani Farnusch, Markus Kaiser, Renier A. L. van der Hoorn, Fredrik Levander, Erik Andreasson. Leaf apoplast of field- and greenhouse-grown potato analysed by quantitative proteomics and activity based protein profiling. Manuscript

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* Equally contributing authors

The contribution of **Kibrom B. Abreha** the papers included in this thesis was as follows:

I Planned the experiment together with co-authors, performed the practical work and data analysis, and writing of the manuscript.

II Planned the experiment together with co-authors, performed the practical work, and participated in data analysis and writing of the manuscript.

III Planned the experiment together with co-authors, performed the practical work, and participated in the analysis and writing of the manuscript.

IV Planned the experiment together with co-authors, performed the practical work and data analysis, and writing of the manuscript.

V Planned the experiment together with the co-authors, performed part of the practical work, and participated in data analysis and writing of the manuscript.

VI Planned the experiment together with co-authors, performed the practical work and data analysis, and writing of the manuscript.

Related works by **Kibrom B. Abreha** but not included in this Thesis:

I Erik Andreasson, **Kibrom B. Abreha**, Svante Resjö (2017). Isolation of Apoplast. In: Isolation of Plant Organelles and Structures: Methods and Protocols (eds N.L. Taylor & A.H. Millar), pp. 233-240. Springer New York, New York, NY.

II Tina Boddum, Bela Molnar, Sharon R Hill, Göran Birgersson, Bill S Hansson, **Kibrom B. Abreha**, Erik Andreasson and Ylva Hillbur. *Contarinia nasturtii* host selection. Manuscript

III Tewodros M. Zewdie, Bayeh Mulatu, **Kibrom B. Abreha**, Habte Tekie, Mohammed Yessuf, Erik Andreasson, Erik Alexandersson. The influence of phosphite on *P. infestans* and synergism with conventional fungicides in field-grown potato and tomato in Ethiopia. Manuscript

Abbreviations

ABPP	activity based protein profiling
Avr	avirulence
BABA	β-aminobutyric acid
BABA-IR	BABA- induced resistance
BLAST	Basic Local Alignment Search Tool
ETI	effector triggered immunity
ETS	effector triggered susceptibility
FAO	Food and Agricultural Organization
GO	Gene ontology
HR	hypersensitive response
LC/MS	liquid chromatography–mass spectrometry
NBS-LRR	nucleotide binding site-leucine rich repeats
NGS	next Generation Sequencing
PAMPs	pathogen-associated molecular patterns
PCA	principal components analysis
PRR	pattern recognition receptor
PTI	PAMP trigerred immunity
RK	receptor kinases
RLP	receptor-like proteins
Rpi-genes	Resistance genes against *Phytophthora infestans*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STEM	Short Time-series Expression Miner

1 Introduction

Potato (*Solanum tuberosum*, 2n = 4x = 48), a member of *Solanaceae* family, is one of the most important food crops in the world. The crop has high yield potential and grows in a wide agro-ecological range delivering more than 380 million tons (MT) total annual production (FAO, 2016). Increasing annual potato production in the developing world underlines a growing role of this crop in global food security. However, potato suffers from a multitude of biotic and abiotic stresses, especially in the face of a changing climate.

Ever since causing the Irish potato famine, in the 1840's, late blight disease caused by the notorious oomycete *Phytophthora infestans* is recognised as the most destructive disease in potato production. The estimated 5 billion US dollars annual losses due to *P. infestans* (Haverkort *et al.*, 2009) and its wide geographical distribution makes the pathogen one of the most economically significant oomycetes (Kamoun *et al.*, 2014). Use of fungicides to control *P. infestans* is not affordable or environmentally sustainable. Moreover, evidence shows isolates of the pathogen can develop fungicide resistance (Chowdappaa *et al.*, 2014; Grunwald *et al.*, 2001; Gisi & Cohen, 1996). Therefore, developing potato cultivars resistant against *P. infestans*, using resistance genes (*R*-gene) from wild species, is considered the most sustainable solution to address this problem. However, the pathogen can also overcome *R*-gene based resistance (Fry, 2008). To ensure durability of *R* genes mediated resistance, pyramiding of multiple *R* genes into a potato genotype is now considered the best strategy. This, enables the plant to recognize multiple effectors of the pathogen, and should decrease the risk of pathogen escape from this multigenic recognition. A multigene strategy has already been used in the new commercial potato cultivar Sarpo Mira (Rietman *et al.*, 2012), which is so far showing stable resistance against *P. infestans*.

Wild *Solanum* species carry traits that can be used to improve potato resistance against biotic stresses, including *P. infestans*. Various resistance genes against *P. infestans* (*Rpi*-genes) have been identified in wild *Solanum* species and some of them have been successfully introduced into potato cultivars (Rodewald & Trognitz, 2013). Due to the pathogen's ability to adapt and overcome host resistance (Haas *et al.*, 2009; Fry, 2008), more resistance sources and a deeper understanding of plant-pathogen interactions are needed to ensure durability of such resistance in potato. Understanding of the interactions between the host and the pathogen using state of the art '-omics' and molecular biology tools will facilitate the efforts of breeders to develop stable resistance in commercial cultivars of potato. Little is known about the effects of the potato-*P. infestans* interactions and introduction of *R*-genes into the crop, on host preference of herbivores. Due to ability of the pathogen to overcome host resistance, it is crucial to enhance this resistance by using resistance inducers such as β-aminobutyric acid (BABA) (Bengtsson *et al.*, 2014a; Liljeroth *et al.*, 2010; Olivieri *et al.*, 2009). Furthermore, understanding the status of the innate immunity activation in agricultural fields can help end-user decision making for timely and appropriate application of disease control measures.

The overall aim of this thesis was to enhance our understanding of *Solanum* species interactions with *P. infestans*. Specifically to 1) evaluate the resistance to *P. infestans* in wild *Solanum* species 2) find out if BABA can induce resistance in a wild species 3) demonstrate possible effects of potato-*P. infestans* interactions on herbivore behaviour, 4) to unravel the status of plant innate immunity activation in the field, and 5) to investigate stress response of *Solanum* species growing under field conditions.

Resistance to *P. infestans* of the three wild *Solanum* species (*S. dulcamara*, *S. nigrum*, and *S. physalifolium*) collected from southern Sweden was characterized. A study of BABA-induced resistance in *S. physalifolium* showed epigenetic inheritance of this induced resistance against *P. infestans*. A transcriptomics approach was used to understand the interactions of the three wild *Solanum* species and three potato clones with *P. infestans*. Moreover, the effects of potato- *Phytophthora* interactions on host plant choice and performance of a generalist moth (*Spodoptera littoralis*) was investigated. By using the presence of pathogenesis related proteins (PR1, PR2-3) as markers, the frequency of innate immunity activation in *S. dulcamara* and *S. nigrum* in natural populations and *S. tuberosum* in agricultural conditions was

investigated. Finally, to understand plant stress response, quantitative proteomics coupled with Activity Based Protein Profiling (ABPP) was used to investigate differences in the apoplastic proteome between greenhouse- and field-grown potato.

2 Background

2.1 Potato (*Solanum tuberosum L.*)

Cultivated potato (*Solanum tuberosum*, 2n = 4x = 48), traces its origin from the wild species of *Solanum brevicaule* in the Andean highlands of South America (Spooner *et al.*, 2005). The other cultivated potato species is the diploid *Solanum phureja* (or Group Phureja), growing in the eastern valleys of the Andes. This species is selected for early maturity (Bradshaw & Ramsay, 2005). Archaeological evidences suggest domestication of potato ~10,000 years ago in present-day Peru and Chile. Spanish explorers introduced potato into Europe in the 16th century (Ames & Spooner, 2008). Today, potato is the third most important food crop in the world, after wheat and rice. Potato is produced in over 150 countries in the world, covering a wide range of agro-ecological zones. The total area of potato cultivation exceeds 19 million ha, delivering an annual world production of more than 380MT (FAO, 2016). Currently, mainland China is the largest potato producer followed by Russia, India and the United States of America.

A more steady production in developed countries is common, for example, in Sweden potato is an irreplaceable part of the diet (Eriksson *et al.*, 2016). In contrast, the total potato production in developing countries is increasing (Gastelo *et al.*, 2014; Haverkort *et al.*, 2009). Since the majority of humans live in developing countries, increasing potato production in these countries highlights the growing significance of this crop in generating income and as an important contribution to global food security.

Potato is a starch-accumulating tuberous crop and hence a good source of dietary carbohydrates. Compared to the major cereal crops, potato produces more dry-matter and protein per unit growing area. The nutritive value analysis

of potato tubers indicate that it is also a source of good quality protein that can complement the lysine deficiency in cereals (Friedman, 1996). Moreover, potato is an excellent source of minerals including potassium, phosphorus, magnesium and vitamin C, B-1, B-3, and B-6 (Camire *et al.*, 2009; Prokop & Albert, 2008); as well as antioxidants like flavonoids, phenolics, and anthocyanins (Brown, 2005). However, potato also contains gluco-alkaloids like solanin that can be poisonous to humans (Friedman, 2006; Friedman, 1996). In order to keep gluco-alkaloids at low levels, potato tubers should be stored in a dark cool place and peeled before cooking (Prokop & Albert, 2008).

However, whilst there are many benefits to growing and consuming potatoes, as a crop it is susceptible to a wide range of biotic and abiotic stresses. For instance, early blight (*Alternaria solani*), blackleg disease (*Dickeya solani)*, viral infections (e.g. *potato virus Y)* as well as yellow potato cyst nematode *(Globodera rostochiensis)* and potato tuber moth (*Phthorimaea operculella*) are known to cause significant yield losses in the crop (Eves-van den Akker *et al.*, 2016; Burra *et al.*, 2015; Odilbekov *et al.*, 2014; Kroschel *et al.*, 2013; Gray *et al.*, 2010). However, the damage caused by *Phytophthora infestans* in potato production is unparalleled (Kamoun *et al.*, 2014; Haverkort *et al.*, 2009). Host plant resistance is an effective, environmentally friendly and sustainable method to protect potato from *P. infestans*.

2.2 Wild *Solanum* species

Solanum is an economically significant genus of flowering plants. It contains more than 1500 species, including important crops like potato, tomato, and eggplant. Although mainly considered as weeds and usually poisonous to humans and animals, wild *Solanum* species can also be used for food, source of medicine or as ornamentals thus are important source of income, e.g. in Africa (Samuels, 2015; Katambo, 2007). Moreover, wild *Solanum* species can be used as a source of resistance traits to improve the disease resistance of cultivated related species. Conversely they could also be potential alternative hosts of pathogens. Some wild *Solanum* species, for example *S. sisymbriifolium*, *S. physalifolium* and *S. sarrachoides,* are known to be susceptible to *P. infestans* and could play important role as natural reservoirs of the pathogen inoculum (Deahl *et al.*, 2005; Andersson *et al.*, 2003; Flier *et al.*, 2003).

2.2.1 Sources of resistance trait

There are more than 4,000 edible varieties of potato, most of which are growing in the Andean highlands of Peru (http://cipotato.org/potato/).

However, outside of South America, potato has a narrow genetic diversity. This is partly due to limited genetic variance in the germplasm introduced into new areas and inbreeding depression (Potato Genome Sequencing, 2011). Moreover, farmers tend to prefer few high yielding clones adapted to local growing conditions and that deliver marketable products. Intensive potato breeding efforts are aimed at improving disease resistance of the crop. However, the success of conventional potato breeding for resistance to late blight disease is limited by its clonal propagation (Bradshaw *et al.*, 2006). Conventional potato breeding for resistance against *P. infestans* takes longer than 10 years (Slater *et al.*, 2014). However, *P. infestans* contains fast evolving effector genes, located in the gene sparse and transposon rich region of the genome (Haas *et al.*, 2009), enabling the pathogen to evade host resistance (Malcolmson & Black, 1966). To cope with *P. infestans*, developing resistant potato cultivars with several different resistance sources is an attractive method in breeding. Therefore, finding resistance traits particularly genes conferring resistance against *P. infestans* (*Rpi*-genes) in wild *Solanum* species and transfer of these genes into potato is to date the most pursued breeding strategy to improve potato resistance.

Wild *Solanum* species are the major source of resistance traits used in potato breeding. Since the discovery of major resistance (*R*-) gene against *P. infestans* in *Solanum demissum*, introduction of these *R*-genes into potato is considered a compelling method to protect the crop (Reddick, 1934). To reduce yield losses resistance genes against *P. infestans* (aka, *Rpi*-genes) are cloned from various wild *Solanum* species and transferred into commercial potato cultivars (Rodewald & Trognitz, 2013; Vleeshouwers *et al.*, 2011; Malcolmson & Black, 1966). So far, 27 *Rpi*-genes conferring resistance against *P. infestans* have been cloned from wild *Solanum* species of South American origin (Rodewald & Trognitz, 2013). However, all the resistance genes cloned so far belong to the CNL class and are from the wild *Solanum* species adapted to South America; hence they may not recognize all strains of the pathogen. Because of its ability to overcome host resistance, *P. infestans* is known as 'the plant *R*-gene destroyer' (Fry, 2008). Therefore, there is a continued interest to find novel *Rpi*-genes that can be introduced into potato individually or by pyramiding. Whilst *Rpi*-genes from native South American *Solanum* species have been well studied, the genetic potential of native European wild *Solanum* species has so far received little attention and the genetic resource of these species remains untapped.

2.2.2 Wild *Solanum* species in Sweden

Three species of *Solanum*, which are potential alternative hosts of *P. infestans*, grow in natural habitats and agricultural fields in south Sweden. These are *Solanum dulcamara* L., *Solanum nigrum* L. and *Solanum physalifolium*.

S. dulcamara L., named after the latin dulcis (= sweet) and amarus (= bitter), is widely known as bittersweet nightshade. *S. dulcamara* is a semi-woody perennial species. Since it has a predominantly climbing growth habit, it is also known as climbing nightshade, and its branches may grow up to 2 meters or more. Originally from Europe, *S. dulcamara* is widely distributed and found in diverse habitats including seashores, dense thickets, roadsides, hedgerows, and along the banks of ponds. The leaves are dark green and alternately arranged along the branches at different internode lengths. Often, leaves within the same plant, even on the same branch, exhibit different shapes. For instance, it is not uncommon to find leaves with entire or serrated margins and leaves with or without stipules on the same branch. Flowers are purple with yellow anthers that produce oval or round shaped red berries (Figure 1).

S. dulcamara is a diploid (2n = 2x = 24) out-crossing species (Golas *et al.*, 2010a). It is also a clonal species reproduced by stem cuttings. Despite its limited role in *P. infestans* epidemiology (Golas *et al.*, 2010c), *S. dulcamara* is considered an alternative host of the pathogen (**Paper I**) and (Flier *et al.*, 2003). Using AFLP markers and next-generation sequencing derived SNPs in a crossing population of *S. dulcamara* segregating for *P. infestans* resistance, loci for two putative *R*-genes (*Rpi-dlc1* in chr-9 and *Rpi-dlc2* in chr-10) have been identified (Golas *et al.*, 2013; Golas *et al.*, 2010b). In RNA-seq analysis of *P. infestans* infected leaves of this species we also found *R*-like genes (**Paper III**).

S. nigrum L. (black nightshade), native to the old world Eurasia, is widely distributed in Europe, Asia, Africa, and North America. It is a self-pollinating annual species, weed problem in potato fields (Defelice, 2003). *S. nigrum* plants have decumbent or erect stems, ovate and lanceolate leaves with entire to sinuate-dentate margins, and can grow up to 70 cm high (Edmonds & Chweya, 1997). Flowers are whitish and berries turn purple-black when ripened (Figure 1).

S. nigrum is a highly variable species which can be found in di-tetra- and hexaploid cytotypes (Venkateswarlu & Rao, 1972). The species contains high levels of glycoproteins, including solanine, solamargine, solasonine in its leaves, berries and stem. The medicinal value of *S. nigrum* was known as early

as 870 AD (Katambo, 2007) and it has largely been investigated for its pharmaceutical value. Glycoproteins (Heo & Lim, 2005) and lunasin, a 43-amino acid polypeptide (Jeong *et al.*, 2010), isolated from *S. nigrum* show anticancer property by promoting apoptosis of tumor cells and preventing oxidative DNA damage, respectively.

Despite *S. nigrum* is considered poisonous, its leaves are consumed as vegetables in some countries (Edmonds & Chweya, 1997). *S. nigrum* was considered a non-host to *P. infestans* (Vleeshouwers *et al.*, 2000), but strains of the pathogen that can infect genotypes of this wild species were recently found in Poland and the Netherlands (Lebecka, 2008; Flier *et al.*, 2003). Monogenic dominant inheritance of resistance to *P. infestans* was shown using a crossing population of *S. nigrum* between susceptible and resistant individuals (Lebecka, 2009). This species harbours *R1* orthologs. *R1* is a member of the CC-NBS-LRR resistance gene class, conferring resistance against *P. infestans* (Gyetvai, 2010), by recognition of the Avr1 effector (Du *et al.*, 2015).

S. physalifolium (2n = 2x =24) also known as hairy nightshade is an annual and self-pollinating species, native to South America but widely distributed. Usually *S. physalifolium* plants grow as small herbs, with light green stems, leaves that are ovate to ovate-lanceolate to trullate, small whitish flowers, and berries that are dark green, purple or brownish-green (Edmonds & Chweya, 1997) (Figure 1). This species is a common weed problem in agricultural fields in Sweden. Recently, *S. physalifolium* has been identified as possible alternate host of *P. infestans* in Sweden, due to its high sensitivity to this pathogen (Andersson *et al.*, 2003). Compared to isolates from potato leaves, isolates of the pathogen from *S. physalifolium* leaves showed shorter latency period and higher sporangia numbers when inoculated on potato leaves (Gronberg *et al.*, 2012). Therefore, *S. physalifolium* might increase aggressiveness of the pathogen (Gronberg *et al.*, 2012) and enhance pathogenicity of the *P. infestans* population in the area.

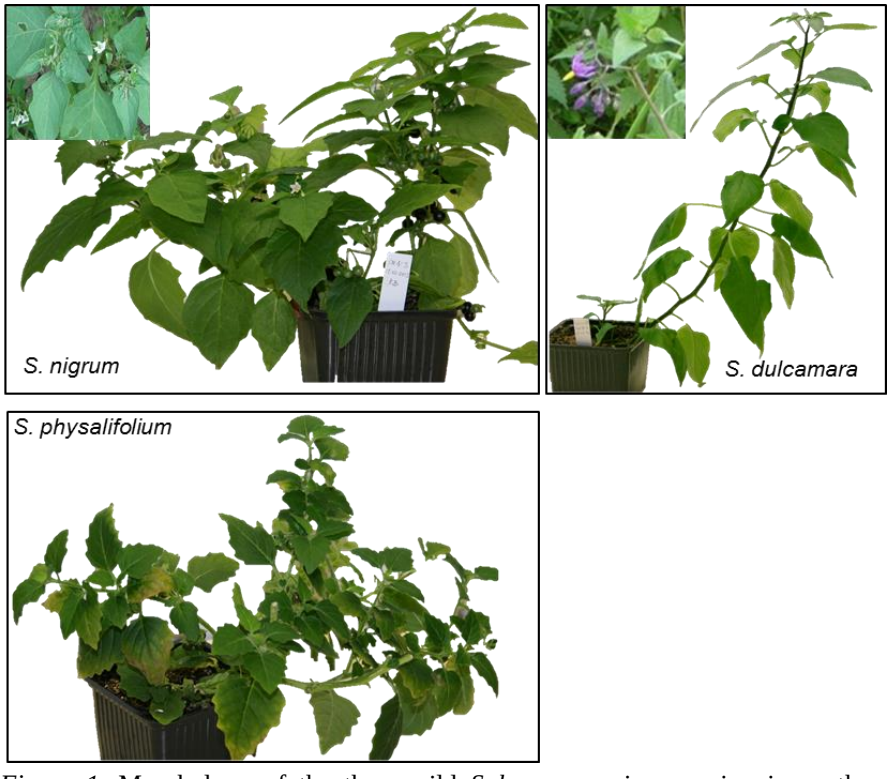

Figure 1. Morphology of the three wild *Solanum* species growing in southern Sweden: *Solanum nigrum*, *S. dulcamara* and *S. physalifolium*.

2.3 *Phytophthora infestans*

2.3.1 The pathogen and mating types

Phytophthora infestans (Mont.) de Bary is an oomycete causing potato late blight and tuber blight. The origin of *P. infestans* is still a debatable topic. Using sequences of nuclear genes and mitochondrial loci, (Gomez-Alpizar *et al.*, 2007) suggested an Andean origin of *P. infestan*s. However, phylogenetic analysis based on microsatellite markers and sequences of four nuclear genes, including longer sequences of the β-tubulin gene also used in the previous study (Gomez-Alpizar *et al.*, 2007), suggested a Mexican origin of *P. infestans* (Goss *et al.*, 2014). Therefore, the origin of *P. infestans* still awaits a conclusive study.

The first migration of *P. infestans* out of southern America to Europe and USA is believed to have occurred in the early 19th century (Goodwin *et al.*, 1994). The pathogen was introduced into the rest of the world through the

international trade of seed potatoes (Fry *et al.*, 1993). *P. infestans* is a heterothallic pathogen with A1 and A2 mating types. Prior to the 1970's the *P. infestans* population was predominantly the A1 clonal lineage (Fry *et al.*, 1993), and coexistence of both mating types had been previously confined to Mexico (Fry, 2008). Second migration event of *P. infestans* was from Mexico to Europe in 1976 (Niederhauser, 1991), which might have introduced the A2 clonal lineage (Hohl & Iselin, 1984). At present, both A1 and A2 mating types coexist, e.g. in the Czech Republic (Mazáková *et al.*, 2006), Russia (Beketova *et al.*, 2015), eastern Estonia (Runno-Paurson *et al.*, 2010), the Netherlands (Fry, 1991), and Tunisia (Harbaoui *et al.*, 2014). This coexistence might enable the pathogen to reproduce both sexually and asexually, which may increase genetic recombination and diversity and promote pathogen survival under adverse conditions (Yuen & Andersson, 2013; Fay & Fry, 1997). Coexistence of the A1 and A2 mating types of the *P. infestans* and oospores production in southern Sweden might suggest sexual reproduction of the pathogen in the area (Yuen & Andersson, 2013; Widmark *et al.*, 2007; Andersson *et al.*, 2003). In Sweden, there is unusually large variation of the *P. infestans* population within the same field and the A1 and A2 mating types exist in almost 50:50 ratios (Gronberg *et al.*, 2012; Sjöholm, 2012).

Due to similarities in terms of absorptive mode of nutrition, growth by hyphal extension, and reproduction through formation of spores, oomycetes were historically identified as fungi (Money, 1998). However, (Harper *et al.*, 2005) using six genes encoding cytoplasmic proteins showed distinct evolution between the fungi and oomycetes. Furthermore, the cell wall of the oomycete is composed predominantly of glucans and cellulose in contrast to the chitin rich wall of fungi (Fry, 2008). Haas *et al.* (2009) revealed that the *P. infestans* genome has 17, 797 predicted protein coding genes with a large number of effectors genes localized in the gene sparse but repetitive sequences and transposable elements rich region of the genome (Haas *et al.*, 2009). This leads to the 'two speed genome' model, where the effectors containing section of the genome is rapidly evolving thus facilitating adaptive evolution of the pathogen (Dong *et al.*, 2015).

2.3.2 Life cycle and infection process

Late blight disease starts from either aerial or soil borne inoculum, the later usually originates from inoculum surviving on infected tubers, or as soil-borne oospores when conditions favour sexual reproduction. Aerial borne sporangia may penetrate plant tissue directly through formation of a germ tube or indirectly through the release of bi-flagellated zoospores (Figure 2). The later

predominantly occurs in high humidity and cool temperatures, whilst direct germination is favoured at higher temperatures with lower relative humidity. Direct or indirect germination is followed by production of a specialised penetration structure, the appressoria, which releases cell-wall degrading enzymes and accumulates turgor pressure to allow the pathogen to breach the host cell wall and enter the plant to initiate infection. The pathogen obtains nutrients from the host by production of haustoria (Birch *et al.*, 2003). The first macroscopic disease symptoms, (small necrotic areas), can be visible two days after penetration (Fry, 2008). Large areas of necrosis appear as the disease progresses. Sporangiophores, bearing sporangia emerging through stomata can be seen on the leaf surface within 3-4 days (Vleeshouwers *et al.*, 2000). Since each sporangium can go on to cause successive cycles of infection, this rapid reproduction of inoculum can lead to complete destruction of the whole field within a few days.

Furthermore, in the presence of both A1 and A2 mating types, sexual reproduction can also occur. The pathogen forms haploid anthridia and oogonia which come together through karyogamy to form diploid oospores. Oospores contribute positively to the long-term survival of the pathogen in the soil through resistance to harsh environmental conditions, as well as to pathogen fitness through sexual recombination. As an inoculum source oospores may directly germinate, produce sporangia and infect stems or leaves that directly contact the soil to further propagate the disease (Figure 2).

To better understand the infection process and biology of the pathogen, many molecular components of *P. infestans* life cycle have been elucidated (Fry, 2008). A large number of *P. infestans* genes showed differential expression during the life cycle, indicating dynamic structural and physiological difference among the life stages (Judelson *et al.*, 2008). Light conditions and spore-associated transcripts, protein kinase Pks1 and transcription factors *Myb2R1* and *Myb2R3*, play crucial role in sporangia formation during the *P. infestans* life cycle (Xiang & Judelson, 2014). A protein kinase of *P. infestans* (Pipkz1) is required for zoospores motility and appressoria formation (Blanco & Judelson, 2005). *P. infestans* requires active cellulose synthesis for production of appressoria and subsequent penetration and successful infection (Grenville-Briggs *et al.*, 2008). But during the infection process, the plant activates its defence related repertoire in order to restrict the pathogen growth, for example, HR in the penetrated epidermal cells, thickening of cell wall and condensing of the nuclei (Vleeshouwers *et al.*, 2000). Thirty-eight members of the bZIP transcription factor family can be found in the *P. infestans* genome, some of which protects the pathogen from oxidative stress (Gamboa-Melendez *et al.*, 2013).

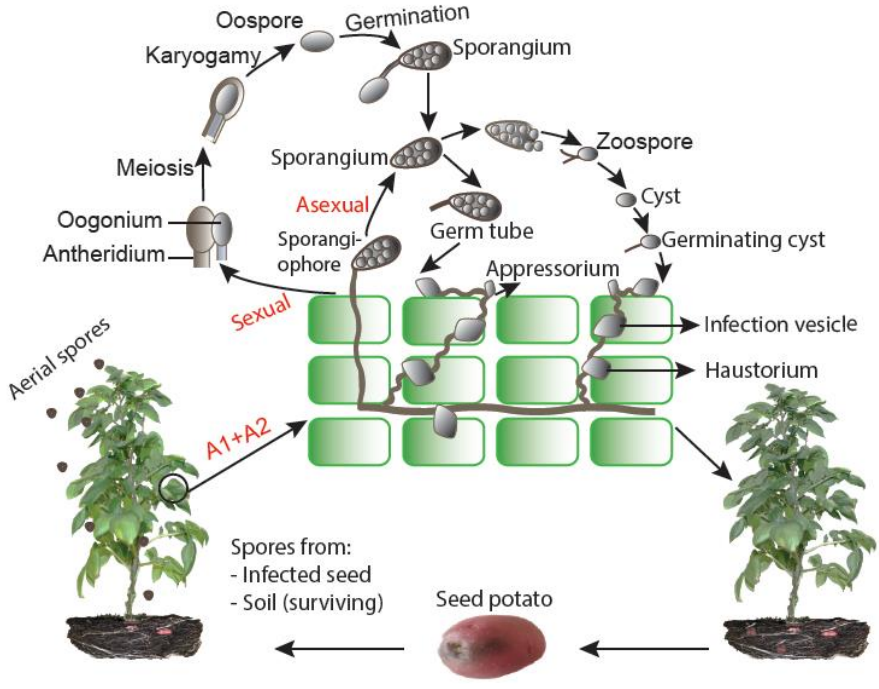

Figure 2. Sexual and asexual life cycle of *Phytophthora infestans*. Illustration by Kibrom Berhe Abreha.

2.4 Plant innate immunity

Through evolution plants have developed sophisticated defence responses to thwart pathogen attack. At the same time, pathogens have co-evolved mechanisms to evade host plant defences. Beyond the constitutive level of defence, plants have distinctive inducible defence mechanisms against a broad spectrum of pathogens (Conrath *et al.*, 2002). Plant innate immunity is discerned into pathogen associated molecular pattern (PAMP) triggered immunity (PTI) and Effector triggered immunity (ETI), described by the so called zig-zag model of plant immunity (Figure 3; Jones & Dangl, 2006). According to the zig-zag model (Figure 3), plants detect PAMPs via pathogen recognition receptors (PRRs), which activate PTI. Some pathogens may release effectors to suppress PTI, leading to effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). However, some plants can recognize effectors of the pathogen via NB-LRR proteins and trigger ETI (Jones & Dangl, 2006). The model is widely used to explain plant-microbe interactions. PTI and ETI are regarded as extremes of the continuous plant defense response to the

infecting pathogen (Pritchard & Birch, 2014; Thomma *et al.*, 2011). PTI and ETI components of potato in response to *P. infestans* infection are identified and their molecular function partially elucidated.

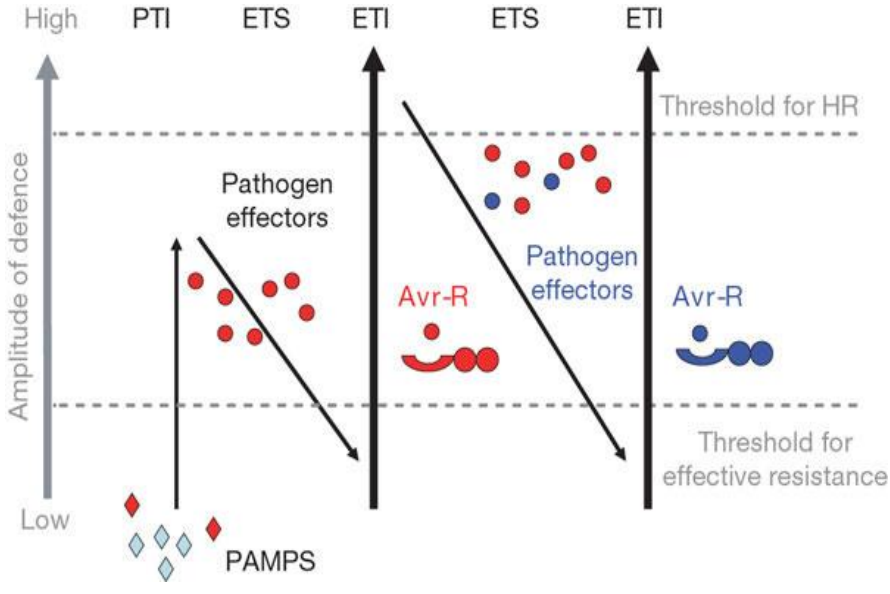

Figure 3. Zig-zag model of plan innate immunity system (Jones & Dangl, 2006).

2.4.1 PAMPs triggered immunity (PTI)

Pathogen-associated molecular patterns (PAMPs) are conserved structural components or molecules of the pathogen. At the onset of an infection attempt, PAMPs are perceived by extracellular domains of host cell surface receptors called pattern recognition receptor proteins (PRR) (Zipfel 2008; Thomma et al. 2011). PRRs are cell-surface localized receptor kinases (RKs) or receptor-like proteins (RLPs) containing a ligand-binding ectodomain, a single-pass transmembrane domain, and sometimes an intracellular kinase domain (Zipfel, 2014). Perception of PAMPs by PRRs activates downstream defence signalling pathways. The leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1/SERK3 is central regulator of plant innate immunity against pathogens (Heese *et al.*, 2007). Thus, recognition of PAMPs triggers the defence signalling cascade resulting in PTI (Figure 3), which is the first line of defence, and is often marked by callose deposition and activation of other general defence responses such as the production of reactive oxygen species (ROS).

The cell wall of *P. infestans* plays a crucial role in the infection process and survival of the pathogen. Silencing of the cellulase synthase genes *CesA1*, *CesA2*, *CesA3*, and *CesA4* disrupts the cell wall of *P. infestans* appressoria and leads to complete loss of pathogenicity of the pathogen (Grenville-Briggs *et al.*, 2008). During the infection process, the cell wall components can be recognized by the plant thus trigger host defence signalling. For example, the *P. infestans* PAMP (PiPE) derived from mycelial wall binds to the Ca^{2+} dependent protein kinase (CDPK), located upstream of NADPH oxidase in plasma membrane, and induces a hypersensitivity response (HR) in potato (Furuichi, 2014). Similarly, infiltration of potato leaves with conserved motif of peptidase 13 (Pep-13), a constituent of the cell wall transglutaminase (TGase) of *Phytophthora* species, increases lipoxygenase, 4-coumarate:CoA ligase, SA, JA, and H_2O_2 accumulation in potato (Halim *et al.*, 2009; Halim *et al.*, 2004; Brunner *et al.*, 2002). A cell wall component of *P. infestans* can increase virulence of the pathogen. For instance, β-glucans isolated from virulent strain of *P. infestans* could inhibit the potato phytoalexin and reduce glucanase accumulation in the host to promote invasion (Andreu *et al.*, 1998).

2.4.2 Effector triggered immunity (ETI)

The PTI results in massive physiological changes important to overcome the infection process. However, to counteract the PTI response from plants, some pathogens secrete effector proteins that are targeted to disrupting PTI defences. This process is called effector-triggered susceptibility (ETS) (Figure 3). However, as a result of the arms race between the plant and pathogen, resistant hosts are endowed with another layer of defence, effector-triggered immunity (ETI) (Figure 3).

Recognition of intracellular effectors, secreted from pathogens into host cells occurs through the activation of major resistance gene (*R*-gene) encoded receptors. *R*-genes trigger host defence signalling cascades resulting in ETI. *R* genes encode resistance proteins most of which are of the nucleotide-binding-leucine-rich-repeat (NB-LRR) type (Rodewald & Trognitz, 2013; Zhang *et al.*, 2013). This recognition of the effector proteins by the NBS-LRRs initiates ETI responses that can specifically inhibit pathogen growth.

P. infestans genome contains two major effector families: ~563 genes encoding RXLR effectors and ~200 genes encoding crinklers (CRN) (Haas *et al.*, 2009). RXLR effectors are secreted proteins with N-terminal region containing the Arg-X-Leu-Arg (in which X represents any amino acid) motif

that is required for delivery of the effectors into host cells (Kamoun, 2006). Fast evolving C-terminal region of the RXLR effectors is required for manipulating host defence (Win *et al.*, 2007). CRN are cytoplasmic effectors, containing conserved N-terminal with ~50-amino-acid LFLAK domain, diversified DWL domain, and 60% of them also have predicted signal peptide (Haas *et al.*, 2009). The C-terminal of CRN effectors contains diversified domain structures (Haas *et al.*, 2009). Some of the RXLR and CRN effectors have been experimentally verified, and their function as virulence (causing disease) and avirulence factors (triggering host plant) have been elucidated.

Compared to expression in growth media, seventy nine RxLR genes encoding *P. infestans* effector proteins including the Avr3a, Avr4, and Avrblb1 (ipiO), were highly expressed during potato infection (Haas *et al.*, 2009). Recognition of the Avr4 by R4 (van Poppel *et al.*, 2008) and Avrblb1 by Rpi-blb1 resulting in ETI have been reported (Vleeshouwers *et al.*, 2008). The cytoplasmic effector Avr3a interacts with E3 Ubiquitin Ligase CMPG1 to suppress the INF1-induced cell death which is a form of PTI (Bos *et al.*, 2010). The suppressing effect of Avr3a can be abolished if recognized by host plants containing the resistance gene R3a (Armstrong *et al.*, 2005). Similarly, the PexRD8 and PexRD3645-1 effectors of *P. infestans* could also suppress this INF1- induced cell death (Oh *et al.*, 2009). Recently, PexRD2 was found to suppress MAPKKK-induced cell death and enhance susceptibility to *P. infestans* (King *et al.*, 2014).

The *P. infestans* Avr2 should be associated with putative plant phosphatase BSU-LIKE PROTEIN1 (BSL1) to be recognized by the R2 protein (Saunders *et al.*, 2012) but only 32aa region in the C-terminal of Avrblb2 is required to initiate Rpi-blb2 mediated HR (Oh *et al.*, 2009). The Rpi-vnt1.2 and Rpi-vnt1.3 but not their truncated versions, indel of 33 amino acids in their N-terminal region, recognize the Avrvnt1 effector (Pel, 2010). However, the pathogen can also exploit components of the plant, known as susceptibility factors, to supress the defence response and promote host invasion. For example, to suppress INF1-mediated cell death, *P. infestans* RXLR effector (Pi02860) needs presence of the potato NPH3/RPT2-LIKE1 (NRL1) protein (Yang *et al.*, 2016). Other RXLR effectors, Pi04089 and Pi04314, are known to interact respectively with host K-homology (KH) RNA-binding protein (KRBP1) and protein phosphatase PP1 catalytic subunits to promote the pathogen leaf colonization (Boevink *et al.*, 2016). A Putative RXLR effector (PITG_03192) promotes disease development by interacting with NAC (NAM/ATAF/CUC) transcription factors and preventing its re-localization

from endoplasmic reticulum (ER) into nucleus (McLellan *et al.*, 2013). Recently, Vetukuri *et al.* (2017) identified *P. infestans* effector (PITG_14054) involved in suppression of RNA silencing in *Nicotiana benthamiana*, and it showed increased expression during early infection of a susceptible potato cv. Bintje.

Another major family of effectors is the Crinklers (CRN). 196 genes encoding CRN proteins reside in the sequenced *P. infestans* strain, and showed increased expression during potato infection (Haas *et al.*, 2009). Moreover, CRN proteins are present in abundance during different life stages of the pathogen (Meijer *et al.*, 2014; Resjo *et al.*, 2014), indicating that they could be involved in promoting host colonisation by the pathogen. By mining the ESTs sequences of *P. infestans* extracellular proteins, Torto *et al.* (2003) identified CRN1 and CRN2 expressed during tomato infection. The CRN8 kinase is secreted into the nucleus of the host cell and enhances virulence of *P. infestans* on *N. benthamiana* (van Damme *et al.*, 2012). The extracellular, cytoplasmic and nuclear localization of the effectors suggest that multiple host targets localized in the respective cellular compartments could be simultaneously targeted by the pathogen. In a resistant genotype, the recognition of PAMPs and effector proteins trigger downstream defence signalling cascade which results in PTI or ETI, restricting pathogen growth.

2.4.3 *Solanum* species stress response in field conditions

It is well known that plant responses to potential pathogens and/or herbivores may effect subsequent defence responses (Zamioudis & Pieterse, 2012), and host choice and performance of herbivores (Zakir *et al.*, 2013; Jallow *et al.*, 2008; Anderson & Alborn, 1999). Moreover, abiotic stresses also affect defence responses to microbes and herbivores; a cross-talk between biotic and abiotic stress response pathways is well known (Rejeb *et al.*, 2014). Due to compounding stress factors, which may be largely absent in controlled conditions, field-grown plant defence responses to these stresses are complex (Cramer *et al.*, 2011).

The zig-zag model of plant-microbe interactions (Jones & Dangl, 2006), assumes a single plant-pathogen interaction. However, the model does not consider the continuous presence and recognition of multiple pathogen-associated molecular patterns (PAMPs) and effectors as well as the abiotic stresses in field and natural populations (**Paper V**). Moreover, in field conditions other interactions such as plant-plant interactions may affect plant

fitness and the composition of communities (Brooker, 2006). This in turn is likely to have a knock-on effect on plant defence responses to pests, pathogens and herbivores.

2.4.4 Apoplast: the frontier in plant-pathogen interaction

The apoplast is a compartment of plant tissue comprising the extracellular space and cell wall. The soluble fraction within this compartment known as the apoplastic fluid contains a myriad of proteins involved in biological processes related to maintaining cell wall structure, plant-pathogen interactions, responses to abiotic stresses, and nutrient transport (Andreasson *et al.*, 2017; Delaunois *et al.*, 2014; Alexandersson *et al.*, 2013). During the course of infection, an array of pathogen effector proteins are secreted, some of which target processes inside the cell and some of which target the apoplast. *P. infestans* apoplastic effectors such as protease inhibitors, cysteine rich proteins, and nucleotide pyrophosphatase/phosphodiesterase (NPP1) family members were in higher abundance during early infection of potato (Haas *et al.*, 2009). *P. infestans* also releases small extracellular proteins like elicitins INF1, INF2A, INF5 and INF6 (Du, 2014) into the apoplast. Furthermore, many proteins involved in plant defence like pathogenicity related proteins (PR1), P69B, and serine hydrolases are secreted into the apoplast by the host, presumably in response to perceived attack by potential pathogens (Ali *et al.*, 2014; Sueldo *et al.*, 2014). Hundreds of proteins with differential abundance can be found in the apoplastic secretome of potato infected with *P. infestans* (Ali *et al.*, 2014). This highlights the importance of the apoplastic compartment to understand the potato-*P. infestans* pathosystem.

2.5 The -Omics of potato-*Phytophthora* pathosystem

The perception of *P. infestans* signatures by potato and the ability of the pathogen to evade host defence indicates a complex network of interactions between the plant and the pathogen (Birch *et al.*, 2003). Recent studies on plant-pathogen interactions are benefiting from the advancements in molecular biology methods and fast developing sequencing platforms (Burra *et al.*, 2016). The -omics techniques (transcriptomics, proteomics, and metabolomics) play a crucial role in aiding our understanding of the underlying molecular events taking place during the infection process. Moreover, use of these techniques facilitates identification of candidate proteins for functional analysis.

2.5.1 Potato-*P. infestans* interaction transcriptome

Sequencing the potato genome unravelled that the crop's ~844-megabase (Mb) genome contains predicted 39,031 protein coding genes (Potato Genome Sequencing, 2011). A transcriptome analysis of 32 potato tissues, including leaves inoculated with *P. infestans*, provided a database of tissue-specific expression of transcripts (Massa *et al.*, 2011). Moreover, the *P. infestans* genome has been sequenced, and is known to be ~240 Mb containing 17,797 protein-coding genes (Haas *et al.*, 2009). RNA-seq analysis in two *P. infestans* strains revealed transcriptome differences among life stages hyphae, sporangia, sporangia undergoing zoosporogenesis, motile zoospores, and germinated cysts (Ah-Fong *et al.*, 2017). Such genomic and transcriptome sequences of the plant and the pathogen are useful sources to understand the pathosystem and crop response to multiple stresses.

A large number of potato and *P. infestans* genes are up- or down-regulated during the infection process, and the total set of transcript changes is termed the interaction transcriptome (Birch *et al.*, 2003). Previously, microarray analysis has been the predominant technique used for transcriptome profiling during compatible and incompatible interactions (Sierra *et al.*, 2010; Wang *et al.*, 2005). However, due to its capacity in detecting low abundance transcripts, differentiating biologically critical isoforms, and allowing the identification of genetic variants the Next Generation Sequencing (NGS) of RNA (RNA-seq) techniques is now preferred over microarray analysis (Zhao *et al.*, 2014). Moreover, due to the continuous reduction of sequencing prices and improvements in data handling methods, most of the recent transciptome profiling studies are using RNA-seq techniques.

Large numbers of differentially expressed transcripts were identified in potato-*P. infestans* infection (Ali *et al.*, 2014). As pointed out by the authors, the different sets of expressed transcripts may indicate the biotrophic and necrotrophic phases of the infection processes. This type of output is important to identify resistance gene candidates in either or both phases of the interaction. Prior to transcriptome assembly, sequence reads of the pathogen from RNA-seq data of infected potato leaves could be extracted by mapping the dataset to the *P. infestans* genome and filtering sequence reads which mapped uniquely to the genome (**Paper III**). In doing so, RNA-seq identifies a set of RXLR effectors of *P. infestans* expressed in infected potato (Gao *et al.*, 2013).

2.5.2 Proteomics of the potato-*Phytophthora* pathosystem

Detection of more than 20,000 expressed genes in a transcriptome studies (Massa *et al.*, 2011) suggests that large number of proteins are involved in plant response to biotic and abiotic stresses. Proteomics studies identified life stage specific proteins of the pathogen, including the CRN2 (Ebstrup *et al.*, 2005) and phosphorylated proteins of RXLR and CRN effector families (Resjo *et al.*, 2014). Proteins involved in cell wall modifications, pathogenesis,

defence responses, and proteolytic process were identified (Meijer *et al.*, 2014). Grenville-Briggs *et al.* (2010) also identified appressorial and mycelial cell wall proteins, several of them classified as PAMPs and CRN effectors. Moreover, amino acid biosynthesis genes such as methionine synthase threonine synthase were with higher abundance in *P. infestans*, during appressorium formation and potato infection (Grenville-Briggs *et al.*, 2005). Such studies may provide valuable insights to understand establishment of infection process and pathogenicity of the pathogen, and may help to accurately predict developmental stages of the pathogen which could be targeted with chemical or biological control methods. However, the above studies have been performed on *in vitro* grown *P. infestans* and may not reproduce the proteome profile of the pathogen when grown *in planta*.

Potato proteins which are either involved in recognizing the pathogen or signalling transduction are localized in different compartments of the plant cell. The proteome of cell wall, cytoplasmic (Lim *et al.*, 2012) and apoplastic fluid (Ali *et al.*, 2014) components of potato leaf tissue have been profiled. Secreted proteins, containing N-terminal signal peptides, residing in the apoplastic fluid may play a crucial role in plant-pathogen interactions. The methods to isolate and identify these proteins have been discussed in a review by Alexandersson *et al.* (2013). Simultaneous isolation of the proteins in the cell wall, cytoplasm, and apoplastic fluid would be of great interest to get a more global proteome profile. Some proteins could be isolated whilst on their secretion pathway and contamination of a sample with materials from other compartments is inevitable. A differential centrifugation approach has been used to separately isolate cell wall and cytoplasmic proteins of the same potato leaf samples. However, even at low centrifugal forces (1500× g), cell wall proteins were contaminated by proteins from other organelles (Lim *et al.*, 2012). Due to tissue maceration after pathogen infection, a less disruptive and reproducible method should be used to isolate proteins in different sub-cellular compartments. For precise proteomics analysis of specific organelles, laser micro-dissection has been used to study layers of epidermal cells from *Arabidopsis* leaves (Faltert *et al.*, 2015). However, non-destructive *in planta* secretome sampling methods are yet to be developed.

2.6 BABA-induced resistance

Induced resistance can be described as potentiation of basal defence mechanisms of plants against biotic and abiotic stresses (Alexandersson *et al.*, 2016). A non-proteinaceous amino acid β-aminobutyric acid (BABA) is a potent inducer of plant defence against biotic and abiotic stresses (Jakab *et al.*, 2001). Application of BABA induces both local and systemic resistance in different pathosystems and reduced potential pathogen infection in potato (Alexandersson *et al.*, 2016; Bengtsson *et al.*, 2014a; Liljeroth *et al.*, 2010;

Olivieri *et al.*, 2009; Altamiranda *et al.*, 2008; Si-Ammour *et al.*, 2003). A combination of BABA with the commercial fungicide Shirlan can reduce the total fungicide usage by 20-25%, whilst still adequately protecting potato against late blight under field conditions (Liljeroth et al. 2010). Thus, BABA may play crucial role in reducing fungicide use against *P. infestans*.

The BABA induced resistance (BABA-IR) against pathogens is related to an elevated expression of basal defence system in plants, a process known as priming (Ton & Mauch-Mani, 2004). Treatment of Arabidopsis with BABA leads to a faster and higher expression of defence related genes from the salicylic acid pathway and plants show enhanced defences against *P. syringae* (Slaughter *et al.*, 2012). In addition, increased expression of Jasmonic acid-dependent genes, the (lipoxygenase, LOX-9; and PR-4), enhanced resistance of BABA-treated grapevine (*Vitis vinifera*) against the oomycete downy mildew (*Plasmopara viticola*) (Hamiduzzaman *et al.*, 2005). The phytohormones, jasmonic acid (JA) and salicylic acid (SA) are important in plant defence response to multiple stresses. BABA treatment also enhances general defence responses such as callose deposition and accumulation of PR1 in potato (Floryszak-Wieczorek *et al.*, 2015). Direct restriction of *P. infestans* growth after treatment of potato with BABA has also been observed (Floryszak-Wieczorek *et al.*, 2015; Bengtsson *et al.*, 2014a). Moreover, BABA-induced resistance transduces into the next generation as shown in potato (Floryszak-Wieczorek *et al.*, 2015) and its wild relative *S. physalifolium* (**Paper II)**. BABA-IR is inherited epigenetically; hence, the information from parents to offspring is transferred without changes in the DNA sequence. It has been reported that chromatin modification and DNA-methylation in Arabidopsis (Luna *et al.*, 2012; Slaughter *et al.*, 2012), play important role in transgenerational stability of BABA-IR against *Pseudomonas syringae*.

Potato cultivars show differences in efficacy of BABA-induced resistance against *P. infestans* (Liljeroth *et al.*, 2010; Olivieri *et al.*, 2009; Altamiranda *et al.*, 2008). The mechanisms of action, transgenerational stability of BABA-IR, and molecular determinants of genotype sensitivity to BABA treatment remain to be elucidated.

2.7 Effects of potato-*P.infestans* interactions on herbivore behaviour

Plant responses to pathogen attack involve activation of a myriad of defence signalling pathways. During the *P. infestans* infection process, a large number

of plant genes are up/down regulated, which may lead to changes in abundance of large number of proteins (Ali *et al.*, 2014; Bengtsson *et al.*, 2014b; Burra *et al.*, 2014). Furthermore, *P. infestans* infection alters the volatile ((E)-2-hexenal, 5-ethyl-2(5H)-furanone and benzene-ethanol) and non-volatile (phytoalexins, glycoalkaloids and phenolics) metabolite composition of potato plants (Laothawornkitkul *et al.*, 2010; Andreu *et al.*, 2001).

The changes in plant appearance due to infection, as well as the plant defense response can affect host plant choice and performance of insects. For instance, inoculation of potato with *P. infestans* alters host choice behavior of the polyphagous moth *Spodoptera littoralis*, which is an invasive pest species in southern Europe (**Paper IV**). The C2H2 zinc finger transcription factor induced by *P. infestans* in potato was also induced by the generalist tobacco hornworm (THW, *Manduca sexta* L.) and the specialist Colorado potato beetle (Lawrence *et al.*, 2014). Moreover, the tomato resistance gene (Mi-1 gene) is known to confer resistance to potato aphid (*Macrosiphum euphorbiae*), whitefly (*Bemisia tabaci*), and root-knot nematodes (*Meloidogyne* spp.) (Kaloshian, 2004). This indicates that there is a shared plant defence pathways against multiple biotic stresses. Therefore, it is crucial to extend pathogen resistance studies by assessing potential effects in non-target organisms.

3 Aim and Objectives

The overall aims of this thesis were 1) to enhance the molecular understanding of the interactions between *Solanum* species and *P. infestans*, by investigating transcriptome of the interaction, induced resistance against the pathogen in these species and evaluating the potential effects of the pathosystem on behaviour of a herbivore, and 2) to study the stress response of *Solanum* species growing in agricultural fields and natural conditions to help design and apply disease control measures. The specific objectives of this thesis were to:

- Evaluate the resistance to *P. infestans* of the three wild *Solanum* species in Sweden: *S. dulcamara*, *S. nigrum* and *S. physalifolium* (**Paper I**)

- Determine the direct effects and non-genetic inheritance of BABA-induced resistance against *P. infestans* in *S. physalifolium* (**Paper II**)

- Analyse the transcriptome of three wild *Solanum* species and three potato clones with varying resistance towards *P. infestans* (**Paper III**)

- Find out if potato-*P. infestans* interactions and introduction of an *Rpi*-gene into a crop affects host plant preference of the generalist insect herbivore, *Spodoptera littoralis* **(Paper IV)**

- Probe the level of activation of innate immunity in *Solanum* species growing in natural conditions and agricultural fields (**Paper V**)

- Investigate the apoplastic proteome of potato grown in greenhouse and field conditions, and identify putative peptide biomarkers to help link studies in these growing conditions (**Paper VI**)

4 Results and Discussion

4.1 *Solanum-P. infestans* interactions and response to stress in field condition

In order to study the interactions of different *Solanum* species with *P. infestans* and unravel the biology of host plant stress response under natural conditions, several experiments were performed (Figure 4). Seeds of three wild *Solanum* species (*S. dulcamara*, *S. nigrum* and *S. physalifolium*) growing in southern Sweden were collected, grown and screened for resistance against *P. infestans*. Using *S. physalifolium* as a study system, direct and transgenerational BABA-induced resistance was investigated. To understand *Solanum-Phytophthora* interactions transcriptome analysis was performed in the three wild *Solanum* species and three potato clones (Desirée, Sarpo Mira and SW93-1015) with varying resistance level against the pathogen. Furthermore, to understand the *Solanum* species response to stresses in natural conditions, the presence or absence of pathogenicity related (PR) proteins were used as markers to estimate the frequency of activation of innate immunity in the field. Finally, the apoplastic proteome of field and greenhouse grown potato (Bintje) was analysed using quantitative proteomics and activity based protein profiling (Figure 4), to get insight into functional status of the proteins.

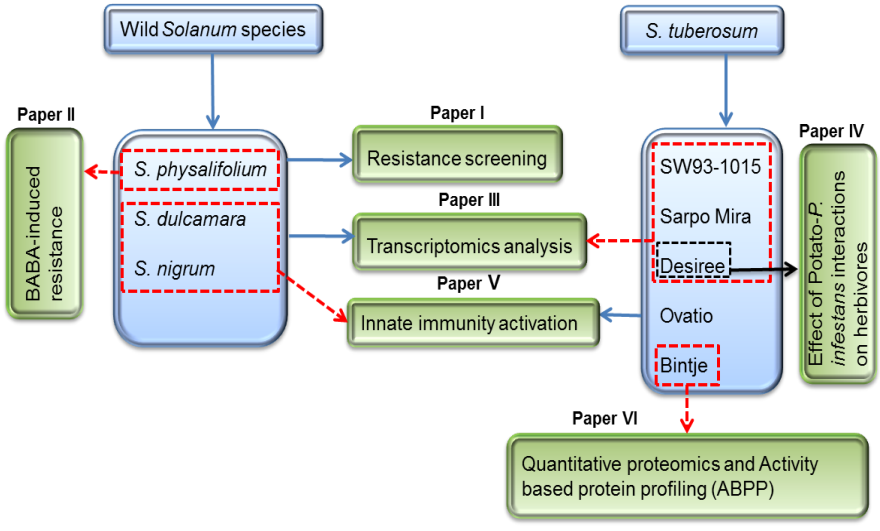

Figure 4. Overview of the relationship between plant material, experiments performed and papers in this thesis. The central aim of this work was to understand the interactions between *Solanum* species and *P. infestans*. Furthermore, activation of innate immunity as a proxy for a general response of plants to biotic stress in natural conditions was explored.

4.2 *Phytophthora* resistance in Swedish wild *Solanum* species (Paper I, II)

Phytophthora infestans is infamous as the causal agent of the great Irish famine in 1840's, and continues to cause huge yield losses in potato production, globally. Wild *Solanum* species are source of novel *P. infestans* resistance traits that can be used in potato breeding. The first step towards identifying the required resistance traits in these species for potato breeding programs is to identify which species act as alternate hosts of the pathogen. As a result of continuously evolving host-pathogen interactions, the unusually diverse *P. infestans* population structure found in Sweden (Sjöholm, 2012) might lead to variable defence mechanisms in the *Solanum* species. Since the *Solanum* species present in Sweden are native to Northern Europe, the resistance properties of these plants could be well adapted to the colder conditions prevalent in northern Europe. Therefore, we investigated resistance to *P. infestans* in the three wild *Solanum* species: *S. dulcamara*, *S. nigrum* and *S. physalifolium* growing in natural habitats in southern Sweden (**Paper I**).

To ensure sustainability of resistance, it is crucial to augment one host resistance source with other resistance genes or with other mechanisms such as induced resistance. Plants with induced resistance show enhanced or earlier activation of defence reactions against subsequent pathogen infection attempts. A non-proteinaceous amino acid β-aminobutyric acid (BABA) can induce plant resistance against multiple pathogens, including potato resistance against *P. infestans* (Bengtsson *et al.*, 2014a; Liljeroth *et al.*, 2010). However, all the previous studies on BABA-induced resistance were performed on cultivated plants. Studying the role of BABA in resistance induction in wild species, directly collected from natural populations, is crucial to understand the importance of the induced resistance in nature. Furthermore, the study of this resistance inducer in a natural system will allow us to assess the evolution of induced resistance. Therefore, we have investigated if BABA can induce resistance against *P. infestans* and the transgenerational effect of this BABA-IR in *S. physalifolium* (**Paper II**). This wild *Solanum* species is susceptible to the pathogen (Gronberg *et al.*, 2012; Sjöholm, 2012) with no known resistance to *P. infestans* (**Paper I**).

We collected seeds of *S. dulcamara*, *S. nigrum* and *S. physalifolium* from plants growing in natural habitats in southern Sweden. Ten accessions from three populations of *S. physalifolium* (**Paper I** and **Paper II**), 164 accessions of *S. dulcamara* from 12 populations, and 75 accessions from 20 *S. nigrum* populations were used in this study (**Paper I**).

Resistance tests against *P. infestans* were performed using both detached leaf assays and field assessments (**Paper I** and **Paper II**). After removing leaves for detached leaf assays, plants were acclimatized for one-week in greenhouse and transferred into the experimental garden located at Alnarp (55° 66'N, 13° 08'E). Experiments were performed at this garden in 2012, 2013 and 2014. During each experiment quantitative (lesion area) and qualitative (resistance classification) measurements were taken at 7 days after inoculation with *P. infestans* (**Paper I**).

Resistance screening with material from controlled conditions showed that lesions were significantly larger in *S. physalifolium* compared to those in *S. nigrum* and *S. dulcamara* (**Paper I**, Figure 3). Moreover, all the *S. physalifolium* accessions showed a susceptibility reaction (S phenotype) to *P. infestans* (**Paper I**, Figure 4). In 2013 we planted 48 clonally propagated plants of two *S. physalifoium* genotypes at the experimental garden. These plants were naturally heavily infected by *P. infestans.* These plants shed infected

leaves, but rather than succumbing to full infection and subsequent death of the plant (the typical response of susceptible potato cultivars), these *S. physalifolium* were able to regrow new leaves (**Paper I**, Figure 2). Shedding of the blighted leaves and regrowth in this species can be a defence mechanism against the pathogen. In 2003, it was reported for the first time that *S. physalifolium* is an alternative host of *P. infestans* in Sweden (Andersson *et al.*, 2003). This finding was later followed by a study that shows *P. infestans* strains maintained on *S. physalifolium* leaves have higher aggressiveness upon subsequent infection of potato than the same strains maintained on potato leaves (Gronberg *et al.*, 2012). Our study was the first to screen the resistance of accessions from different populations of *S. physalifolium*. In the field experiment in 2013, all the *S. physalifolium* plants were heavily infected, which is in agreement with the previous studies (Gronberg *et al.*, 2012; Sjöholm, 2012). Defoliation of blighted leaves could be survival mechanism but the fallen leaves may increase local soil inoculum highlighting the significance of *S. physalifolium* in *P. infestans* epidemiology in Sweden. Given the susceptibility of this plant to *P. infestans*, we have used *S. physalifolium* to study transgenerational stability of BABA-induced resistance (**Paper II**).

In contrast to *S. physalifolium*, all 75 *S. nigrum* accessions were either asymptomatic (R phenotype) or developed a necrotic lesion smaller or equal to the point of inoculation (R^N phenotype) (**Paper I**, Figure 4 and 5). *S. nigrum* was not naturally infected by *P. infestans* in 2012, which indicates that in Sweden this species has a high level of resistance to *P. infestans*. *S. nigrum* was referred to in previous studies as a non-host of *P. infestans* (Vleeshouwers *et al.*, 2000; Platt, 1999; Pieterse *et al.*, 1994). So far no *P. infestans* isolate from southern Sweden has been shown to infect *S. nigrum* (Andersson *et al.*, 2003), however, isolates of the pathogen that can infect *S. nigrum* have been found in Poland (Lebecka, 2008). *R*-gene based resistance and the presence of putative *R*-genes was reported previously in this species (Lebecka, 2008; Flier *et al.*, 2003). Moreover, we identified *R*-gene like sequences in transcriptomic analysis of *S. nigrum-P. infestans* interactions (**Paper III**). This indicates that this species may be a good source of novel resistance alleles against *P. infestans*, which may be potentially mined for use in European breeding programs.

In 2012, we found diverse resistance phenotypes in *S. dulcamara*. This diversity was consistently noted both in material from controlled conditions and from the experimental garden. In addition to the three phenotypes described earlier (R, R^N, and S) we identified a fourth resistance phenotype (S^L- a lesion larger than the point of inoculation) (**Paper I**, Figure 2) in *S. dulcamara*. Despite identification of these diverse resistance phenotypes, no

natural infections were observed in *S. dulcamara* during the seasons 2012-2014 in the Alnarp experimental garden. This is consistent with previous reports that it is a rare event to find natural *P. infestans* infections in *S. dulcamara* (Flier *et al.*, 2003; Cooke *et al.*, 2002). In comparison to *S. physalifolium*, the lesions in *S. dulcamara* were smaller, even in the most susceptible interaction.

Due to the diverse resistance phenotypes observed in the *S. dulcamara* accessions, we expanded the experiment by adding new accessions in 2013. The new accessions were screened for resistance against *P. infestans* in controlled conditions and then transferred into the experimental garden. In total, in 2013 and 2014, we screened 164 *S. dulcamara* accessions in the experimental garden, representing 34 sibling groups, obtained form 12 populations in southern Sweden (**Paper I**, Figure 1). The results show that indeed there are four resistance phenotypes (R, R^N, S^L, and S) in *S. dulcamara* however the proportion of these phenotypes changed across the years (**Paper I**, Figure 5). Sibling accessions of this species showed different resistance phenotypes (**Paper I**, Table 2). Moreover, in *S. dulcamara*, the lesion size was negatively correlated with many of the performance parameters measured in 2013 at the experimental garden (**Paper I**, Figure 8).

In a previously reported resistance screening of European *S. dulcamara* accessions, it was rare to find both resistance and susceptible accessions within the same collection site (Golas *et al.*, 2010b). Therefore, the resistance variation in our material that shows differences within a population, and even between sibling accessions, is highly novel. *S. dulcamara* is an out-crossing species Golas *et al.* (2010a). Thus the differences in resistance to *P. infestans* within the sibling groups (plants generated from seeds of the same wild parent) could be a result of the out-crossing nature and recombination ability of this species (**Paper I**). The diverse resistance phenotypes in this species may also indicate that diverse resistance mechanisms against *P. infestans* are present in *S. dulcamara* (Huang *et al.*, 2005; Vleeshouwers *et al.*, 2000). Previously in this species, two loci containing putative resistance genes, *Rpi-dlc1* and *Rpi-dlc2*, were mapped to chr-9 and chr-10 respectively (Golas *et al.*, 2013; Golas *et al.*, 2010b). By isolating genomic DNA from one of the accessions, and designing primers based on the predicted NBS-LRR gene sequence, we have cloned the putative gene and confirmed that it has 100% sequence similarity with the reference BAC-sequence. Functional analysis of this putative resistance gene (*Rpi*-gene) against *P. infestans* is currently underway.

P. infestans contains fast evolving effector genes, located in a highly dynamic and expanded region of its transposon rich genome (Haas *et al.*, 2009). The two-speed architecture of the genome allows the pathogen to carry out faster adaptive evolution (Dong *et al.*, 2015). As a result, beyond using host

resistance, it is important to understand other control mechanisms that can be used to develop integrated late blight disease management systems in potato. For example, the use of BABA can reduce the need for so many fungicide applications since it has been suggested that this BABA-induced resistance can be used in combination with other management practices to reduce potential effects of *P. infestans* (Liljeroth *et al.*, 2010). To understand this induced resistance we introduced *S. physalifolium* as a study system in relation to plant resistance inducers (**Paper II**). Since the plant material is directly collected from natural populations it is also important to understand the basic biology of this induced resistance.

To find out if BABA can induce resistance against *P. infestans*, *S. physalifolium* plants were sprayed with BABA and three days later tested for resistance against the pathogen using detached leaf assays (**Paper II**). Using *Arabidopsis thaliana,* (Slaughter *et al.*, 2012) showed a transgenerational effect of BABA-induced resistance against the bacterial pathogen, *Pseudomonas syringae*. To test for a similar transgenerational stability of BABA-induced resistance against *P. infestans* in wild species, seeds were collected and used to generate offspring from the sprayed and unsprayed *S. physalifolium* plants. These plants were then tested for resistance against the pathogen (**Paper II**).

BABA treatment significantly reduced lesion area in two of the three genotypes (**Paper II,** Figure 2A and 2B). As previously reported in cultivated potato (Bengtsson *et al.*, 2014a; Liljeroth *et al.*, 2010), BABA can induce resistance against *P. infestans* in *S. physalifolium*. Seeds of BABA treated genotypes that showed direct BABA-induced resistance as well as control plants, were collected and used to generate next generation plants (S1). Before BABA treatment, these S1 plants were tested for resistance against *P. infestans* and descendants of one of the genotypes showed higher levels of resistant to *P. infestans* than descendants of control plants (**Paper II,** Figure 3), suggesting a transgenerational effect of BABA treatment. It was also previously reported that potato cultivars showed different degrees of inducibility after BABA-treatment (Liljeroth *et al.*, 2010). We hypothesize that the differences in BABA responsiveness among *S. physalifolium* plants might be due to differences in the surface structure and chemistry of the leaves (Balmer *et al.*, 2015; Pastor *et al.*, 2014). BABA-induced resistance can be transferred into the next vegetative progeny of potato (Floryszak-Wieczorek *et al.*, 2015) and in bacteria inoculated descendants of Arabidopsis generated from seeds of treated plants (Slaughter *et al.*, 2012). Our study, for the first time, confirmed that BABA can induce resistance against *P. infestans* in a wild species directly originated form seeds in wild populations. Furthermore, this induced resistance can be transferred into the next generation. Induction of plant defence typically involves changes in gene expression, protein abundance and metabolites, and thus IR may have a fitness cost (Alexandersson *et al.*, 2016). However, BABA-induced resistance did not affect seed traits (weight of berries and weight per

seed) under field conditions, in our study. This indicates that it may not significantly affect the fitness of *S. physalifolium* plants (**Paper II**). Further studies on this wild species may give meaningful insight into the molecular mechanisms of BABA-induced resistance in economically important crops; especially with regards to susceptibility genes and the potential role of induced resistance in nature.

4.3 Understanding the *Solanum-P. infestans* interactions and effects on a generalist moth (Paper III and IV)

Screening of the wild *Solanum* species for *P. infestans* resistance showed that *S. physalifolium* is susceptible and *S. nigrum* is resistant whereas *S. dulcamara* showed variation in resistance to *P. infestans* (**Paper I**). To enhance our understanding of the plant response in the three wild *Solanum* species and cultivated *S. tuberosum* against *P. infestans*, transcriptome analysis was performed on inoculated leaves of these species (**Paper III**).

Based on the *P. infestans* resistance screening using detached leaf assays one individual accession from each wild *Solanum* species, and three potato clones with varying resistance levels against the pathogen were selected for transcriptome analysis (**Paper III**). RNA was isolated from this material and RNA-seq data generated using next generation sequencing (Illumina HiSeq2000 platform). Sequence reads belonging to *P. infestans* were identified by mapping our obtained sequence data to the publically available *P. infestans* genome using TopHat2 (Kim *et al.*, 2013), and were analyzed separately. The remaining RNA-seq data was *de novo* assembled using trinity (Grabherr *et al.*, 2011) (**Paper III**).

To identify transcript families expanded or depleted in response to the pathogen both quantitative and qualitative groups were created. The quantitative group was assembled from sequence related to a gradient vector based on the results of the resistance assay and the qualitative group from sequences related to resistant vs. susceptible species and clones. Significantly different numbers of transcript families was found depleted in the resistance vs susceptible groups as well as among the wild *Solanum* species and the three potato clones (**Paper III**). Furthermore, functional annotation of the expanded transcript families using Gene ontology (GO) for functional characterisation analysis (Li *et al.*, 2003), identified terms associated to plant defense. GO terms such as protein phosphorylation, defense response, hypersensitive response, host programmed cell death induced by symbiont and aspartic-type endopeptidase activity were expanded in the resistance group (**Paper III**; Figure 4; Figure S2). Expanded transcript families in the susceptible group were populated by terms such as lipoprotein biosynthetic process, signal transduction, protein acylation, transmembrane transport and nitrogen biosynthesis (**Paper III,** Figure4; Figure S2). A putative susceptible factor, the

cytokinin-regulated kinase 1 (CRK1) (Schafer & Schmulling, 2002) was found in this group. However, in the susceptible group we also found a few enriched GO terms associated with plant defence response, which might reflect the basal PAMP-triggered immunity (PTI) present in the susceptible hosts (Jones & Dangl, 2006). In addition, transcript families containing susceptibility factors and genes were identified in the susceptible group.

The *Solanum* genome contains hundreds of resistance gene (*R*) homologues, mostly containing the nucleotide-binding-leucine-rich-repeat (NB-LRR) domains (Witek *et al.*, 2016; Andolfo *et al.*, 2014; Jupe *et al.*, 2013). Therefore, we mined our data for *R*-gene like sequences to find out the number and type of these genes expressed during the *Solanum-Phytophthora* interactions. By identifying sequences containing NB-LRR domains (Jupe *et al.*, 2012) and BLAST analysis to the 112 reference *R*-genes from the Plant Resistance Genes database (Sanseverino *et al.*, 2013); different types of *R*-gene homologues (CNL, TNL, RLP, and RLK) were identified in the wild *Solanum* accessions and potato clones (**Paper III**, Table 3). The results indicate that it is possible to identify potentially functional resistance genes against *P. infestans* (*Rpi*-genes), by employing RNA-seq based transcriptome analysis of infected leaves.

We also analyzed sequences in our dataset that were identified as from *P. infestans* in order to find the pathogenicity factors used by the pathogen to promote disease in these different host species and clones. Analysis of the *P. infestans* transcripts identified a total of 7769, 2612, 1471, 892, and 73 *S. physalifolium*, *S. nigrum*, SW93-1015, Desirée, and *S. dulcamara*, induced transcripts respectively (**Paper III**, Table 5). *P. infestans* uses pathogencity factors, RXLR and crinkler effectors, to colonize and manipulate host cells. Different numbers of RXLR effectors, Crinkler effectors (CRN) and elicitins were identified in our dataset (**Paper III**, Table5; Table S8). However, the number of *P. infestans* transcripts and pathogenicity factors identified did not follow the resistance gradient. For instance, the number of putative pathogenicity factors identified in the dataset from the resistant hosts (*S. nigrum* and SW93-1015) was larger than in the dataset from the susceptible cv. Desirée (**Paper III**, Table 5). This might highlight different resistance mechanisms and strategies of the pathogens to overcome these resistances.

The perception of *P. infestans* leads to signaling transduction and phenotypic changes, which include changes in the metabolic profile of the plant. In the transcriptome analysis study (**Paper III**), we identified *R*-gene like sequences and putative *P. infestans* pathogenicity factors. During potato-*Phytophthora* interactions, large numbers of transcripts and proteins show differential abundance (Ali *et al.*, 2014). Moreover, *P. infestans* infection alters the volatile and non-volatile profiles of potato (Laothawornkitkul *et al.*, 2010; Andreu *et al.*, 2001). To reduce potato yield losses caused by *P. infestans* introduction of

R genes is considered as one of the most powerful management strategies (Fry, 2008). However, studies investigating the effects of inoculation with *P. infestans* and introduction of *R*-genes into potato on off-target organisms, such as insects are lacking. Therefore, we studied the effects of *P. infestans* inoculation and introduction of *Rpi-blb1*, from the *Solanum bulbocastanum*, against *P. infestans* on behavioral responses of the generalist insect herbivore *Spodoptera littoralis*, which is invasive in several ecosystems (**Paper IV**).

Oviposition preference of adult *S. littoralis* for either *P. infestans*-inoculated or uninoculated (control) plants of cv. Desirée and *Rpi-blb1* containing Desirée (A01-22) was tested in net cages. Female oviposition preference was tested in the following four two-choice bioassays: 1) inoculated vs uninoculated Desirée, 2) inoculated vs uninoculated clone A01-22, 3) cv. Desirée vs clone A01-22, both uninoculated, and 4) cv. Desirée vs clone A01-22, both inoculated. For the larval performance test, leaves of *P. infestans*-inoculated and uninoculated plants of cv. Desirée were used to feed first instar larvae. Egg and larvae weight data was collected to make host-preference and larva performance comparisons (**Paper IV**).

We confirmed that the *Rpi-blb1* gene confers resistance against the *P. infestans* strain SE-03058 (**Paper IV**). In the two-choice test between inoculated and uninoculated cv. Desirée, as well as inoculated and uninoculated A01-22, a significantly higher proportion of *S. littoralis* eggs were laid on the *P. infestans* inoculated plants (**Paper IV**, Figure 2). Previously it has been reported that in potato, *P. infestans* infection may lead to changes in the volatile and non-volatile profiles of potato (Laothawornkitkul *et al.*, 2010; Andreu *et al.*, 2001). Therefore, these volatile differences between inoculated and non-inoculated plants may explain the preference of the *S. littoralis* females oviposit on inoculated plants. A two-choice test between uninoculated plants of Desirée and A01-22 did not show significant differences, indicating importance of the pathogen infection and/or plant response to the infection on host choice behavior of the *S. littorlais*. After *P. infestans* inoculation, a significantly higher percentage of eggs were found on the inoculated susceptible cv. Desirée than on the inoculated A01-22 (**Paper IV**, Figure 3). The cv. Desirée and A01-22 are genetically identical except for the introduced *Rpi-blb1* gene into A01-22. Major *Rpi*-gene-based resistance leads to effector-triggered immunity (ETI), and *Rpi-blb1* gene expression increases after *P. infestans* inoculation, this specific defence response is lacking in the susceptible cv. Desirée (Jones & Dangl, 2006). So that, we can conclude that introduction of the *Rpi*-gene into potato affects the host preference of *S. littoralis* only after inoculation of the plant with *P. infestans*.

In spite of the host preference difference to *P. infestans* inoculated and non-inoculated cv. Desirée, there was no larval weight differences between these hosts, which may indicate that the oviposition site decision of *S. littoralis* may

not be directly associated with performance (**Paper IV**, Figure 4). As shown in a meta-analysis (Gripenberg *et al.*, 2010), host preference may not be associated with performance especially in generalist insect species. Our study showed that use of *Rpi*-genes to control late blight disease in potato production might also reduce the generalist insect herbivores infestation.

4.4 Activation of *Solanum* defence response in greenhouse and field conditions (Paper V, VI)

In nature and in agricultural fields, plants are continuously interacting with a multitude of above- and below-ground microbial populations and are at the same time challenged by abiotic stresses. However, most of the studies aimed at understanding the plant-microbe interactions are conducted in laboratory conditions. The predictability of laboratory based studies for field performance remains questionable. Moreover, understanding the activation of plant innate immunity in agricultural fields is crucial to allow informed decisions, for example, to maximise the efficiency of control methods. Therefore, we studied the activation of innate immunity in *Solanum* species growing in agricultural fields and natural populations (**Paper V**). To increase the predictability of studies conducted under controlled conditions we compared the apoplastic proteome of potato plants grown in greenhouse and field conditions (**Paper VI**).

To investigate the status of plant innate immunity activation, we isolated over 500 apoplastic samples of non-diseased potato (from agricultural fields) as well as wild *S. dulcamara* and *S. nigrum*, using the protocol described previously (Andreasson *et al.*, 2017; Alexandersson *et al.*, 2013). The samples were collected from three consecutive years, June-August, in Sweden. Apoplastic fluid was cleaned and proteins separated by SDS-PAGE (**Paper V**). The presence of pathogenesis-related PR proteins (PR1, a marker for salicylic acid (SA) induction; and PR2+3, as markers for Jasmonic acid (JA) related pathways), were used to indicate plant immunity activation in the gel analysis.

Generally, our results showed immunity-activation in only 36.4% of all the field samples (**Paper V,** Figure 1A). PR1 was present in 16.5% and PR2+3 in 32.7% of the samples. This low PR presence frequency directly contrasts with predictions from the zig-zag model based on laboratory data. Such predictions suggest that PTI should be frequently expressed in the presence of PAMPs, and that since plants are continuously exposed to microbes, they would be continually exposed to PAMPs in the field (**Paper V**). We found an increased presence of PR1 later in the growing season (**Paper V**, Figure 1C). This could reflect an increasing density of microbes and thus increased presence of the PAMPs that trigger PTI as previously hypothesised in (Copeland *et al.*, 2015).

Comparisons among the species showed that there was higher presence of PR2-3 in *S. dulcamara* than in *S. tuberosum* or *S. nigrum* (**Paper V**, Figure 1B). *S. dulcamara* can be attacked by herbivores and the higher presence of these PR proteins may reflect induction of JA by such attacks (Kazan & Lyons, 2014), even if we did not detect visible symptoms on any of the leaf material collected. The presence of PR1 was more common in the *S. tuberosum* plants grown in agricultural fields than the wild *Solanum* species collected from natural populations (**Paper V**, Figure 1A). This might suggest that induction of JA due to insect biting in wild populations has reduced the SA based defense response. Antagonistic interaction between JA and SA mediated defence signaling pathways was previously reported in the resistance of Arabidopsis to Cucumber Mosaic Virus (Takahashi *et al.*, 2004). Thus, such antagonism is likely to exist in other plant species such as members of the *Solanum* Genus.

In this study (**Paper V**), we used five potato clones that differ in resistance to *P. infestans*: Bintje = PTI; Desirée = PTI; Ovatio = PTI; Sarpo Mira = PTI + ETI; SW93-1015 = ETI + PTI. This sampling allowed us to investigate activation of different layers of innate immunity in asymptomatic potato growing in agricultural fields. The susceptible hosts are assumed to only be able to activate basal defence responses (PTI) since they either do not contain functional R gene alleles or contain alleles known to be overcome by all races of the pathogen. In contrast, the resistant hosts are presumed to be able to activate both PTI and ETI. Although we suggested that activation of ETI will be increased due to the presence of *P. infestans* in surrounding fields, the presence of PR proteins, in our gel analysis was similar between the susceptible and resistant groups. Based on the above results, we propose an extension of a zig-zag model depicting innate immunity activation in plants growing in natural conditions. The model denoted 'Pyramide' suggests that activation of innate immunity (PTI/ETI), in plants growing in field conditions, can be suppressed by effectors from the infecting pathogen (ETS). It can also be actively or passively down-regulated in the absence of disease (**Paper V**, Figure 2).

Most molecular studies aimed to understand plant resistance to biotic and abiotic stresses are conducted in controlled conditions. But, how well can these controlled experiments predict plant performance in (agricultural) field conditions? Therefore, we compared the apoplastic proteomes of potato plants grown in both greenhouse and field conditions using quantitative proteomics and activity based protein profiling (ABPP) (Figure 5). Apoplastic fluid was isolated by vacuum infiltration-centrifugation (Andreasson *et al.*, 2017; Alexandersson *et al.*, 2013) and used for quantitative analysis or activity based protein profiling (ABPP). To generate quantitative proteomics data, apoplastic proteins were in-solution digested with trypsin and subjected to mass spectrometry. The generated data was used for peptide identification and quantitative analysis to determine peptide abundance (Ali *et al.*, 2014; Burra *et*

al., 2014). Part of the apoplastic fluid was used to label the active proteome in an ABPP assay. This technique uses fluorescence probes to irreversibly bind to the active residue of distinct protein classes, and proteins can be detected using SDS-PAGE and in-gel fluorophore scanning (van der Hoorn *et al.*, 2011). To identify the active proteins detected in ABPP, proteins were co-immunoprecipitated with Streptavidin beads, displayed by SDS-PAGE, subjected to in-gel tryptic digestion and then analyzed by mass spectrometry. A comparison between peptides identified in quantitative and ABPP-LC/MS/MS was also performed (Figure 5). In order to create a basis for identification of robust peptide biomarkers, we investigated differences in the apoplastic proteome of potato growing in two experimental sites (Mosslunda and Borgeby) in Southern Sweden. We also investigated the effects of fungicide treatment over two years at the Mosslunda site, and since we collected samples across the growing season (June-July-August), we were able to compare seasonal changes within and between locations. (**Paper VI**).

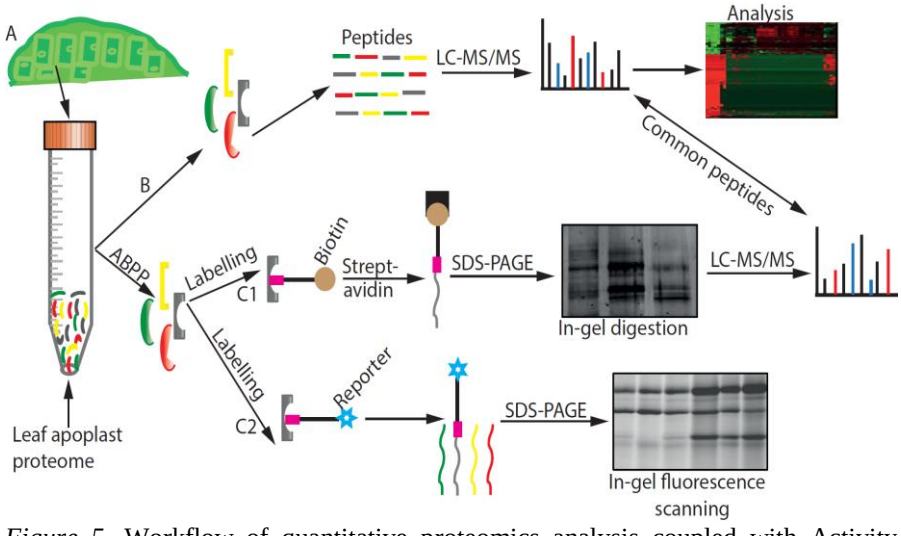

Figure 5. Workflow of quantitative proteomics analysis coupled with Activity Based Protein Profiling (ABPP). (**A**) apoplastic fluid was isolated by vacuum infiltration-centrifugation and aliquoted until used for quantitative analysis or ABPP. (**B**) Apoplastic proteins were in-solution digested with trypsin and subjected to mass spectrometry. (**C1**) Part of the apoplastic fluid was used to label the active proteome in an ABPP assay using fluorescence probes followed by detection of the proteins through SDS-PAGE. (**C2**) Characterization of the active proteins detected in-**C1** was performed using biotinylated probes. (**Paper VI**). Illustration by Kibrom B. Abreha.

Field-grown and greenhouse-grown samples grouped separately in a principal component analysis (PCA), and a large number of peptide/proteins showed

different abundance in both conditions (**Paper VI**, Figure 2). MapMan pathways analysis (Thimm *et al.*, 2004), showed that a large number of the proteins associated with plant responses to biotic and abiotic stresses were in higher abundance in field than greenhouse conditions (**Paper VI**, Figure 3). A similar result was reported in a recent comparative proteomics analysis of Arabidopsis plants grown in field and controlled conditions (Ruhe *et al.*, 2016). Among the proteins found with higher abundance in field condition were 63 proteins associated with proteolysis, 40 proteins involved in cell wall synthesis or degradation, 19 proteins classified as pathogenesis related proteins (PR-proteins), 15 peroxidases and 25 proteins involved in defence signalling.

Fungicide application is the most common strategy to reduce yield losses due to pathogens. Foliar application may, affect the apoplastic proteome of the plants. These effects may be direct or indirect, (e.g.by changing the microbial population in the phyllosphere). To understand the fungicide effect, a two-way comparison was performed but no differentially abundant proteins was detected between fungicide treated and untreated plants (**Paper VI**, Data not shown).

Samples from two growing sites, Mosslunda and Borgeby, were grouped together in the PCA (**Paper VI**, Figure 5A). In agreement with this, in a PCA analysis an overlap of apoplastic proteome samples from plants growing in two different sites was reported (Ruhe *et al.*, 2016). Despite the PCA showing similarity between the samples, we were still able to identify 314 peptides from 234 proteins that were differentially abundant in the growing sites (**Paper VI**, Supplementary Figure S3). In evaluating the year effect, in spite of an overlap, samples from the same year grouped together (**Paper VI**, Figure 5B). 205 peptides corresponding to 156 proteins showed differential abundance between 2011 and 2012 in Mosslunda (**Paper VI**, Supplementary Figure S3).

A multi group comparison was also performed and this revealed that 320 peptides from 240 proteins were differentially regulated in at least one month of the June-July-August in a growing season. This allowed us to identify peptides and/or proteins co-regulated across the growing season. Furthermore, expression profile clustering analysis identified co-regulated proteins across the season and showed that 108 peptides increased in abundance in July and August compared to the early season sample in June (**Paper VI**, Figure 6C).

The above quantitative analysis gives information about the relative abundance of a given peptide or protein in a proteome sample. In order to find out if some of these proteins were functionally active, we used activity based protein profiling. Serine and glycosyl hydrolase protein families are commonly found in the apoplast (Alexandersson *et al.*, 2013) and both families were among the most abundant in our samples (**Paper VI**, Figure 1). Therefore, using ABPP we studied the activity profile of serine hydrolase and β-glucosidase proteins in

the apoplast. Our analysis showed a differential activity state of these protein families in field and greenhouse samples (e.g. the presence or absence of some proteins; or changes in the intensity of the protein signals) between field and greenhouse samples (**Paper VI**, Figure 7). To further characterize these proteins, protein bands were excised from SDS-PAGE and subjected to mass spectrometry analysis (**Paper VI**, Figure 8). This analysis detected a group of serine hydrolases: P69E (PGSC0003DMP400056894), P69E (PGSC0003DMP400007008), P69F (PGSC0003DMP400006964), peroxidase (M1AY17), Subtilase (PGSC0003DMP400011990) and Carboxypeptidase (PGSC0003DMP400054112) were among the active proteins in the samples (Paper VI, Figure 8). Moreover, member of the β-glucosidase family such as Beta-galactosidase (PGSC0003DMP400004621), Beta-glucosidase (PGSC0003DMP400015895), Beta-mannosidase (PGSC0003DMP400009956) and Alpha galactosidase (PGSC0003DMP400018078) were also detected as active proteins (Paper VI, Figure 8).

To identify potential peptide biomarkers we used three different approaches. Firstly we combined the results from our quantitative proteomics and ABPP-LC/MS-MS assays. Using this approach, we were able identify peptides that were both differentially regulated between field and greenhouse and that were were functionally active (**Paper VI**, Table 4). Secondly, we wanted to identify biomarkers that show stability across different conditions, to be able to more accurately predict plant performance in both growing conditions. Thus, we mined our data for peptide biomarkers that are were not differentially abundant in greenhouse or field conditions, and that showed stability across different conditions. Quantitative comparisons were made between field and greenhouse conditions, two growing sites, and between years and months within a growing season in the same experimental site. The analysis identified 750 stable peptides (**Paper VI**, Supplementary Table S3). We defined field physiology markers as peptides that were higher in abundance in the field than in the greenhouse, and the abundance of which, was not dependent on year or growing site.

5 Conclusions and future perspectives

In this thesis, *Solanum* species growing in Sweden were evaluated for resistance against *P. infestans* and BABA-induced resistance was studied in one of these wild species. Transcriptome analysis was performed to enhance our understanding of the interactions between *Solanum* species and the pathogen. We studied the effects of potato inoculation with *P. infestans* on the host choice behaviour and performance of *S. littoralis*. Moreover, the stress response of the *Solanum* plants growing in both natural and field conditions was investigated by predicting the activation of innate immunity and identifying active apoplastic proteins as biomarkers of these responses. The main conclusions and future perspectives are:

- The wild *Solanum* species growing in Sweden vary widely in resistance against *P. infestans*. *S. physalifolium* is susceptible and *S. nigrum* is resistant against the pathogen whereas there is a wide resistance-variation among *S. dulcamara* accessions.

- The diverse resistance phenotypes identified in *S. dulcamara* suggest variable resistance reactions against *P. infestans*. Further characterization of the resistance phenotypes using cytological methods would provide invaluable insights into the specific mechanisms of pathogen infection and host defence.

- BABA can induce resistance against *P. infestans* in *S. physalifolium* (a naturally susceptible host). This induced resistance can be transferred into the next generation. However, direct and transgenerational BABA-induced resistance is dependent on host genotype. Molecular studies of the mechanisms of action of BABA and identification of factors that determine the differences in BABA-inducibility among the

genotypes are needed in order to consider application of BABA-induced resistance to enhance plant defence responses.

- Unravelling how the BABA-induced resistance is carried into the next generation, i.e. understanding the mechanism of epigenetic inheritance, would help facilitate potential use of plant materials with induced resistance in agricultural production. In the future, this strategy may be applied together with host resistance or with reduced fungicide applications.

- Transcriptome analysis of *P. infestans* inoculated leaves of three wild *Solanum* species and potato clones (Desirée, SW93-1015, and Sarpo Mira) with varying resistance levels towards the pathogen revealed transcript families expanded or depleted depending on whether the interaction was resistant or susceptible. Furthermore, different numbers of *R*-gene like sequences and *P. infestans* transcripts were identified reflecting the variability of interactions between the pathogen and different hosts. Characterization of potential genes related to plant resistance and specific effectors from pathogen can be useful to identify molecular targets of the pathogen used to promote host invasion.

- Potato-*P. infestans* interactions can alter the host choice behaviour of a generalist moth *Spodoptera littoralis*. Introduction of *Rpi*-genes from wild *Solanum* species into potato can reduce the load of *P. infestans* as well as *S. littoralis*. Studies on the effects of *Solanum*-*P. infestans* interactions and introduction of cloned *Rpi*-genes on other herbivores and microbes are advised in order to increase applicability of the host resistance to broad range of natural enemies. Furthermore, identification of the volatile cues involved in the tri-trophic interaction can be an important input for example in pulling away the herbivore from potato fields.

- In contrast to the perception that plants can constantly activate defence response in natural conditions, only one-third of the *Solanum* plants growing either in agricultural fields or in natural populations showed activation of innate immunity. Generally, the rate of innate immunity activation is higher towards the end of the season which may be linked with increased PAMPs pressure later in the growing season. Surveying the above and below-ground (micro)biota may further elucidate the

nature of this innate immunity activation in agricultural fields and nature.

- Associating the differential innate immunity activation in field conditions across the growing season would be crucial to further connect it with efficiency of disease control measures, for example application of defence inducers or fungicides.

- A large number of proteins related to plant response to biotic and abiotic stresses increased in abundance in field conditions compared to greenhouse conditions. Serine hydrolases and β-glucosidases, showed differential activity profiles in greenhouse and field conditions as well as across the growing season within the same field. Functional analysis of the proteins with differential abundance in different growth conditions may shed light into the possible biological role of the identified proteins in plant response to these conditions.

- Peptide biomarkers with potential roles in predicting plant performance in field and greenhouse conditions have been identified. Confirming the applicability of these peptides in predicting plant performance would provide valuable insights into the concept of application of peptide biomarkers. To do this, further studies screening a larger number of genotypes are needed to make accurate predictions of plant performance in field and controlled conditions.

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