Defence related molecular signalling in Potato

New perspectives from “- Omics”

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Defence related molecular signalling in Potato. New perspectives from “- Omics”.

Abstract

Potato production is hampered by several pathogens and is subjected to intense chemical based disease control, use of which also has undesirable consequences. Resistance breeding programs have also shown limited success. Hence, there is a need to develop durable disease resistance. Omics-techniques enable new layers of knowledge regarding molecules and their interactions mediating defence, which can contribute to identification of durable resistance sources.

A novel network-based approach was used to improve the existing annotation of gene probes on the genome based microarray. Approximately 8000 unannotated probes received a new annotation. This improved annotation was used to assess genome wide changes in transcripts and proteins in response to treatments with resistance inducers, β- amino butyric acid (BABA) and Phosphite based salt (Phi). Five thousand transcripts were significantly regulated 48 hours after 10 mM BABA treatment while one was regulated with 1 mM BABA. In coherence, 10 mM BABA but not 1 mM induced protection to the hemibiotroph *Phytophthora infestans*. No transcript was significantly regulated 48 hours after Phi treatment. Time course analysis revealed that Phi exerts a transient effect, as significant transcriptomic changes were observed only 3, 6 and 11 hours after treatment. In contrast, plants showed resistance to *P. infestans* even at 120 hours after Phi treatment. Phi and BABA dependent “Induced state” is not restricted to transcripts related to plant defence, as transcripts related to abiotic stress and primary metabolism were altered, while biotic stress and cell wall related proteins also increased in abundance.

Furthermore, an *in vitro* based blackleg disease screening assay was developed to investigate Potato – *Dickeya solani* interactions. We show that salicylic (SA) and COI1 are necessary for defence in shoots and tubers to this necrotroph. We also screened a crossing population and identified “potential” *D. solani* susceptibility genes related to transcriptional regulation. We also show that while SA is necessary to restrict lesion development and pathogen growth in response to the necrotroph *Alternaria solani*, COI1 affects pathogen growth only. Transcriptomic analysis indicated that rapid defence response to *A. solani* involves biotic, abiotic and oxidative stress related transcripts regulated by SA and COI1. We identified a citrate binding protein, which is also induced by resistance inducers, as an SA-repressed susceptibility factor to *A. solani*. Finally, proteomics of PAMP triggered immunity revealed upregulation of oxidative stress proteins while proteins related to oxidative stress tolerance, GTP binding activity were specifically upregulated in effector triggered immunity interactions.

Keywords: Phosphite, *Phytophthora*, Transcriptomics, Proteomics, *Alternaria*, *Dickeya*, SA, JA, PTI, ETI

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Dedication

Dad, Mom, Yogya and Saraswathi, this one´s for you!

"KARMANYE VAADHIKA RASTE
MAA PHALESHU KADACHANA
MAA KARMA PHALAHE TURBHURMA
TE SANGOSTVA KARMANE"

'You only have the right to work, but not to its fruits.
Let not the fruits of action be your motive, nor let your attachment be to inaction'

- Ch.2, Verse 47, THE BHAGVAD GITA

Contents

List of Publications 8

1 Introduction 12

2 Background 14
2.1 Potato cultivation and challenges 14
2.1.1 Late blight disease 18
2.1.2 Early blight disease 19
2.1.3 Issues with current method of Early and Late blight disease control 20
2.1.4 Blackleg disease 22

2.2 Opportunities 24
2.2.1 Induced resistance 24
2.2.2 Genetic sources of resistance 28
2.2.3 Durable disease resistance facilitated by “Omics” based understanding of plant defence 32

3 Aims and Objectives 35

4 Results and Discussion 37
4.1 BABA and Phosphite (Phi) mediated induced resistance (Paper I and II)
4.1.1 BABA and Phi treatment reduces *P. infestans* infection in Potato 37
4.1.2 Phi distribution *in planta* 38
4.1.3 Improved annotation of gene probes spotted on the genome-based Potato microarray 39
4.1.4 Transcriptomic and proteome (secretome) activity associated with induced resistance 40

4.2 Improved elucidation of plant defence in order to identify genetic sources of resistance (Paper III- Paper VI)
4.2.1 Development of an *in vitro* assay to screen for blackleg disease 44
4.2.2 Identification of potential blackleg disease susceptibility factors based on trait association with RNAseq data of a crossing population in Potato 46

4.2.3 Role of salicylic and jasmonic acid dependent COI1 in Blackleg and Early blight disease 49
4.2.4 Comparative proteomics of PAMP (PTI) and Effector triggered immunity (ETI) in Potato 55

5 Conclusions
5.1 BABA mediated induced resistance 62
5.2 Phi mediated induced resistance 62
5.3 Potato-*Dickeya solani* interactions 63
5.4 Potato-*Alternaria solani* interactions 64
5.5 Comparative proteomics of PAMP (PTI) and Effector triggered immunity (ETI) in Potato 65

6 Future Perspectives 66

References 70

Acknowledgements 83

List of Publications

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

I Bengtsson, T., Weighill, D., Proux-Wéra, E., Levander, F., Resjö, S., **Burra, D.D**., Moushib, L.I., Hedley, P.E., Liljeroth, E., Jacobson, D. and Alexandersson, E., 2014. Proteomics and transcriptomics of the BABA-induced resistance response in Potato using a novel functional annotation approach. *BMC genomics*, *15*(1): 315

II **Burra, D.D**., Berkowitz, O., Hedley, P.E., Morris, J., Resjö, S., Levander, F., Liljeroth, E., Andreasson, E. and Alexandersson, E., 2014. Phosphite-induced changes of the transcriptome and secretome in Solanum tuberosum leading to resistance against Phytophthora infestans. *BMC plant biology*, *14*(1),:254.

III **Burra, D. D**., Mühlenbock, P., Andreasson, E. (2015), Salicylic and jasmonic acid pathways are necessary for defence against *Dickeya solani* as revealed by a novel method for Blackleg disease screening of *in vitro* grown Potato. *Plant Biology*, *17*: 1030–1038

IV **Burra, D.D**., Kushwaha, K.S., Alexandersson. E., Mühlenbock. P., Andreasson, E. Potential blackleg disease susceptibility factors revealed in potato by RNAseq analysis of a crossing population (Manuscript)

V Odilbekov, F*****., **Burra, D.D***., Rosahl, S., Hedley, PE., Morris, J., Zeigler, J., Liljeroth, E., Andreasson, E. Positive role of Salicylic acid COI1 signaling in early response to *Alternaria solani* in Potato (Manuscript)

VI **Burra, D.D**., Lenman, M, Levander, F, Resjo, S., Andreasson, E, Comparative proteomics of three different immunity reactions in Potato (Mansucript)

Papers I-III are reproduced with the permission of the publishers.

* Equally contributing authors

The contribution of Dharani Dhar Burra to the papers included in this thesis was as follows:

I Contributed in the development of the Parallel OrthoMCL algorithm. Analysed the results and wrote the manuscript together with co-authors.

II Planned and performed the experiments. Analysed the data and wrote the manuscript together with co-authors.

III Planned and performed the experiments. Analysed the data and wrote the manuscript together with co-authors.

IV Planned and performed the experiments. Analysed the data and wrote the manuscript together with co-authors.

V Performed a part of the experiments, analysed the data and wrote the manuscript together with co-authors.

VI Planned and performed the experiments. Analysed the data and wrote the manuscript together with co-authors.

Related articles that Dharani Dhar Burra has been a part of but are not included in this thesis.

I. **Burra, D.D**., Vetukuri, R.R., Resjö, S., Grenville-Briggs, L.J. and Andreasson, E., 2016. RNAseq and Proteomics for Analysing Complex Oomycete Plant Interactions. Current issues in molecular biology, 19:73-88

II. Liljeroth, E., Lankinen, Å., Wiik, L., **Burra D.D**., Alexandersson, E., Andreasson, E., Potassium phosphite combined with reduced doses of fungicides provides efficient protection against Potato late blight in large-scale field trials. Submitted.

1 Introduction

Potato is the fourth largest crop in terms of area under cultivation and is the third most consumed food crop in the world. It is relatively easy to cultivate, and it is well adapted to grow in most parts of the temperate and subtropical world. Potato also offers balanced combination of nutrients sufficing human dietary requirements (King and Slavin, 2013). In comparison to major food crops, Potato requires the lowest volume of water to produce 100 kcal of energy (Water, 2009). Potato is therefore ideally suited to feed the rapidly growing population. However, achieving this full potential has been curtailed due to major losses incurred by various diseases, hence subjecting the crop to intense fungicide treatment (Osteen and Fernandez-Cornejo, 2013). Although chemical based protection has contributed in maintaining the amounts of Potato worldwide for consumption, excessive and continuous use has also lead to undesirable effects such as high cost and energy consumption, increased health risks, emerging pathogen resistance etc. Furthermore, efficient chemical control does not exist for diseases like Potato blackleg. Consequently, alternative methods for disease protection and genetic sources of durable resistance need to be developed.

The objective of this thesis is to improve understanding of molecular aspects of defence and immunity in Potato. This improved knowledge can significantly contribute to development of alternative and durable disease resistance strategies. Technical and analytical improvements in "-Omics" based approaches and the Potato genome sequence have offered the possibility for improved understanding of defence and immunity (Visser et al., 2014). One line of investigation in this thesis was aimed at transcriptomic analysis of Potato plants treated with two different resistance inducers, β-amino butyric acid (BABA) and Phosphite based salts (Phi). These compounds have previously been shown to activate plant defence and induce protection to *Phytophthora infestans*; however knowledge about transcripts and proteins that

mediate this resistance in Potato is limited. In addition, defence-related proteins secreted in response to resistance inducer treatment were analysed using a proteomics approach.

Another line of research in this thesis deals with improving the current understanding of the role of plant hormones, salicylic (SA) and jasmonic acid (JA) in molecular defence responses to blackleg disease caused by bacteria *Dickeya solani* and early blight disease caused by the fungi *Alternaria solani* in Potato. In relation to *Dickeya solani*-Potato interactions, work in this thesis describes the development of an *in vitro* disease screening assay which enables large scale screening of blackleg disease symptoms. This work was further built upon by combining the *in vitro* system with transcriptomics and trait association, performed on a crossing population to identify potential susceptibility factors that play a role in rendering Potato plants susceptible to blackleg disease. With regards to *Alternaria solani*-Potato interactions, we have identified a susceptibility factor, and elucidated the role of SA in defence responses to necrotrophic pathogens. The final part of research work in this thesis is related to employing proteomics to identify proteins that are involved in mediating PAMP triggered immunity (PTI) and effector triggered immune (ETI) responses in Potato. In this study we identified proteins regulated by both PTI and ETI responses, proteins specifically regulated by PTI and ETI were also identified. In addition, comparative investigation of proteins regulated by ETI interaction of different resistance (R) – avirulence (Avr) gene pairs that has not been performed previously, was also performed.

Findings from the above studies have provided systems-level information of Potato defence and immunity in response to economically important pathogens. Through these studies, numerous molecules associated with defence and immunity have been identified. This enhanced molecular information could benefit plant resistance breeding and protection strategies.

2 Background

2.1 Potato cultivation and challenges

Potato is a tuber bearing crop that belongs to the genus *Solanum* in the *Solanaecae* family. In addition to Potato, this genus also contains other economically important crops such as tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*). Earliest records indicate that Potato is native to the Andes region spanning from eastern Venezuela to northern Argentina and to the lowland regions of south-central Chile. Potato was domesticated in the Andes region of Southern Peru 10,000 years ago, that led to the formation of various groups of *Solanum tuberosum*, such as the diploid group of Phureja and the tetraploid group Andigena (Ames and Spooner, 2008, Bradshaw and Ramsay, 2005). During the domestication process, the Andigena group was adapted for tuber production in long day conditions in Chile, while Phureja group was selected for faster tuber development for cultivation in the Andes region (Bradshaw and Ramsay, 2005). Cultivation of Potatoes outside South America was first carried out in the Canary Islands in Europe (Figure 1). Potato became popular, rapidly spread into rest of Europe (Figure 1) and further on into rest of the world. Currently, it is the fourth largest food crop in terms of growing area (Mullins et al., 2006). Potato production over the past 50 years has been comparatively steady in the developed world. This is in contrast to the developing world, where there has been a distinct increase in Potato production (Figure 2A) (Gastelo et al., 2014). At least until 2005, Potato production in the developing world showed an increasing trend in comparison to other crops such as maize and rice (Figure 2B) (Walker et al., 2011). An increasing trend in production combined with the large proportion of the world´s population in the developing world has made Potato, the third most important food crop in terms of global consumption (Gastelo et al., 2014).

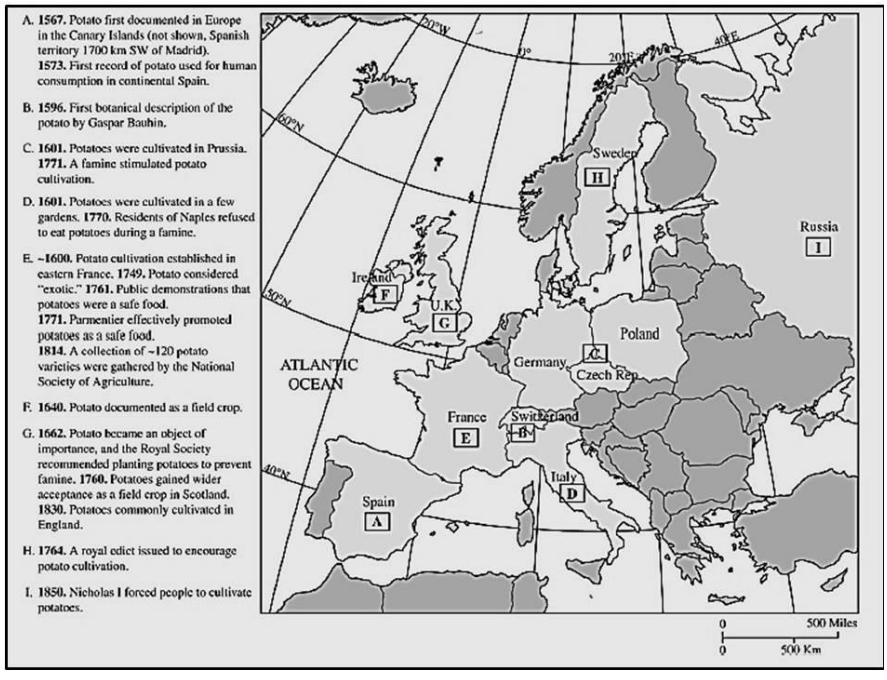


Figure 1. Overview of Potato introduction in Europe. Republished with permission from American Journal of Botany from "DNA from herbarium specimens settles a controversy about origins of the European Potato", Ames and Spooner, American Journal of Botany 95.2 (2008). Represented with permission from the Botanical society of America.

A number of reasons render Potato production and consumption different from other major food crops. It is grown over 125 countries across the world (Mullins et al., 2006), and it has a short growth season and hence offers the flexibility of crop rotation (Gastelo et al., 2014). In comparison to other food crops, Potato needs less volume of water to produce 100 kcal of nutritional energy (Figure 3A) (Water, 2009). This makes it an ideal crop in the current scenario, wherein the world is simultaneously faced with challenges of population growth and water scarcity.

In comparison to major food crops, Potato also offers improved and balanced nutrition (as indicated by higher completeness score in Figure 3B), such as higher levels of dietary intake of amino acid lysine and minerals like phosphorous and potassium (Figure 3B). Potatoes also produce the most amount of starch per hectare than any other food crop; it is also second to soybean in terms of the amount of protein produced per hectare (OERKE, 2006).

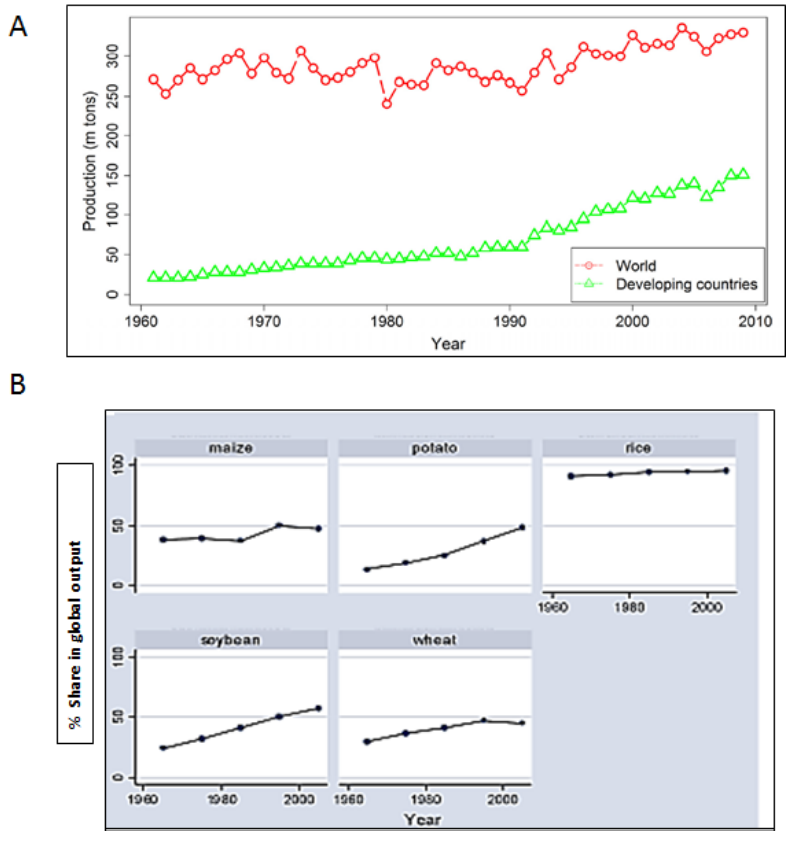


Figure 2. (A) Global Potato production between 1961 – 2009 (Manuel Gastelo, Ulrich Kleinwechter and Merideth Bonierbale. 2014. Global Potato Research for a Changing World. International Potato Center (CIP), Lima, Peru. Working Paper 2014-1. 43 p © International Potato Center (CIP), 2014). (B) % Share of developing country output to global Potato production in comparison to major crops (Walker, T., Thiele, G., Suarez, V. and Crissman, C. 2011. Hindsight and foresight about Potato production and consumption. International Potato Center (CIP), Lima, Peru. Social Sciences. Working Paper 2011-5. 43p. © International Potato Center (CIP), 2011).

This great potential of feeding the growing population however has been curtailed due to crop loss caused by pathogen attacks. Potato crop is susceptible to pathogen attack at every stage of its growth. A large number of fungal, bacterial and viral species can cause severe damage to the crop. In the United States, 50 fungal, 10 bacterial and 30 viral species have been identified to cause economic losses in Potato production (Oerke et al., 2012). These problems are complex to combat because Potato is a vegetatively propagated crop.

A

Product	Average water need per kg product	Average water need per 100 Kcal
Rice	1,111 litres	80 litres
Corn	870 litres	25 litres
Wheat	1,429 litres	45 litres
Potato	200 litres	20 litres
Apples	333 litres	67 litres
Olives	500 litres	43 litres
Beef	16,667 litres	741 litres

B

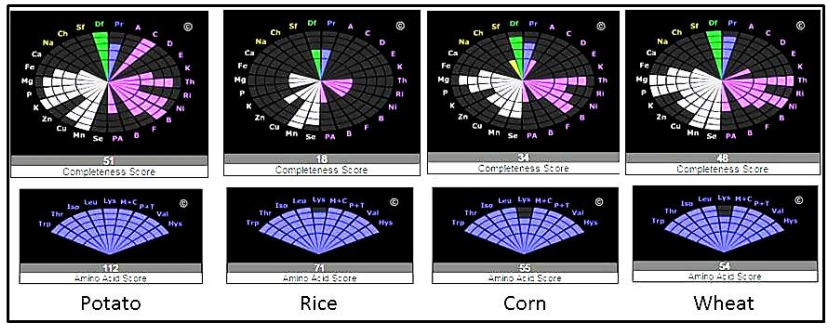


Figure 3. (A) Comparison of average volume of water required as input for some of the major food items; UNESCO world water development report 2009. (B) Nutrition information of major food crops Top panel represents mineral content while bottom panel represents amino acid content. Data obtained from http://nutritiondata.self.com/.

The actual and estimated loss potential due to pathogens has been calculated to be much higher than other food crops (Figure 4A) (OERKE, 2006). Almost 24% of Potato crop in Northwest Europe and 50% in central Africa is estimated to be lost due to pathogen damage. A major proportion of this damage has been linked to late blight disease caused by the oomycete *Phytophtora infestans* and early blight disease caused by *Alternaria solani* (OERKE, 2006). The fact that both these diseases affect Potatoes grown worldwide (Figure 4B) makes them a global concern.

A

Crop	Attainable production [M t]	Weeds		Animal pests		Pathogens		Viruses	
		Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual
Wheat	785·0	23·0 (18–29)	7·7 (3–13)	8·7 (7–10)	7·9 (5–10)	15·6 (12–20)	10·2 (5–14)	2·5 (2–3)	2·4 (2–4)
Rice	933·1	37·1 (34–47)	10·2 (6–16)	24·7 (13–26)	15·1 (7–18)	13·5 (10–15)	10·8 (7–16)	1·7 (1–2)	1·4 (1–3)
Maize	890·8	40·3 (37–44)	10·5 (5–19)	15·9 (12–19)	9·6 (6–19)	9·4 (8–13)	8·5 (4–14)	2·9 (2–6)	2·7 (2–6)
Potatoes	517·7	30·2 (29–33)	8·3 (4–14)	15·3 (14–20)	10·9 (7–13)	21·2 (20–23)	14·5 (7–24)	8·1 (7–10)	6·6 (5–9)
Soybeans	244·8	37·0 (35–40)	7·5 (5–16)	10·7 (4–16)	8·8 (3–16)	11·0 (7–16)	8·9 (3–16)	1·4 (0–2)	1·2 (0–2)
Cotton	78·5[a]	35·9 (35–39)	8·6 (3–13)	36·8 (35–41)	12·3 (5–22)	8·5 (7–10)	7·2 (5–13)	0·8 (0–2)	0·7 (0–2)

B

Pathogen	Common name	Principal regions affected
Bacteria		
Erwinia Carotovora	Blackleg, softrot	Worldwide
Ralstonia solanacearum	Wilt, brownrot	Asia, Africa and South America
Clavibacter michiganensis subsp. sepedonicus	Ring rot	Worldwide
Streptomyces scabies	Commn scab	Worldwide
Fungi and Oomycetes		
Spongospora subterranea f. sp. subterranea	Powdery scab	Temperate regions
Synchytrium endobioticum	Wart	Worldwide
Alternaria solani	Early blight	Worldwide
Golovinomyces orontii	Powdery mildew	Latin america , Europé
Rhizoctonia solani	Black scurf	Worldwide
Macrophomina phaseolina	Charcoal rot	When soil temperature exceeds 28°C
Boeremia foveata	Gangrene	North America, Europé, Asia and Oceania
Fusarium spp.	Dry rot, wilt	Worldwide
Phytophthora infestans	Late blight	Worldwide

Figure 4. (A) Higher potential and actual pathogen induced % crop loss calculated for Potatoes (marked in red; Crop losses to pests, Oerke 2006, The Journal of Agricultural Science. Representation with permission from Cambridge University Press) (B) List of major Potato diseases and the regions affected (Figure represented from Crop Production and Crop Protection: Estimated Losses in Major Food and Cash Crops, E. C. Oerke, H. W. Dehne, F. Schönbeck and A. Weber, 1994).

The focus of the present work is restricted to three of the diseases mentioned in Figure 4B, namely late blight disease (caused by *Phytophthora infestans*), early blight disease (caused by *Alternaria solani*) and blackleg disease (caused by *Erwinia carotovora* and the emerging *Dickeya species*).

2.1.1 Late blight disease

Late blight disease is a serious problem in Potato production; it is caused by the oomycete *Phytophthora infestans*. *P. infestans* was responsible for causing the Great Irish Famine in Ireland between 1845 and 1852, wherein a large part of the predominantly Potato dependent Irish population either starved to death or emigrated out of the country, since the entire Potato produce was wiped out by *P. infestans*. Over the last two decades, it has been responsible for causing approximately 3.5% loss to the total yield in the United States alone (Nowicki et al., 2011). *P. infestans* is a fast growing pathogen and if left unprotected an entire field can be destroyed within 7-10 days. The pathogen has a short life cycle that starts from sporangia produced in infected tissue. During favourable conditions such as temperature between 15°C – 20°C and high humidity, each sporangium releases numerous zoospores that infect and develop into mycelia;

these in turn germinate into sporangiophores containing a new set of sporangia. During unfavourable conditions, the pathogen can also develop mycelia from sporangia that will in turn eventually give rise to new sporangia. On the leaf, the infective zoospores form special structures called haustoria that penetrate through intercellular spaces and obtain nutrients from the host plant; this characterizes the biotrophic phase and occurs 2-3 days post infection. This phase is followed by a necrotrophic phase that is characterized by secretion of enzymes and toxins causing cell death, resulting in death of the plant (Figure 5A) (Nowicki et al., 2011). Use of resistant potato as a disease control measure has yielded limited success as a sustainable solution. Several reasons can be attributed to this; perhaps one key reason is the genome architecture of *P. infestans*. The genome architecture facilitates powerful adaptive evolution (Dong et al., 2015), allowing the pathogen to quickly adapt to many resistant plants. In cases where in the source of resistance is multi-genic, introgression of those resistant genes into elite cultivars often leads to linkage drag resulting in yield penalty. Furthermore, there are issues concerning conventional breeding, for instance the process is time consuming and laborious, making it difficult to develop and introduce resistant varieties quickly (Vleeshouwers et al., 2011). New strategies such as cis/trans-genesis, stacking of resistance genes in combination with genome selection and inbreed diploids offer opportunities for development of sustainable resistance. Despite this, fungicide use is still is the most common and effective method for disease control (Kromann et al., 2014).

2.1.2 Early Blight Disease

Early blight is a devastating disease that damages leaves and tubers of the Potato crop; it is caused by the fungi *Alternaria solani.* Yield losses to early blight vary enormously , however losses ranging between 20-50% in tuber yield have been generally reported (Odilbekov et al., 2014). Although the disease occurs at a wide range of temperatures with varying severity, high humidity is often a pre-requisite. Infection spreads via conidia, which form germ tubes that penetrate host epidermal cells through the formation of appressoria; alternatively the conidia can also form hypha that can enter the leaf through natural openings (Figure 5B) (Chaerani and Voorrips, 2006). However, unlike *P. infestans*, penetration into host tissue is quickly followed by production of toxins and chemicals that degrade host cell wall. Cell death lesions on leaves are characterized by concentric rings that are distinctly different from that of the symptoms caused by *P. infestans*. Lesions expand during the course of infection that ultimately leads to defoliation. Spores of *A. solani* have the ability to overwinter on infected crop debris that renders

disease control difficult. However, proper cultural practices such as removal of plant debris that hinders overwintering can help prevent disease occurrence and spread (Kemmitt, 2002). Breeding for resistant plant varieties has been hampered due to the absence of strong resistance sources or genetic markers. Several studies have shown that resistance to *A. solani* is either under the control of several genes (multi-genic) or is linked to important traits such as plant maturity and age (Chaerani and Voorrips, 2006). Due to these factors, chemical control via repeated fungicide application is routinely used and has been relatively efficient in controlling the disease.

2.1.3 Issues with current method of Early and Late blight disease control

A common theme that connects both late and early blight disease is the use of fungicides as an effective measure for disease control. This makes the Potato crop subject to intense chemical treatment, it is estimated that without chemical protection, 75% of Potato production would be lost to various diseases (OERKE, 2006). In the United States alone, Potato cultivation area under fungicide treatment increased from 24% in 1966 to 85-98% between 1994 and 2010, Potatoes in combination with fruits and vegetables accounted for 90% of fungicide use between 1966 and 2010 (Osteen and Fernandez-Cornejo, 2013). Although, chemical based protection has contributed in maintaining the availability of Potato for consumption worldwide, excessive usage has led to development of pathogen strains that are resistant to fungicides. For instance, in 2014, *blue-13* was the most predominant *P. infestans* genotype all across Europe (Figure 5C); this strain is particularly difficult to manage since it is resistant to phenylamide class of fungicides. With regards to *A. solani* too, genotypes with a mutation (F129L) in the *cytochrome b* gene, that renders them insensitive to Quinone outside inhibitors (QoI) fungicides have been identified in Europe, including Sweden (Leiminger et al., 2014, Odilbekov, 2015).

Excessive use of chemical based protection has raised concerns about health safety as well as economic and energy efficiency. These issues stand particularly exacerbated in the European context because 40% of total fungicide use worldwide on Potatoes occurs in Europe (Oerke et al., 2012). With regards to economic burden caused by fungicide use, it has been calculated that in the Netherlands, cost of fungicide use amounts to as high as 370 €/hectare (Haverkort et al., 2008). A 2014 estimate suggests that approximately 472 €/hectare is spent on fungicide treatment of Potato crops in Sweden (*Personal communication: D. Eriksson, U. Carlson-Nilsson, R. Ortíz and E. Andreasson*). High costs associated with fungicide use has also been

observed in developing nations too, a study in 2011-2012 determined that 27% of the cost in growing a hectare of Potato in Peruvian highlands is incurred due to fungicides (Kromann et al., 2014).

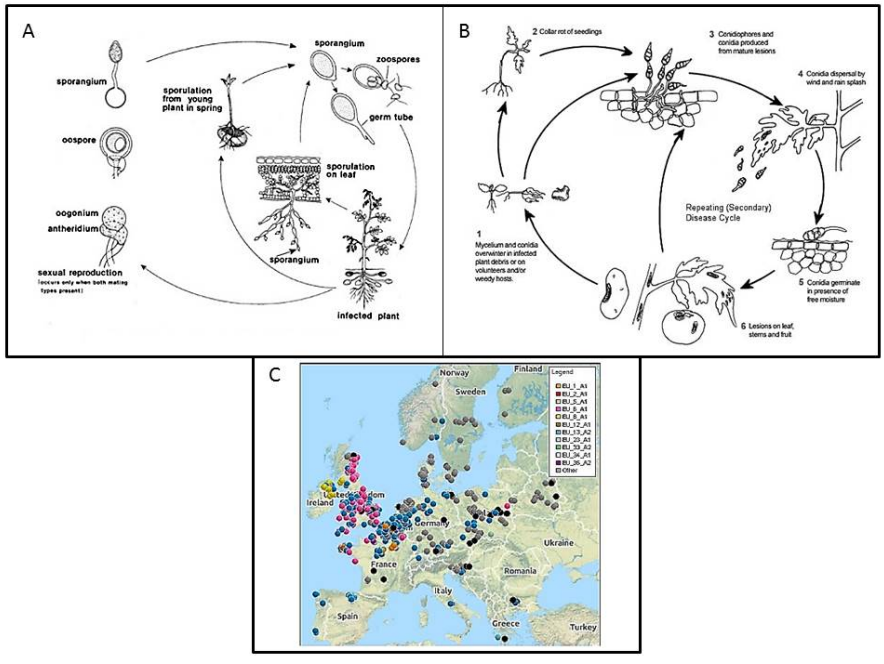


Figure 5. Life cycle of (A) *Phytophthora infestans* (Represented from Schumann, G.L., 1991. The Irish Potato famine and the birth of plant pathology. Plant Diseases: Their Biology and Social Impact. American Phytopathological Society, St. Paul, MN, pp.1-24, © 2016 The American Phytopathological Society), (B) *Alternaria solani* (Represented from Kemmitt, G., 2002. Early blight of Potato and tomato. The Plant Health Instructor, © 2013 The American Phytopathological Society), (C) Predominance of phenylamide resistant *blue-13* genotype (represented by blue spots) in *P. infestans* populations across Europe in 2014 (Fungicide resistance in Oomycetes, Yigal Cohen, Avia E. Rubin and Mariana Galperin, Unpublished data).

Furthermore, the excessive energy expenditure incurred by fungicide application is also unsustainable; a recent European Union report on Agriculture and Energy Efficiency suggested that Potato cultivation in Europe requires more number of energy saving measures with regards to plant protection in comparison to major crops such as cotton, sunflower and sugar beet that are also grown in Europe (de Visser et al., 2012).

Further damage due to constant fungicide use can however be prevented by development of alternative methods of disease protection such as induced resistance (Borges and Sandalio, 2015) or via improved use of molecular

information about plant resistance mechanisms in breeding programs, both of which will facilitate generation of plants with durable resistance (Visser et al., 2014).

2.1.4 Blackleg Disease

Among the most prevalent Potato diseases listed in Figure 4B, there are some diseases for which the only control method is avoidance. One such example is Blackleg disease caused by the bacteria belonging to the genera *Pectobacterium*, more recently however, blackleg disease outbreak in Northern Europe has been primarily caused due to the highly virulent bacteria belonging to the genera *Dickeya* (Czajkowski et al., 2011). Potato cultivation, especially in Europe has been facing severe economic losses due to this disease. For instance, 50% of field grown Potato between the years 2003 to 2010 in Switzerland have been rejected due to blackleg disease (Gill et al., 2014). In Israel, 20-25% reduction in yield was noted due to blackleg infections while growers in the Netherlands have reported 30M€ yearly losses due to this disease. In fact, as low as 40 bacterial cells per gram of Potato peel resulted in 15-30 % yield loss in field experiments in the Netherlands (Toth et al., 2011). Both *Dickeya* and *Pectobacterium* species have also been listed in the "Top10" most scientifically and economically important plant bacterial pathogens (Mansfield et al., 2012).

Blackleg causing bacteria are gram negative, pectinolytic bacteria, which spread via infected seed tubers, contaminated soil and field equipment or through irrigated water (Czajkowski et al., 2011). The bacteria start to enter tubers via natural openings, lenticels or wounds. Blackleg-causing bacteria require high humidity to develop. Although bacteria belonging to *Pectobacterium* genera can cause infection at milder temperatures, bacteria from the *Dickeya* genera have the ability to infect at temperatures as high as 28°C. Recent warm and humid weather conditions during summers in Europe have been linked to rise in blackleg disease incidence caused by *Dickeya*. Under favourable conditions, the bacteria multiply and produce pectinolytic enzymes that lead to rotting of the tuber tissue (Figure 6). During the course of infection, bacteria spread upwards into the shoot through the xylem. This spread is also accompanied by production of pectinolytic enzymes that leads to rot, blackening and decaying of the shoot tissue which characterizes typical blackleg symptoms (Figure 6) (Toth et al., 2011). Final stages of the infection are characterized by dampening off of aerial parts of the plant followed by collapse and death. Some reports have also indicated that during unfavourable

conditions, the bacteria have the ability to remain in a quiescent stage and not cause symptomatic infections (Pérombelon, 1992). In many cases, symptoms can be attributed to coordinated infection by both *Dickeya* and *Pectobacterium*, and it is difficult to distinguish infection caused by either of the bacterial species based on symptoms (Toth et al., 2011)

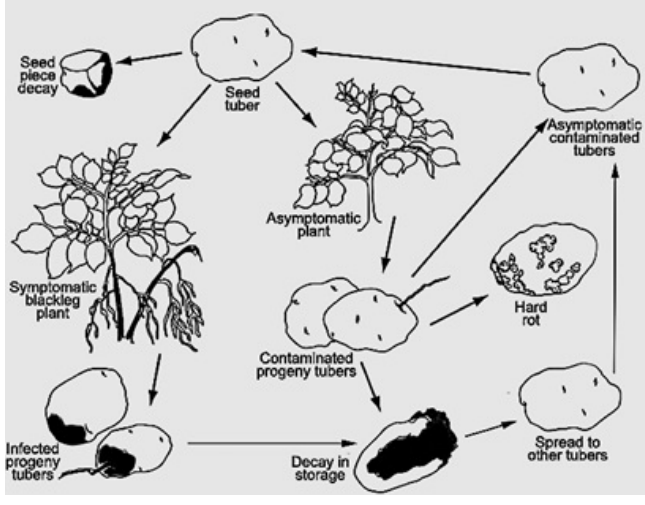


Figure 6. Life cycle of Blackleg-causing bacteria belonging to genera *Pectobacterium* and *Dickeya* (Represented from De Boer, S. H. 2004. Blackleg of Potato. The Plant Health Instructor, © 2016 The American Phytopathological Society).

Sound agricultural practices like the use of disease free seed lots as probably the main intervention, or sanitized equipment can minimize the risk of obtaining the disease. However, once infection is established, it is difficult to control the disease. Alternative disease control methods such as copper sprays, UV treatment of tubers have been tested but to a limited success (Charkowski, 2015). The situation is worsened since no resistant commercial cultivars exist (Czajkowski et al., 2011). Although, resistance to *Pectobacterium* has been observed in wild *Solanum* species, the process of introgression of resistance genes into cultivars has been hindered by several reasons. For example, there is minimal information regarding markers associated linked to disease resistance. In addition, it has also been observed that resistance to disease in the tubers and the shoot do not correlate (Czajkowski et al., 2011). To further add to the problems, development of disease symptoms is dependent on several abiotic factors such as humidity, temperature, soil nutrition status and biotic factors such as presence of antagonistic or oppotunistic microbes in the soil, leading to inconsistencies in disease development, these factors have therefore complicated disease screening (Charkowski, 2015). In fact, existing disease

screening methodologies are inconsistent, laborious and cannot be employed for screening large breeding populations (Rietman et al., 2014). A combination of these reasons, in addition to the overwhelming effect of pectinolytic enzymes produced during infection have resulted in limited molecular knowledge with regards to plant defence and resistance responses (Charkowski, 2015). In the existing scenario, wherein control methods are ineffective, there is a need to improve existing disease screening protocols that can enable better molecular understanding of plant defence responses, which will eventually catalyse the process of obtaining plants with improved resistance.

2.2 Opportunities

It is evident that although chemical based disease control has been effective, it also has led to several undesirable effects and concerns. In order to mitigate these, laws such as the European Union led IPM (Integrated Pest Management) directive (Directive 2009/128/EC) are being adopted, implementation of such laws will lead to limited availability and reduced use of fungicides. Therefore there is a need to explore and develop robust, durable alternative disease control strategies that are less-dependent/ independent on fungicide use.

The work presented here deals with two types of alternative disease control opportunities that are discussed in detail below.

2.2.1 Induced resistance

Induced resistance (IR) has shown the potential to contribute towards development of durable resistance. Numerous compounds, chemical or either biological in origin have the ability to activate and induce defence responses in plants, which further can lead to resistance upon pathogen attack (Walters et al., 2013). Since multiple defence processes tend to be activated, IR leads to protection against a broad range of pathogens. In practice, IR is protective rather than curative, plants upon treatment with resistance inducers show heightened immune activity, upon pathogen infection, the state of heightened immunity leads to stronger defence and resistance (Balmer et al., 2015). Since the efficiency depends on how plants respond to the resistance inducer, there is a strong impact of environmental conditions on activation of IR induced defence responses, this has been one of the reasons attributed to the large variation (20% - 85%) observed in IR mediated protection (Walters et al., 2013).

β- amino butyric acid (BABA), a non-proteinaceous amino acid has shown to induce resistance against various pathogens in several different plant systems with a great degree of success (Justyna and Ewa, 2013). BABA is structurally similar to γ-aminobutyric acid (GABA) (Figure 7), which is an inhibitory neurotransmitter in mammals. In contrast to GABA that is found in plants, BABA has not been detected in plants with the exception of few reports (Justyna and Ewa, 2013, Bengtsson, 2013). Use of BABA as a protective agent was first described in 1963 wherein BABA treated pea plants showed significant reduction in root rot symptoms caused by *Aphanomyces euteiches* (Jakab et al., 2001). Since then, BABA has been shown to provide protection against numerous diseases in many different plant systems both in lab and field studies (Liljeroth et al., 2010, Justyna and Ewa, 2013).

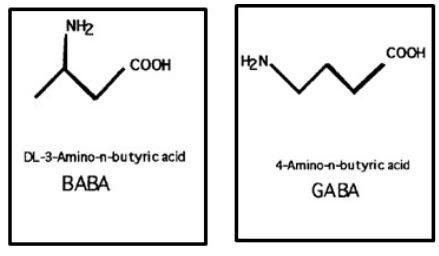


Figure 7. Molecular structures of β- amino butyric acid (BABA) and γ-aminobutyric acid (GABA) (Represented from Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J.P. and Mauch-Mani, B., 2001. β-Aminobutyric acid-induced resistance in plants. European Journal of plant pathology, 107(1), pp.29-37)

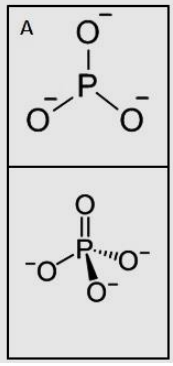
BABA also has been shown to directly inhibit some of these pathogens. Furthermore, depending on the plant species and the concentration used, BABA has also been shown to cause growth defects in plants (Jakab et al., 2001). Plants have been shown to activate an array of defence responses ranging from induction of reactive oxygen species to production of secondary metabolites in response to BABA treatment (Justyna and Ewa, 2013, Liljeroth et al., 2010). Post-infection, BABA treated plants have been shown to activate defence responses faster than untreated plants (Balmer et al., 2015).

Molecules and pathways that mediate BABA-dependent IR have been elucidated in greater detail in the model plant Arabidopsis. It has been suggested that BABA potentiates salicylic acid (SA) and abscisic acid (ABA) dependent defence responses that leads to increased PR-1 expression in response to *Pseudomonas syringae* and enhanced callose deposition in response to *Hyloporenospora arabidopsidis*, leading to improved resistance

(Ton et al., 2005). Recently, it has also been shown that BABA binds to *IBI1* (impaired in BABA-induced disease immunity) receptor that has an aspartyl-tRNA synthetase activity (Luna et al., 2014). It has also been shown in Arabidopsis, that descendants of BABA treated plants display enhanced defence to *Hyaloperonospora arabidopsidis* suggesting that BABA mediated induced resistance can be transgenerational (Slaughter et al., 2012). A similar transgenerational effect of BABA mediated IR against *Phytophthora infestans* was shown in the vegetative progeny of cultivated Potato (Floryszak-Wieczorek et al., 2015).

BABA treated Potato plants also show improved protection to *Phytophthora infestans* (CNHEN, 2002). Field studies have shown that BABA treated Potato plants display 40-50% reduction in lesion size in comparison to untreated plants (Liljeroth et al., 2010). Interestingly, BABA is also non-toxic to *Phytophthora infestans* (Justyna and Ewa, 2013), suggesting that the observed protection can be attributed to BABA dependent induction of Potato defence. In similarity to Arabidopsis, a functional SA regulated defence pathway has been shown to mediate BABA dependent induced response to *P. infestans* in Potato (Eschen-Lippold et al., 2010). Furthermore, it has also been shown that BABA treatment induces reactive oxygen species production, PR-1 and hypersensitive like response (HR-like) in Potato. In addition, differential phenotypic responses and phenolic composition were observed among Potato cultivars in response to BABA treatment (Bengtsson et al., 2014).

Phosphite (Phi) based salts have also been successfully used to induce resistance against several fungal but especially oomycete pathogens (Walters et al., 2013). Phi is structurally similar to Phosphate (Figure 8A), although phosphate is an important source of nutrition for plants; Phi is metabolically inert (Gómez-Merino and Trejo-Téllez, 2015). Soil bacteria however possess enzymes that convert Phi to phosphate (Lopez-Arredondo and Herrera-Estrella, 2012). Salts of Phi are sold commercially as fungicides, bio-stimulants and resistance inducers (Figure 8B) (Thao and Yamakawa, 2009).



B

Product	Company	Country	Active ingredient	Marketed as
Aliette	Bayer Cropscience	Germany	Aluminum phosphite	Fungicide
Nutri-phite	Biagro Western Sales	USA	Phosphite and organic acids	Fertilizer
Ele-Max	Helena Chemical	USA	Phosphorous acid	Foliar fertilizer
ProPhyt	Luxembourg-pamol	USA	Monopotassium phosphite	Systemic fungicide
Nutrol	Lidochem	USA	Potassium phosphite	Fertilizer and fungicide
Phostrol	NuFarm America	USA	Phosphorous acid	Biochemical pesticide
Agrifos	Liquid Fert Pty (Agrichem)	USA	Monopotassium phosphite	Fungicide
Foli-r-fos 400	UiM Agrochemicals	Australia	Monopotassium phosphite	Fungicide
Fosphite	Jh Biotech	USA	Monopotassium phosphite	Fungicide
Lexx-a-phos	Foliar Nutrients Inc	USA	Monopotassium phosphite	Fungicide
Trafos line	Tradecorp	Spain	Potassium phosphite	Fertilizer and defense stimulator
Phytos'K	Valagro	Italy	Potassium phosphite	Biostimulant (registered as EC fertilizer)
Phosfik line	Biolchem	Italy	Phosphorous acid	EC fertilizer
Fosfisan, Vigorsan	Agrofill	Italy	Potassium phosphite	Defense stimulator (registered as fertilizer)
Geros-K	L-Gobbi	Italy	Potassium phosphite	EC fertilizer
Kalium Plus	Lebosol	Germany	Potassium phosphite	EC fertilizer
Frutoguard	Spiess Urania	Germany	Potassium phosphite	EC fertilizer
Foliaphos	Plantin	France	Potassium phosphite	EC fertilizer

Source: Leymonie 2007. EC, European Commission (for identifying chemicals).

Figure 8. (A) Molecular structure of Phosphite (top panel) and Phosphate (bottom panel). (B) Commercial availability of Phosphite based salts (Represented with Permission from Thao, H.T.B. and Yamakawa, T., 2009. Phosphite (phosphorous acid): fungicide, fertilizer or bio-stimulator? Soil Science and Plant Nutrition, 55(2), pp.228-234)

This is because Phi has been shown to have a complex mode of action that consists of both a direct fungicidal effect and an indirect IR effect (Walters et al., 2013). A study on Phi-mediated IR in Arabidopsis against *Hyaloperonospora arabidopsidis* suggested that while at higher concentrations, Phi is directly toxic to pathogen growth, lower concentrations of Phi induce resistance. This study also identified that Phi induced IR is mediated via SA and is independent of jasmonic acid (JA), Abscisic acid (ABA) and ethylene (ET) pathways (Massoud et al., 2012). Although the above study did not identify induction of SA regulated PR1 in response to Phi before pathogen inoculation, PR1 was shown to be induced in non-inoculated Arabidopsis plants in response to Phi in another study, which investigated the protective effect of Phi against *Phytophthora cinnamoni* (Eshraghi et al., 2011). This discrepancy could be attributed to the different concentrations used in both the studies. In the same study, it was also shown that JA and ET defence gene markers were also induced in non-inoculated Arabidopsis plants in response to Phi treatment. Furthermore, using the Arabidopsis-*P. cinnamoni* patho-system, the authors also showed that Phi dependent IR is partially mediated by defence responses regulated by abscisic acid (ABA) and auxin (Eshraghi et al., 2014a, Eshraghi et al., 2014b).

Phi has also been shown to induce protection against *P. infestans* in both leaves and tubers of Potato. In addition, Phi treatment also protects Potato seed tubers against *Fusarium solani* and *Rhizoctonia solani* (Lobato et al., 2008). A later study revealed that this protection to *F. solani* correlated to higher amount of pectin in the tuber cortex, higher activity of polygalacturonase and proteinase inhibitor in the periderm of Phi treated tubers (Olivieri et al., 2012). Harvested

tubers from foliar sprayed Phi plants also showed reduced susceptibility to *Phytophthora infestans, Fusarium solani* and *Erwinia carotovora* (Lobato et al., 2011), although Phi levels were not measured in the tubers of Phi sprayed plants, the observed protection in the tubers could be due to translocation of foliar sprayed Phi into the tubers as shown in another study (Borza et al., 2014). A comprehensive analysis of proteins changing in abundance in leaves of Potato plants treated with Phi revealed that proteins related to SA dependent defence responses were induced, while proteins related to energy and carbohydrate metabolism were down-regulated. In contrast, fewer proteins were induced in response to *P. infestans* in Phi treated leaves suggesting that a major part of Phi-induced IR is dependent on defence gene activation after Phi treatment and before *P. infestans* infection (Lim et al., 2013).

Most of the above studies aimed at understanding BABA and Phi mediated induced resistance responses have yielded information about limited number of transcripts and proteins regulated in response to resistance inducer treatment , however in order to develop durable resistance strategies, a comprehensive systems analysis of molecular nature of induced resistance in Potato is required.

2.2.2 Genetic sources of Resistance

Plants have evolved a dynamic and a robust immune system that enables them to adapt and compete; plant-pathogen interactions are therefore characterized by competing survival strategies employed by both plants and their pathogens that is influenced by various environmental factors (Atkinson and Urwin, 2012). The plant immune system can be divided into two parts – PAMP triggered immunity (PTI) and Effector triggered immunity (ETI) (Figure 9).

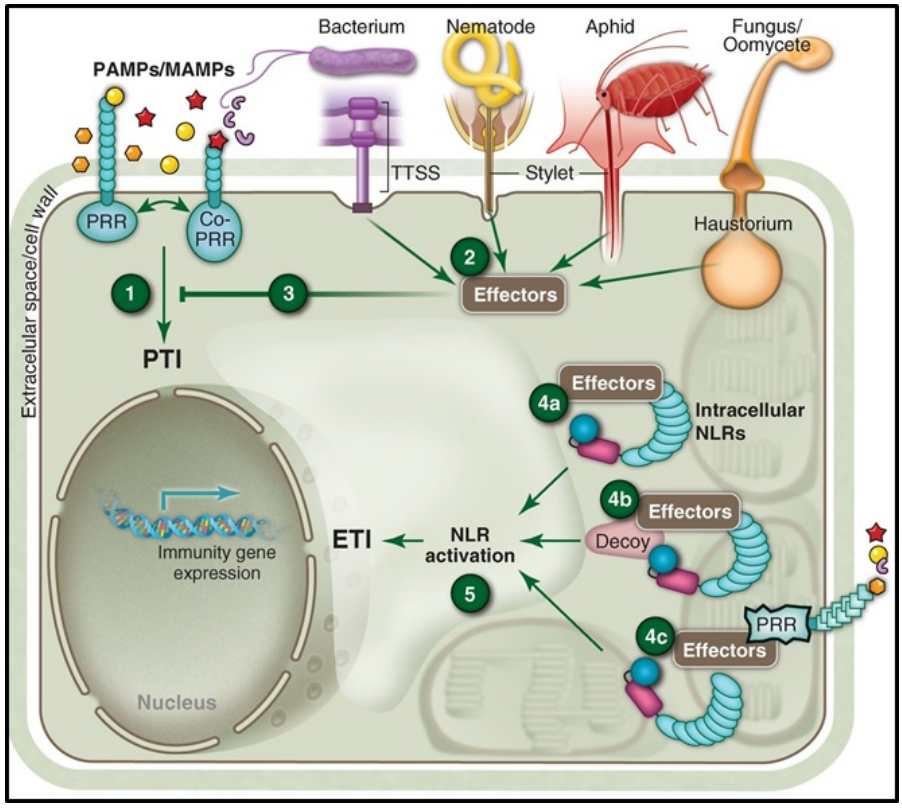


Figure 9. Simplified perspective of the plant immune system (Reproduced with permission from Nature Publishing Group and AAAS from Dangl, J.L., Horvath, D.M. and Staskawicz, B.J., 2013. Pivoting the plant immune system from dissection to deployment. Science, 341(6147), pp.746-751).

Pathogens contain molecules called pathogen associated molecular patterns (PAMPs) that are important for their survival and plants have evolved pathogen recognition receptors (PRRs) that can detect PAMPs. PTI is characterized by the interaction between specific PAMP and PRR molecules (Dangl et al., 2013). This interaction results in the activation of first line of plant defence that consists of production of reactive oxygen species (ROS), mitogen activated protein kinase (MAPKs) activation, production of transcription factors like WRKYs, and activation of downstream defence genes (Bigeard et al., 2015). However, successful pathogens have evolved to circumvent these PTI responses by producing specific molecules called effectors that modify or inhibit PTI responses (Dangl et al., 2013). In turn, plants have also evolved to produce resistance genes (R-genes) whose products detect effector molecules and prevent infection. This effector-R gene interaction is a specific interaction that results in effector triggered immunity

(ETI). The majority of these interactions lead to a hypersensitive response (HR) that is characterized by regulated cell death wherein cells in and around the site of infection are killed "voluntarily" by the plant defence system in order to prevent nutrient uptake and spread of pathogen (Jones and Dangl, 2006). This is a constant cycle in which the pathogen adapts and evolves to circumvent ETI responses by the production of a different effector, in response to which the plants counter-evolve to produce another R-gene specific to the new effector. With regards to Potato, there is a lot that is yet not known about genes and gene networks that constitute different levels of immunity although several R genes have been characterized (Vleeshouwers et al., 2011). In addition, knowledge about the effect of factors such as abiotic stress, simultaneous biotic stresses on this interaction in Potato is also limited.

Breeding programs focused on traits like yield have also influenced R-gene distribution within plant populations. In several cases, this has resulted in cultivated varieties with reduced or lost resistance, while wild plant populations from where these cultivated varieties originate from still possess resistance capabilities (Henry, 2012). Therefore, the focus of numerous resistance breeding programs has been to introgress R-genes that exist in wild populations into cultivated varieties; this strategy has been pursued for long but with limited success. Multiple reasons can be attributed to the observed limited success. One reason for this, is that the success of breeding programs hinges on identification of major genes that lead to clear phenotypic differences (Langridge and Fleury, 2011). This dependence can be linked to limited knowledge regarding various other molecules that constitute plant defence. However, advancement in the plant molecular biology, biochemistry and genetic engineering has indeed helped but to a limited extent. Application of these tools to understand plant defence has revealed that resistance is not always primarily regulated by R-genes. There is a robust network of molecules and their interactions that regulate both R-gene dependent and independent defence mechanisms (Hammond-Kosack and Parker, 2003).

Plant stress hormones like SA and JA regulate defence signalling as well, connecting metabolic processes and plant defence (Berger et al., 2007, Wiesel et al., 2015). For instance, defence responses to *P. infestans* in Potato are dependent on SA and pathways linked to it; surprisingly JA and JA dependent pathways are not essential for these responses (Halim et al., 2007). This is in contrast to defence responses to *Alternaria*. Since the role of SA and JA pathways in defence to *A. solani* in Potato is not known, studies from near relative host, tomato and its pathogen *Alternaria alternata* suggest a more

prominent role of JA and Ethylene in facilitating susceptibility, while SA is necessary for resistance (Jia et al., 2012). Information in Potato regarding the importance of JA and SA in defence responses to blackleg disease caused by *D. solani* is also not known. However, based on studies from Arabidopsis and Tobacco, it has been hypothesized that both SA and JA signalling pathways are an important part of defence to *Pectobacterium* (Davidsson et al., 2013).

Results from the use of molecular methods to understand defence signalling in Potato also indicate the presence of a large network of molecules other than hormones that drive defence responses. An example is the C14 protease that is secreted into the apoplast in Potato in response to *P. infestans* infection, and is under diversifying selection in Potato and other natural pathogen hosts (Kaschani et al., 2010). In addition, it also is targeted by effectors (Bozkurt et al., 2011). Examples of molecules other than R-genes with regards to infection caused by *Pectobacterium* are extensin, phenylalanine ammonia lyase and hydrogen peroxide. It has been shown that in the tubers, induction of extensin and phenylalanine ammonia lyase, and overexpression of hydrogen peroxide can prevent infection spread caused by *Pectobacterium* to a limited extent (Wu et al., 1995, Rumeau et al., 1990). Chitinases and β-1,3-glucanases are also examples of molecules that are part of biotic stress responses and these molecules were found to be constitutively highly expressed in tomato genotypes resistant to *A. solani* in comparison to the susceptible varieties (Lawrence et al., 2000).

The plant immune system produces an array of molecules in response to any kind of biotic stress, regardless of a resistant or susceptible interaction. Some of these molecules are not directly connected to defence but could rather facilitate pathogen infection. Genes producing such molecules can be considered as susceptibility genes (S-genes). Products of S-genes allow compatibility by enabling pathogen detection and penetration, S-genes also negatively regulate plant defence or allow uptake of nutrients by the pathogen (Schie and Takken, 2014). S-genes are examples of successful pathogen adaptation wherein the pathogen targets or modifies plant molecules to fulfil its requirements. Numerous molecules in several plant patho-systems have been identified to act like S-genes. MLO proteins are successful examples of the use of S-genes in plant breeding. Although the precise biochemical function of MLO is unknown, silencing of this membrane localized protein in Barley has resulted in resistance to for example *Blumeria graminis*. More importantly, this resistance has stayed intact under field conditions for a long time and, barley cultivars with *mlo* mutations have been bred and are currently being used

(Acevedo-Garcia et al., 2014). Another example of S-genes that has shown promise to contribute to development of resistant rice cultivars are two sugar transporters namely SWEET11 and SWEET13, which facilitates *Xanthomonas oryzae* susceptibility. These transporters have been shown to interact with copper transporters to mediate susceptibility to the pathogen. Silencing of both the transporters resulted in reduced pathogen growth on the plant (Schie and Takken, 2014). Although modifications of S-genes have also yielded undesired phenotypic effects (Pavan et al., 2010), they can be of practical interest, especially with the advent of new genome editing technologies that have already been successfully applied in Potato (Nicolia et al., 2015).

Indeed, there are numerous examples wherein defence molecules other than R-genes have shown promise in reducing disease; however, the observed reduction has been minor and unsatisfactory in several cases, and this has negatively impacted transfer of this basic knowledge into practice. Unlike R-gene mediated defence which is dependent on the presence or absence of a particular R-gene, molecules discussed above are part of a larger network of interacting molecules. Unfortunately, large scale information of molecules that constitute these networks in Potato is limited. Elucidation of these molecules and their interactions will not only improve the existing knowledge about defence signalling in Potato, but also positively contribute to resistance breeding programs. Moreover, this type of resistance is not dependent on one particular R-gene that can be easily broken, but is instead regulated by several genes and their interactions, hence is a potential source for durable resistance.

2.2.3 Durable disease resistance facilitated by “Omics” based understanding of plant defence

Until recently, the ability to gather large scale information about molecules and their interactions that make up plant defence has primarily been affected by lack of machines and algorithms that could enable this (Francisco et al., 2015). However “-Omics” approaches, that enable genome sequencing, that allow expression analysis of genes in the genome through transcriptomics, that facilitate identification of changes in protein abundances via proteomics have allowed researchers to gain systems-understanding of various plant processes including defence at the molecular level. Although most of these studies were initially focused on model plants such as Arabidopsis, recent technical innovations such as improvements in sequencing/analysing complex genomes and reduction in sequencing costs, has led to a surge in the application of “-

Omics" methods to study non-model crops. This is illustrated by the rate at which plant genomes have been sequenced in the recent years, while between 2000 and 2006, only three plant genomes were successfully sequenced, during the following 7 years, six times more number of plant genomes were sequenced (Bolger et al., 2014). Potato has also benefited from this, as the first draft of its genome was published in 2011 (Consortium, 2011). It was also the first tuber-bearing crop to be sequenced and is considered as the model plant for this group of plants. With the availability of the genome and ongoing innovations in sequencing methodologies, it is now possible to add "multiple-layers" of molecular information to the genome sequence that will help better understand processes such as defence in Potato. Such genome-wide approaches are in fact ideally suited to provide a systems perspective of defence.

Improved understanding of plant defence has numerous benefits. Traditional resistance breeding programs have been time-consuming and are adapted to introgression of limited number of resistance gene (R-gene) targets, for instance it took 46 years to develop *P. infestans* resistant Potato cultivars Bionica and Toluca that contain a single broad spectrum R-gene Rpi-blb2 (Haverkort et al., 2009). However due to the fast paced adaptive nature of pathogens, these resistance sources once introduced could be rapidly overcome. This has occurred with cultivars Bionica and Toluca with evidence of resistance breakdown 3 years after the introduction (Richard G.F. Visser, European Commission – EPSO Conference on crop genetic improvement techniques, 14 July 2015). In order to obtain a more durable resistance solution, the focus now is towards stacking of multiple R-genes for durable resistance, especially with regards to *P. infestans* (Zhu et al., 2012). Moreover, traditional breeding approaches for resistance to *D. solani* and *A. solani* have been comparatively less successful due to the absence of genetic resources with resistance or strong linkage drag. Hence, limited knowledge with regards to molecular aspects of defence in Potato, absence of strong resistance sources complemented with the mismatch between the pace of pathogen adaptation and introduction of crops with R-genes has hindered the success of numerous breeding programs.

Therefore it is evident that detailed and comprehensive molecular knowledge of defence system is necessary to aid in the development of crop varieties with durable resistance. "– Omics" based approaches have provided improved insights into PAMP triggered immunity (PTI), which is one of those essential components of the plant defence system. "- Omics" based understanding of

PTI has revealed that similar processes are induced by different sets of PRR and PAMP interactions, therefore transgenic expression of multiple PRRs in principle can lead to PTI responses to wide variety of pathogens, such an approach scores over current single gene mediated resistance methods that are bound to be overcome by rapidly evolving pathogens, since broad spectrum PTI responses are governed by multiple genes leading to durability in resistance (Huang and Zimmerli, 2014). It is further possible to increase resistance further by combining PRR expression with introgression of R-genes. Another avenue for positive impact of "- Omics" based approaches is in terms of breeding for quantitative resistance, which has been a hurdle due to the polygenic nature of inheritance. Proteomics approaches offer the possibility to identify and breed for targets that are associated with such type of resistance (Kushalappa and Gunnaiah, 2013). Use of "- Omics" approaches to study the plant defence system has also shown that defence processes have a degree of plasticity. They are influenced by environmental factors such as temperature, multi- pathogen stress etc. Addition of multiple-layer information through the use of transcriptomics, metabolomics and proteomics over the genome potentially connects defence genes to their functions under these various environmental factors. For instance, plant resistance can be directly influenced by abiotic stresses, as evidenced by extensive crosstalk between molecular processes that drive both biotic and abiotic stress responses. With the use of transcriptomics, it is possible to identify resistance targets that are independent of abiotic stress hence leading to more durable resistance (Kissoudis et al., 2014). "– Omics" based approaches can also aid in development of alternative disease resistance strategies. A specific example in this regards is IR. Several studies have recently employed the use of "-Omics" to understand the "induced resistance" state of plants, with such knowledge, it should then possible to breed for genetic targets that will enable plants to better respond to priming agents (Balmer et al., 2015).

3 Aims and Objectives

Late blight disease caused by *Phytophthora infestans*, Early blight disease caused by *Alternaria solani* and Blackleg disease caused by *Dickeya solani* have severe economic impact on Potato cultivation worldwide. Therefore, there is a need to develop new disease protection methods such as IR or cultivars with durable resistance. With the aim to facilitate development of alternative and durable disease protection, the focus of this study is to obtain systems level understanding of various aspects of Potato defence. Transcriptomics and proteomics based approaches were employed to understand induced resistance in Potato, similar approach was also used to better understand molecules and signalling pathways involved in Potato defence response to the three most devastating Potato pathogens, namely *Phytophthora infestans*, *Alternaria solani* and *Dickeya solani*.

Individual and specific objectives are listed below:

1. To better understand responses in Potato after treatment with the resistance inducer β- amino butyric acid (BABA) using transcriptomics and proteomics (Paper I)

2. Transcriptomic and proteomic elucidation of defence related molecules and processes regulated by phosphite (Phi) treatment in Potato (Paper II)

3. Development of an *in vitro* based blackleg disease screening assay, and understanding the role of salicylic (SA) and jasmonic (JA) dependent COI1 (Coronatine-insensitive) pathways in Potato defence to *Dickeya solani* (Paper III)

4. Trait-transcriptome association analysis to identify potential susceptibility genes (S-genes) to *Dickeya solani* in Potato (Manuscript IV)

5. Use of transcriptomics to elucidate the role of SA and COI1 dependent defence response to *Alternaria solani* in Potato and identification of SA regulated susceptibility gene (Manuscript V)

6. Comparative proteomics of PAMP and effector triggered immunity in Potato (Manuscript VI)

4 Results and Discussion

4.1 BABA and Phosphite (Phi) mediated induced resistance

BABA (β-amino butyric acid) and salts of phosphite (Phi) have previously been shown to induce resistance against several different diseases in various plant systems. Both these resistance inducers have also shown to induce protection to *P. infestans* in Potato. However, comprehensive knowledge about molecules and molecular networks that mediate this protection in Potato is not known. For the first time, a potato genome-based microarray was used to investigate transcriptomic changes in response to treatment with these resistance inducers. In addition, proteomic analysis of the secreted proteins in the apoplast in response to resistance inducer treatments was also performed to identify defence related proteins that change in abundance during induced resistance.

4.1.1 BABA and Phi treatment reduces *P. infestans* infection on Potato

In order to identify if the concentration at which both the resistance inducers were used, indeed protect plants against *P. infestans* , plants were foliar sprayed either with BABA (1 mM and 10 mM) or with Proalexin (Potassium phosphite; 1.25% v/v) while the controls were sprayed with water. Since, Proalexin is acidic, an additional control in the form of acidified water (pH 5.4) was included. With regards to BABA, plants were foliar sprayed and 2 days later; leaflets were sampled and inoculated with *P. infestans*. To better understand the durability of Phi induced protection, leaflets from plants were sampled for pathogen inoculation 3, 6, 11, 24, 48 and 120 hours after foliar Phi spray. Leaflets from 10 mM BABA treated plants had significantly smaller lesion size at the inoculated site in comparison to their controls (Paper I, Figure I), while 1 mM BABA did not induce protection. A similar effect was observed

on the leaflets sampled from Phi treated plants, lesion size in leaflets from Phi treated plants were significantly smaller than their respective controls at all time points tested (Paper II, Figure I). These results indicate that both these inducers at the concentrations used protect Potato plants against *P. infestans*. Furthermore, we also observed "Hypersensitive response" (HR) like symptoms at the site of pathogen inoculation on leaflets obtained from plants treated with both the resistance inducers, which might suggest activation of plant defence. Control plants that were sprayed with acidified water showed normal susceptibility, suggesting that Phi induced protection is independent of its acidifying property. Since Phi is known to have both direct and indirect modes of action, additional treatments were added to understand the same. In a set of Potato plants, 2 leaves were covered with transparent plastic bags to avoid direct contact with Phi while the rest of the plant was sprayed with Proalexin. These samples (covered leaf) were also inoculated to identify if Phi induced protection was systemic. No difference in lesion size was observed between covered leaf samples and control samples (Paper II, Figure I). To further understand this direct/indirect mechanism of Phi, another set of plants were sprayed with Proalexin, and were washed to get rid of Phi on the surface of the leaflets, dried for a minimum of five hours before pathogen inoculation. These samples (washed) had similar lesion sizes in comparison to Phi treated plants (Paper II, Figure I).

4.1.2 Phi distribution *in planta*

In comparison to BABA, Phi distribution *in planta* is not well characterized. In order to correlate the observed infection phenotype with levels of Phi, leaflets that were sampled for pathogen inoculation were also quantified for Phi levels. As expected, Phi was detected in all Phi treated leaflets; however no significant differences were detected across time points (Paper II, Figure IIA). Surprisingly though, Phi was detected in the covered leaf samples at all time points after Phi application. However there was neither a significant difference in Phi levels in the covered leaf samples between time points, nor between Phi levels of Phi treated and covered leaf samples (Paper II, Figure IIA). This data indicates that Phi is rapidly translocated *in planta* as Phi was detected in covered leaf samples 3 hours after Phi application. This did not correlate to the phenotype observed, because although Phi translocated systemically into covered leaf samples, protection to *P. infestans* was not observed. This suggests that Phi induced protection is local and it does not induce systemic resistance. In addition, no significant differences were identified between Phi

levels in Phi treated and washed samples (data not shown). This further indicates that either the washing process was unable to get rid of topical Phi on the leaflets or that there is rapid uptake of Phi. Several studies previously have reported that Phi interferes with various aspects of phosphate metabolism (Ticconi et al., 2001, Jost et al., 2015). To test this, samples that were processed for Phi level measurements were also analysed for phosphate levels. No significant difference in phosphate levels between Phi treated and covered leaf samples was observed (Paper II, Figure IIB). In addition phosphate levels in water sprayed samples and Phi treated samples was also similar (Paper II, Figure IIB). These observations indicate that Phi does not have a direct antagonistic effect on phosphate levels in these experiments.

4.1.3 Improved annotation of gene probes spotted on the genome based Potato microarray

In order to better characterize transcriptomic activity in response to treatment with resistance inducers, a novel network-based gene annotation method was developed to improve the existing annotation of Potato genes. In this network-based annotation method, OrthoMCL (Li et al., 2003), an algorithm that identifies orthologues across genomes with the lowest false positive rates using a Markov clustering approach, was parallelized. Twenty six sequenced plant genomes, including the Potato genome were subjected to this parallelized version of OrthoMCL, in order to cluster orthologous genes from the genomes analysed. Each cluster essentially represents a gene family. GO terms were downloaded and mapped onto their corresponding genes (for all species where GO terms were available except Potato genes) present in each family/cluster. Using a network based approach, Potato genes in a cluster were then assigned the GO terms of the genes belonging to the same cluster. BLAST analysis was performed between the probes on the array and Potato genes in the clusters. In the best hit scenario, GO terms from Potato genes were transferred onto the corresponding probe. This annotation was compared to the previous probe annotation provided by Solanaceae Genomics Resource (SGR). With the new network-based annotation method, 8616 additional probes received an annotation in comparison to the SGR based annotation (Paper I, Figure II). Furthermore, the number of GO terms per probe which is a measure of the amount of information associated with each probe was also higher with 34 in the network-based annotation method in comparison to 6 with the SGR based annotation. This improved annotation was used to analyse transcriptomic activity in response to resistance inducer treatment.

4.1.4 Transcriptomic and proteome (secretome) activity associated with induced resistance

Several studies in the past have analysed and compared expression of genes between resistance inducer treated and control plants after pathogen inoculation (Balmer et al., 2015), few studies however have addressed genome-wide transcriptomic changes in the absence of pathogen inoculation but specifically in response to the resistance inducer. In this work, facilitated by the improved gene annotation, we analysed transcriptomic responses in response to treatment with BABA or Phi. Potato plants were sprayed with 2 different concentrations of BABA (1mM and 10mM), with Proalexin (Potassium phosphite; Phi; 36 mM) or with tap water. Forty eight hours after BABA application and 3, 6, 11, 24, 48 and 120 hours after Phi application, leaflets were sampled for microarray analysis. The apoplast is the initial battleground wherein molecules from both host and the pathogen interact (Alexandersson et al., 2013) Therefore changes in apoplastic protein profiles defined here as the secretome, after treatment of resistance inducer was also analysed in order to identify defence related apoplastic proteins in Potato.

Transcriptomic analysis revealed that approximately 5400 transcripts were regulated in response to 10mM BABA treatment in comparison to water control, 48 hours after application. In contrast, only 1 transcript was induced by 1mM BABA, which was annotated as a MutT domain protein (DMG400017400) that was also found to be regulated by 10mM BABA treatment. In contrast, no genes were found to be differentially expressed between Phi treated and water sprayed control sampled 48 hours after treatment. Interestingly though, Phi induced protection against *P. infestans* was observed even 5 days after Phi application. Since Phi induced protection was observed at all time points and no transcriptomic activity was identified 48 hours after application, we hypothesized that Phi induces rapid transcriptomic changes that could lead to the observed sustained protection. In order to investigate this, samples treated with Phi, 3,6,11, 24, 48 and 120 hours after application were analysed with Potato genome-based microarrays. Indeed, Phi induced rapid transcriptomic activity as close to 750 transcripts were altered in response to Phi 3 hours after treatment (Paper II, Figure III). Approximately, 5700 and 4300 transcripts were regulated at 6 and 11 hours after Phi treatment (Paper II, Figure III). However this effect on the transcriptome was transient, as no significant differences between water and Phi sprayed samples 24 and 48

hours after treatment were identified (Paper II, Figure III). Interestingly, the MutT domain protein induced by BABA treatment at both the concentrations was not found to be regulated by Phi.

Among the 5400 transcripts regulated by 10 mM BABA treatment, five hundred gene ontology terms were significantly enriched, further clustering of these GO terms revealed two major clusters connected to stress and metabolism and two minor clusters linked to development and stress (Paper I, Figure III). A similar trend of regulation of transcripts related to stress and metabolism was observed among in response to Phi at each time point. Approximately 200 transcripts were found to be induced in all the three time points tested by Phi, analysis of these "core" transcripts also divided them into two clusters associated with response/stress and the other linked to biosynthesis/metabolism (Paper II, Figure IVA). However analysis of transcripts across time points revealed that, transcripts related to biotic and abiotic stress are induced rapidly 3h after Phi treatment and transcripts related to cell wall metabolic processes are repressed 11h after Phi application (Paper II, Figure IVB). This transcriptomic data suggests that there is a strong connection between plant defence and metabolic processes, both of which are influenced by BABA and Phi mediated induced resistance response. Among the transcripts regulated by 10 mM BABA, GO terms such as plant-type hypersensitive response and incompatible interaction defence response were identified. Identification of these gene ontology terms is in line with the "HR" like symptoms observed in pathogen inoculated BABA treated plants. Similarly "HR" like symptoms were identified on Phi treated *P. infestans* inoculated leaflets too; however GO term positive regulation of programmed cell death instead of hypersensitive response, which is also a type of pathogen induced programmed cell death was identified (Coll et al., 2011). Interestingly, GO term incompatible interaction defence response was not identified among Phi regulated transcripts, indicating different mechanisms of induced resistance mediated by both the inducers. However, stress related GO terms such as innate immune response, response to wounding, response to biotic stimulus, defence response to fungus and bacterium were enriched among transcripts regulated by both Phi and BABA, and this suggests that induced resistance activated by both these compounds is mediated via biotic stress response induction. In addition, terms such as response to oxidative stress, response to endoplasmic reticulum stress, response to salt stress etc. were also enriched among transcripts that were induced by both 10 mM BABA and Phi, indicating that both treatments also influence oxidative and abiotic stress responses. Both BABA and Phi also regulated transcripts such as chorismate mutase and

arogenate dehydratase that are associated with aromatic amino acid biosynthesis, therefore changes in amino acid metabolism also contribute to induced resistance. In fact, aromatic amino acid levels have been shown to play a key role in defence induction and amplification in Arabidopsis (Zeier, 2013). The overlap between transcripts regulated by Phi and BABA is considerable and decreases from 53% between transcripts regulated by Phi 3h after application and 10 mM BABA to 30% between transcripts regulated by Phi 11 hours after application and 10 mM BABA. However, it must also be noted that the transcriptomic dataset with regards to Phi consists of 3 time points while the one with BABA consists of only a single time point. Regardless, transcripts regulated by both BABA and Phi show similar levels of induction (Paper II, Figure V), therefore through this comparative transcriptomic analysis, we have identified a common set of transcripts and their expression levels that constitute induced resistance in Potato plants. Additionally, Phi treatment also resulted in induction of transcripts that can be directly related to pathogen defence, transcripts annotated as SNKR2GH5 protein, MYC2, multiple transcripts associated to NAM, MAP kinase, Protein TIFY 9, all these transcripts were not identified in the BABA regulated transcriptome, although they could be induced by BABA at a different time point.

Plant hormones also play a vital role in defence (Robert-Seilaniantz et al., 2011). With regards to Potato, the importance of SA and its pathway in BABA-dependent induced resistance has already been demonstrated. However not much is known about the role of coronatine insensitive (COI1) receptor involved in JA sensing and downstream signalling activation in BABA dependent IR in Potato, and with regards to Phi , the role of both SA and COI1 in Potato is not known. In order to investigate this, SA deficient transgenic plants (*NahG*), JA insensitive transgenic plants (*coi1*) and JA deficient transgenic plants (*aoc* and *opr3*) were either sprayed with Phi or water, 48 hours after spray, whole plants were inoculated with *P. infestans*, Phi induced protection was observed in all plants regardless of the type of transgenic plant, while all the water sprayed plants showed susceptibility (Paper II, Figure VI). This indicates that both SA and COI1 compensate for each other in Phi mediated IR, and only affecting both these components might influence Phi dependent induced resistance. With regards to other plant hormones, GO terms related to Gibberellic acid and Abscisic acid was observed only among transcripts regulated by 10 mM BABA treatment and not in the Phi regulated transcriptome.

Analysis of the secreted protein fraction revealed that 10mM BABA induced production of PR-1, a well characterized marker for plant defence (Paper I, Figure V). In fact, a faint band corresponding to PR-1 was also observed in samples treated with 1mM BABA (Paper I, Figure V). Further proteomic investigation of the secreted protein fraction from leaflets that were sampled 48 hours after BABA treatment revealed that 24 and 91 proteins changed in abundance in response to 1 mM and 10 mM BABA, respectively (Paper I, Table V and Table VI). Twelve proteins that changed in abundance in response to 1 mM BABA treated samples were also found to change significantly in samples treated with 10 mM BABA. These proteins such as acidic class II 1, 3-beta glucanase (PR-2), thaumatin, Pathogen-and wound-inducible antifungal protein CBP20 and Pathogenesis-related leaf protein 6 are typical markers of plant defence activation (Loon et al., 2006). Significant induction of a single transcript, but increased abundance of 24 proteins in response to 1mM BABA, indicates that at lower concentrations, response to BABA might largely be governed by post-transcriptional regulation.

An alginate-lyase motif containing Citrate binding protein *(CBP; CUST_23400_PI426222305)* also increased in abundance in response to 10 mM BABA treatment, induction of the transcript associated with this protein was also observed in the BABA regulated transcriptome, furthermore the same transcript was also observed to be induced in response to Phi 6 hours after treatment. It´s induction was also identified in *P. infestans* inoculated Potato plants (Ali et al., 2014). The fact that this protein does not have an Arabidopsis homolog, and no previous report indicates its direct role in plant defence, makes it an ideal target for further functional characterization.

Although transcriptomic changes 48 hours after Phi treatment were not identified, analysis of the secreted protein fraction revealed 67 proteins that significantly changed in abundance 48 hours after Phi application (Paper I, Table 1), which might explain the long lasting effect of Phi, as observed from the *P. infestans* infection assay of Phi treated plants. Changes were observed among proteins related to cell wall metabolism such as ceramidase, aspartic proteinase nepenthesin-1 and alpha-galactosidase. These proteins increased in abundance in response to Phi treatment. In contrast, aspartic proteinase nepenthesin-1 and alpha-galactosidase were found to be down-regulated in the secreted protein fraction of 10 mM BABA treated samples. Three proteins related to cell wall metabolism encoding chitinases that are known to play an important role in pathogen defence (Loon et al., 2006) were significantly increased in abundance to both Phi and BABA treatment. Phi treatment also

resulted in increased abundance of proteins related to stress, proteins annotated as Peroxidases such as Class III peroxidase and peroxidase N changed in abundance in Phi treated leaflets. These proteins also changed in abundance in BABA treated leaflets. Other stress related proteins that increased in abundance to Phi treatment specifically were a Kunitz trypsin inhibitor and a polygalacturonase inhibiting protein; both these proteins have previously been shown to protect plants against pathogens (Giulia De Lorenzo et al., 2001, Loon et al., 2006, Chen, 2008).

Transcriptomic and proteome investigations suggest that the effect of BABA and Phi is not restricted to plant defence processes, both the resistance inducers also have a complex effect on multiple plant processes such as primary metabolism, cell wall modification and abiotic stress, indicating changes in these processes in addition to activation of defence represents the "induced state" of defence in the plant.

4.2 Improved elucidation of plant defence in order to identify genetic sources of resistance (Paper III- Paper VI)

Another focus of research presented in this thesis is to perform large scale exploration, and thereby improve current knowledge regarding molecules and molecular networks that mediate defence responses to various biotic stresses in Potato. The aim is to use this information to identify and prospect for genes that have the potential to offer durable resistance. Hence, a combination of plant pathology, molecular biology and "- Omics" based approaches were employed to better understand defence responses in Potato, to blackleg-causing bacteria *Dickeya solani*, to early blight causing fungi *Alternaria solani* and to effector molecules of late blight causing oomycete *Phytophthora infestans*.

4.2.1 Development of an *in vitro* assay to screen for Blackleg disease

Blackleg disease resistant Potato cultivars do not exist and breeding programs focused on resistance to blackleg disease have shown limited success. One factor that has complicated the breeding process is the unavailability of disease screening assays that can consistently screen many numbers of plants in a relatively short span of time. In addition, existing assays are based on either greenhouse or field grown plants; this makes the procedure laborious and inconsistent. Blackleg disease symptom development is also dependent on environmental factors; and existing screening assays are performed in

conditions wherein several environmental variables cannot be controlled for, and this has led to inconsistencies in disease development and scoring.

In order to address several of the above issues, the aim was to develop a blackleg disease screening assay based on *in vitro* grown Potato plants. Use of *in vitro* plants results in reduced space, which will lead to accommodating and testing many more number of plants. Furthermore, infection conditions for the assay were standardized and regulated in order to limit the effect of environmental variables.

To validate plant responses obtained in the *in vitro* method with existing disease screening assays, a standard greenhouse infection protocol was first used to assay blackleg disease response of different Potato genotypes to blackleg-causing bacteria, *Dickeya solani* and *Pectobacterium atrosepticum*. Seven days post infection, rot spread on the shoots of cv. Désirée, cv. Bintje and cv. Sarpo Mira was significantly lower than cv. Magnum Bonum and clone SW93-1015 in response to *D. solani* infection (Paper III, Figure 1). A similar result was also observed in response to *P. atrosepticum* wherein both cv. Désirée and cv. Bintje had significantly lower spread of rot in comparison to the breeding clone SW93-1015 (Paper III, Figure II). Previous reports have shown that cv. Désirée and cv. Bintje are moderately resistant to blackleg disease, hence our results from the greenhouse assay are in coherence with previous identifications (Bains et al., 1999). However, in cv. Sarpo Mira that is known to contain several *P. infestans* R-genes (Rietman et al., 2012), response to blackleg disease was not known, the result from the greenhouse assay suggests that cv. Sarpo Mira is moderately resistant to blackleg disease caused by *D. solani*. In contrast, the other *P. infestans* resistant clone SW93-1015 is susceptible to blackleg disease caused by both D. *solani* and *P. atrosepticum*. In addition, cv. Magnum bonum that is known to be moderately resistant to *Alternaria solani* (Odilbekov et al., 2014) is also susceptible to blackleg disease caused by *D. solani*. Although fewer genotypes were tested with *P. atrosepticum*, this data indicates that the genotypes used in this assay respond similarly to both the blackleg disease causing bacteria. Responses observed in different genotypes were used as the reference set to compare and validate the findings of the *in vitro* based screening assay.

In vitro based testing of clone SW93-1015, cv. Magnum Bonum, cv. Désirée and cv. Sarpo Mira with *D. solani* revealed that clone SW93-1015 and cv. Magnum Bonum obtained significantly higher infection scores in comparison to cv. Désirée and cv. Sarpo Mira (Paper III, Figure 4). A similar trend was

observed after inoculation of *in vitro* grown Potato plants with *P. atrosepticum*, clone SW93-1015 obtained higher infection scores in comparison to cv. Bintje and cv. Désirée (Paper III, Figure 5). Results from the *in vitro* infection assay suggest that clone SW93-1015 and cv. Magnum Bonum are significantly more susceptible to blackleg-causing bacteria in comparison to cv. Désirée, cv. Sarpo Mira and cv. Bintje. These results are in coherence with the observations from the established greenhouse assay. Furthermore, the *in vitro* assay is robust enough to be used for testing disease responses with different blackleg-causing bacteria as indicated by the case wherein both *P. atrosepticum* and *D. solani* were used. However, there is a distinct difference in symptom development between the *in vitro* and the greenhouse based assay, symptoms in the *in vitro* assay are smaller and localized in comparison to those in the greenhouse assay. This might be attributed to the difference in the plant architecture and the development of shoot tissue. Regardless, the difference between genotypic responses to infection is similar between both the assays.

4.2.2 Identification of potential blackleg disease susceptibility factors based on trait association with RNAseq data of a crossing population in Potato

The *in vitro* based assay offers the possibility to screen large plant populations in a relatively short span of time. Therefore, this assay was used to identify disease response of a population obtained by crossing moderately resistant cv. Désirée with the susceptible clone SW93-1015 to *D. solani*. A total of 36 genotypes were analysed from this crossing. *D. solani* inoculation of *in vitro* grown genotypes and their parents revealed a continuum in disease development (Manuscript IV, Figure 1). Further statistical analysis indicated that genotypes L1, L34, L32 and SW93-1015 obtained significantly higher infection scores in comparison to genotypes L30, L37, L21, L9, L24, L4 and cv. Désirée that attained lower infection scores. A continuum in disease development rather than distinct separation between susceptibility and resistance indicate that resistance to *D. solani* is controlled by multiple genes. These results are in similarity to previous reports that have suggested multi-genic control of resistance to necrotrophic pathogens (Rowe and Kliebenstein, 2008).

Another factor that has hindered development of blackleg disease resistant plants is the inability to identify and study plant defence response after infection. Blackleg-causing bacteria are necrotrophs; the infection process is characterized by production of plant cell wall degrading enzymes (PCWDEs)

that are produced by the pathogen during the course of infection, hence resulting in cell wall degradation and maceration. Consequently, it becomes difficult to identify plant defence responses, due to the overwhelming effect of PCWDEs (Charkowski, 2015), because plant responses obtained during this phase are usually associated to processes related to cell death but not directly to defence. Recent evidence also suggests that the blackleg-causing *Pectobacterium* has the ability to manipulate host defence signalling during early stages of interaction (Davidsson et al., 2013). Therefore, in order to identify plant defence, processes that are activated immediately after pathogen inoculation and before the onset of PCWDE production have to be identified; however this time-frame is not well characterized in this plant patho-system.

In order to circumvent the problems caused by the effect of PCWDEs, a different approach was tested to identify components of plant defence that might be involved in *D. solani* response. In this approach, the transcriptome of each genotype from the crossing population that was screened for *D. solani* response was sequenced and the RNA was obtained from un-inoculated genotypes. The idea was to associate transcript presence before infection with resistance phenotype, i.e. to identify for pre-formed mechanisms. Since genotypes L1, L34, L32 and SW93-1015 responded significantly differently to *D. solani* in comparison to genotypes L30, L37, L21, L9, L24, L4 and cv. Désirée, these genotypes were divided into two groups: one containing susceptible genotypes (L1, L34, L32 and SW93-1015) and the other containing moderately resistant genotypes (L30, L37, L21, L9, L24, L4 and cv. Désirée). Statistical analysis was performed to identify transcripts with significant differences in expression levels between the two groups. This analysis yielded 21 transcripts that were found to be present at higher levels in the genotypes belonging to the susceptible group while lowly expressed in the genotypes belonging to the moderately resistant group (Manuscript IV, Figure 2). Since all the identified transcripts were highly induced in the susceptible genotypes, they were termed as potential susceptibility genes (S-genes). The top hits of nine out of the 21 transcripts were annotated as either long noncoding RNAs (lncRNA), transcripts with transposon activity or as transcripts with nucleotide binding activity (Manuscript IV, Table 1).Hence a majority of these potential S-genes might be related to transcriptional regulation. Several recent reports have indicated the involvement of lncRNAs and transposons in plant defence regulation (Li et al., 2014, Zhu et al., 2014). Interestingly, the top hits of two more transcripts were found to have leucine repeat rich domains (Manuscript IV, Table 1) ,which are known to facilitate pathogen perception in plants (Padmanabhan et al., 2009).

To establish the role of the identified candidate transcripts as S-genes, they have to be functionally validated. The most logical approach would be to overexpress and knock down their expression in Potato and characterise the resulting transgenic plant´s response to *D. solani*. But the process of obtaining stable transgenic Potato plants is expensive and time-consuming. Therefore in order to perform a quick validation and establish the role of these transcripts in facilitating blackleg disease susceptibility, we have developed a method based on blackleg disease development in *Nicotiana benthamiana*. *N. benthamiana* is the most closely related model plant to Potato and is amenable to virus induced gene silencing (VIGS) assays. The strategy is to identify *N. benthamiana* homologs of S-genes, transiently silence these genes using VIGS and infect the silenced *N. benthamiana* plants with *D. solani* and phenotype the symptoms.

To perform the above, a disease screening assay that involves infecting and phenotyping *N. benthamiana* plants was developed. *N. benthamiana* seeds were sown and grown in the greenhouse. A 10 ul pipette tip was used to create an incision in the middle section of the shoot, 100 ul of *D. solani* (strain IPO2222; $5*10^9$ CFU/ml) was pipetted into the incision, 3 litre plastic bags were sprayed with tap water on the inside and inverted over the top of the inoculated plants. Different infection-related symptoms were scored 3 days post inoculation. These symptoms did not change after 3 days. Preliminary results indicate that *N. benthamiana* plants are sensitive to *D. solani* inoculation and four different types of symptoms were consistently identified: total plant collapse with shoot blackening, leaf wilting/blackening accompanied with shoot blackening, leaf discoloration/blackening with mild blackening of shoots and leaf flaccidity with no blackleg symptoms on the shoot (Manuscript IV, Figure 3). The aim now is to develop a scoring scheme based on the identified symptoms. The sensitivity of *N. benthamiana* plants aptly suits the requirement of testing S-genes, as inoculation of *D. solani* in plants silenced in S-genes would result in milder symptoms compared to wildtype plants.

The concept of altering S-genes in order to obtain resistance has been recently gaining momentum as a strategy for resistance breeding (Schie and Takken, 2014). However, not much is known about S-genes in the Potato - *D. solani* patho-system. In this work we have identified novel putative S-genes that potentially regulate susceptibility in Potato to blackleg disease. With improvements in gene editing techniques in Potato (Nicolia et al., 2015), it is possible to inactivate these genes in elite Potato cultivars so as to obtain

blackleg disease resistance without introducing any foreign DNA, especially as genes that confers complete resistance to blackleg disease are not known, hence the approach based on alteration of S-genes is suited for generating *D. solani* resistant Potato varieties.

4.2.3 Role of Salicylic and Jasmonic acid dependent COI1 in Potato Blackleg and Early blight disease

Plant defence is characterized by activation and repression of a complex web of molecules and processes. Important components of this web are plant hormones such as salicylic acid (SA) and jasmonic acid (JA) that have been shown to play a crucial role in plant defence. However the precise role of these hormones in mediating defence responses depends on the specific plant-pathogen interaction. Several reports investigating defence response in Arabidopsis against a range of pathogens have indicated contrasting roles for both these hormones. The general consensus is that in response to biotrophic pathogens, SA dependent defence responses are necessary for resistance in Arabidopsis, while in response to necrotrophic pathogens, JA plays a prominent role in resistance in Arabidopsis (Glazebrook, 2005). However major exceptions to this have been identified not only in Arabidopsis, but also in other plant species (Robert-Seilaniantz et al., 2011).

With regards to *Pectobacterium,* previous reports in Arabidopsis showed that JA insensitive *coi1* mutants are significantly more susceptible to *Pectobacterium carotovorum ssp carotovorum* in comparison to wildtype and SA deficient *NahG* plants (Norman-Setterblad et al., 2000). Another study in tobacco showed that exogenous application of SA leads to enhanced resistance to *P. carotovorum subsp. carotovorum* (Palva et al., 1994). A recent review also suggested that defence to *Pectobacterium* may be dependent on molecules such as WRKY70 that act in facilitating crosstalk between SA and JA signalling pathways (Davidsson et al., 2013).

Since the role of SA and COI1 in Potato - *D. solani* interactions is not known, *NahG* Potato plants that are SA deficient and *coi1* plants that are JA insensitive were tested using the *in vitro* blackleg disease screening system. Both *NahG* and *coi1* plants obtained significantly higher infection score in comparison to wildtype cv. Désirée (Paper III, Figure 6). These results indicate that SA as well as JA dependent COI1 mediated defence responses are necessary for resistance to *D. solani*. This result is in contrast to data from necrotrophic

pathogen interactions with Arabidopsis wherein COI1 in comparison to SA has generally been shown to play a more prominent role in defence. The increased susceptibility phenotype was further substantiated by analysing the expression of an SA dependent marker belonging to StPR-1 (pathogenesis related protein) and a JA dependent marker from StLOX (lipoxygenase) in plants sampled 3 days post inoculation. Pathogen inoculated plants in the *in vitro* testing system develop symptoms 4 to 5 days after inoculation. Hence, 3 days post inoculated plants were sampled in order to identify early defence responses and to prevent identification of gene expression changes associated with symptom development. StPR-1 and StLOX were induced in inoculated cv. Désirée plants (wildtype) (Paper III, Figure 7) confirming the involvement of both SA and JA dependent COI1 in defence to *D. solani*. A previous study in tobacco has shown that exogenous application of SA leads to improved protection against *P. carotovorum subsp. carotovorum* and also to increased PR-1 expression (Palva et al., 1994), which is in coherence with the results obtained in this study. Another study that investigated Arabidopsis interaction with a necrotrophic fungus *Botrytis cinerea* has also shown SA dependent PR-1 expression in response to BABA treatment resulting in improved protection (Zimmerli et al., 2001), these results are in coherence with the observation of enhanced susceptibility in *NahG* plants and SA dependent PR-1 expression in inoculated cv. Désirée plants (wildtype) in response to the necrotroph *D. solani*. With regards to StLOX expression, our results are also in coherence with for example , another study wherein LOX overexpressing pepper plants were resistant to another necrotroph *Alternaria brassicicola* in comparison to *lox* loss of function pepper mutants (Hwang and Hwang, 2010). In contrast, a study investigating defence responses in Potato cell lines treated with a lipopolysaccharide fraction from *Pectobacterium atrosepticum* did not observe LOX induction (Desender et al., 2006). The use of intact plants and bacterial cultures in this study could contribute to the differences in results, furthermore this also indicates the advantages offered by the *in vitro* disease screening assay to investigate plant defence components involved in interaction with blackleg-causing bacteria. In the JA signalling pathway, StLOX is involved in JA production while COI1 is necessary for sensing JA and activation of downstream signalling (Turner et al., 2002). The fact that StLOX is induced in *D. solani* infected cv. Désirée plants, and infected *coi1* plants show enhanced susceptibility and reduced expression of StLOX indicates that both JA production and JA sensing play a role in defence to *D. solani*. Interestingly, we also observed repression of StLOX in SA deficient Potato plants (Paper III, Figure 7), indicating that SA might also be involved in regulating JA biosynthesis in response to *D. solani* infection in Potato. Based on the above

results from the *in vitro* assay and gene expression analysis, it is evident that coordinated role of both SA and JA signalling is necessary for defence against the necrotrophic pathogen *D. solani* in Potato.

One of the other factors that complicate resistance breeding to blackleg-causing bacteria is the observation of poor correlation between resistance/susceptibility in the shoot and in the tubers (Czajkowski et al., 2011). In order to identify if this poor correlation relates to SA and JA dependent COI1 signalling, tubers of cv. Désirée (wildtype), SA deficient *NahG* and JA insensitive *coi1* plants were inoculated with *D. solani*. Tubers of both the hormone transgenic plants had significantly higher amount of rot tissue in comparison to the tubers of cv. Désirée (wildtype) (Paper III, Figure 8). These results indicate that as in the case of the shoot, SA and COI1 signalling is necessary for defence to soft rot caused by *D. solani*. Similar hormone dependent defence regulation in different tissues has also been identified in other patho-systems such as in maize interaction with *Colletotrichum graminicola* (Balmer et al., 2013). These results indicate that the poor correlation between blackleg in the shoot and soft rot in tubers is not due to differences in SA and COI1 defence signalling.

Another necrotrophic pathogen that has caused severe problems to Potato cultivation is the fungus *Alternaria solani.* In similarity to *D. solani*, role of SA and JA dependent COI1 defence in response to *A. solani* in Potato has not been studied previously. We employed a combination of pathogenicity assays, pathogen biomass and hormone level quantification assays and transcriptomics to elucidate the role of SA and COI1 dependent defence signalling in the Potato-*A. solani* interaction.

A. solani inoculation of the hormone transgenic plants and their background cv. Désirée (wildtype) revealed that SA deficient *NahG* plants had significantly larger lesion size due to pathogen inoculation in comparison to *coi1* and wildtype cv. Désirée plants (Manuscript V Figure 1). In order to substantiate that SA levels are responsible for the observed infection phenotype, SA was measured in control and inoculated plants 24, 72 and 120 hours after pathogen inoculation. As expected, SA was not induced in either the control or inoculated *NahG* plants, whereas SA was induced in inoculated plants of cv. Désirée (wildtype) and *coi1* plants across time points (Manuscript V Figure 2). These results are in coherence with another study wherein tomato *NahG* plants were significantly more susceptible to *A. alternata* (Jia et al., 2012). It has also been shown that SA treatment of tomato plants induces resistance to *A. solani*

by activating signals related to systemic acquired resistance (Spletzer and Enyedi, 1999). A prominent role of JA and COI1 in plant defence to necrotrophic pathogens has been previously proposed, hence in order to further investigate this role in Potato defence to *A. solani*, pathogen biomass was quantified from inoculated leaflets of hormone transgenic and wildtype cv. Désirée plants at an earlier time point, i.e. 5 days after inoculation. Surprisingly, inoculated *coi1* plants had higher pathogen biomass in comparison to wildtype cv. Désirée plants (Manuscript V Figure 3) whereas, as expected inoculated *NahG* plants also had higher pathogen biomass in comparison to wildtype cv. Désirée plants (Manuscript V Figure 3). No differences were observed between pathogen biomass of inoculated *NahG* and *coi1* plants (Manuscript V Figure 3). These results indicate that while SA is responsible for restricting pathogen growth and symptom development, COI1 plays a role in restricting pathogen growth early in the process but does not seem to influence symptom development. In order to identify the importance of JA in this interaction, JA levels were measured, and these results revealed that JA was induced in all inoculated plants (Manuscript V Figure 4). This indicates that JA induction is a response to *A. solani*, and a previous report has shown that JA induction can be triggered by toxins produced by *A. alternata*, in fact the same study also showed that JA regulates cell death induced by *Alternaria alternata f. sp. lycopersici* (AAL) toxins (Zhang et al., 2011). These results indicate that development of lesions in response to inoculation is largely dependent on SA, as only plants lacking SA displayed significantly larger lesions. Results from pathogen biomass quantification, however, indicate that early pathogen growth restriction is dependent on COI1 and independent of JA production. This is in contrast to a study on Tomato–*Alternaria alternata* interactions, which showed that COI1 is involved in susceptibility (Jia et al., 2012). None the less, importance of COI1 in mediating resistance has been shown in several other studies in Arabidopsis (Robert-Seilaniantz et al., 2011). Furthermore, our results underline that the role of COI1 in defence is dependent on the plant-pathogen interaction. In order to identify the role of other plant hormones in this interaction, we also measured Auxin (IAA) and Abscisic (ABA) in inoculated and control plants. No significant differences were observed in inoculated wildtype cv. Désirée, *NahG* and *coi1* plants (Manuscript V Figure 5) showing that IAA and ABA are not induced in response to *A. solani*.

In order to obtain molecular knowledge of defence responses mediated by SA and COI1 in this interaction, RNA from *A. solani* inoculated and control leaflets of wildtype cv. Désirée, *coi1* and *NahG* plants was extracted 24, 72 and

120 hours post inoculation and was analysed using a Potato genome-based microarray. Higher numbers of transcripts were induced in response to *A. solani* 24 hours after inoculation in wildtype cv. Désirée plants in comparison to inoculated *coi1* and *NahG* plants (Manuscript V Figure 6). Analysis of transcripts induced rapidly after inoculation revealed induction of transcripts related to wound responses such as JAZ1 and JA induced WRKY, induction of defence and immune related transcripts such as a TGA transcription factor and NPR1-1, induction of transcripts related to oxidative stress and cell death such as lipoxygenase and ethylene response factor 4. All these transcripts have been shown to be regulated by both SA and COI1 in various other studies (Thines et al., 2007, Neill et al., 2002, Wang et al., 2008, Köster et al., 2012, Fonseca et al., 2009), suggesting that rapid transcriptomic activity is characterized by activation of processes that are regulated by the interplay between both SA and JA dependent COI1 defence processes. Further analysis also revealed that transcripts related to "organonitrogen compound metabolic processes" such as RGA1 and PIP5K5 were repressed (Manuscript V Table 1); these transcripts have been shown to link nitrogen metabolism and cell wall modifications (Steffens and Sauter, 2009, Ischebeck et al., 2010), indicating that inoculated wildtype cv. Désirée might undergoe changes in cell wall architecture that limits pathogen growth and symptom development.

A larger overlap in the number of transcripts and associated processes at later time points, i.e. 72 and 120 hours post *A. solani* inoculation was observed in wildtype cv. Désirée, *NahG* and *coi1* plants (Manuscript V Figure 7 and 8). However, stress related gene ontology terms were identified among transcripts regulated by *A. solani* only at 120 hours post infection in wildtype cv. Désirée (Manuscript V Table 1). Induction of transcripts such as basic PR-1, PR-4 type protein and 1-aminocyclopropane-1-carboxylate synthase 2, multiple transcripts coding chitinases, lipoxygenase and 12-oxophytodienoate reductase 1 suggests that transcriptomic responses during later stages of infection are in fact characterized by JA and Ethylene dependent responses, this observation is also in coherence with induction of JA in response to pathogen inoculation (Manuscript V, Figure 4).

Processes such as "response to cyclopentenone" and "response to auxin stimulus" were specifically altered in inoculated *NahG* and *coi1* plants at later time points while the same were not observed in inoculated wildtype cv. Désirée plants (Manuscript V Table 1). Transcripts coding for multiple glutathione S-transferases and a transcript coding a Twi1 protein, linked to cycloepentenone responses, were specifically induced in hormone related

transgenic plants during later time points after inoculation. Cyclopentenones are known to be produced during pathogen induced breakdown of cellular fatty acids (Farmer et al., 2003), indicating increased levels of pathogen induced stress in hormone transgenic plants which is in coherence with the observations from the pathogenicity assays. GO term "Auxin stimulus response" was specifically enriched among transcripts repressed in infected *coi1* and *NahG* plants during later time points of infection. A previous report has shown that repressed auxin signalling promotes susceptibility in Arabidopsis to necrotrophic pathogens (Llorente et al., 2008). Several transcripts coding SAUR proteins and a transcript encoding Auxin response factor 7 all associated with auxin signalling were repressed, however IAA levels (Manuscript V Figure 5) were not changed after inoculation in neither of the inoculated plants. None the less, these results suggest that susceptibility in inoculated *NahG* and *coi1* plants could be linked to downregulated auxin signalling.

Since these results suggest that SA plays a crucial role in mediating defence against necrotrophic fungi *A. solani*, we set out to search for novel transcripts that are regulated by SA during defence. We identified a transcript annotated as Citrate binding protein *(CBP;CUST_23400_PI426222305)*, this transcript was highly induced in inoculated *NahG* plants in comparison to respective mock-inoculated controls at 72 and 120 hours, and CBP expression did not differ significantly between inoculated and control plants of either wildtype cv. Désirée or *coi1* plants (Manuscript V Figure 9A). This data indicates that SA probably represses CBP expression as SA induction in inoculated wildtype cv. Désirée and *coi1* plants does not lead to high CBP expression, while absence of SA in inoculated *NahG* plants leads to CBP expression. Little is known about the biological and functional characteristics of this transcript. CBP is specifically present in many land living plants but not in Arabidopsis. It is predicted to have an alginate-lyase motif that is thought to facilitate breakdown of carbohydrates and sugars. Furthermore CBP in *Hevea brasiliensis* has been previously shown to be involved in transport of citrate (Rentsch et al., 1995).

We have previously identified this protein to be secreted into the apoplast in susceptible Potato-*Phytophthora infestans* interaction, and we also identified CBP in the secreted protein fraction of BABA treated Potato plants. In order to characterize CBP´s role in *A. solani*-Potato interaction, transgenic Potato plants that either overexpressed CBP or RNAi silenced CBP were generated. In response to *A. solani*, CBP overexpressing lines had significantly larger lesion sizes in comparison to CBP RNAi plants or wildtype cv. Désirée plants

(Manuscript V Figure 9B), suggesting that CBP facilitates susceptibility in Potato to *A. solani*. The importance of citrate as nutrient source for necrotrophic bacteria during infections has been previously illustrated (Kieu et al., 2012). From this data it can be hypothesized that citrate is used during *A. solani* infection and a part of SA mediated defence is to prevent citrate availability for the pathogen thereby leading to reduced pathogen growth and infection.

4.2.4 Comparative proteomics of PAMP (PTI) and effector triggered immunity (ETI) in Potato

Plant immune system can be divided into two major layers, PAMP triggered immunity (PTI) that is characterized by general plant defence induction upon PAMP detection effector triggered immunity (ETI) which is the second line of defence that is more specific and is in response to effector triggered susceptibility. However, not much is known about the differences in proteins that mediate these two interactions, and if different R-gene-effector interactions involve different downstream proteins. Some studies in this regards have been previously performed in Arabidopsis and Tomato but largely on the transcriptomic level. For instance, transcriptomic investigations in Arabidopsis indicated a large overlap in PTI and ETI signalling components (Tsuda and Katagiri, 2010). However, several other studies addressing functions of specific effector molecules have suggested that PTI and ETI responses are not mutually exclusive; PAMPs can also trigger ETI while effectors are not restricted to ETI responses (Thomma et al., 2011). A study in tomato has shown that there are several genes that are specific to ETI and PTI, while there was also a considerable overlap between these two processes, furthermore this study also resulted in the identification of an ETI specific serine/threonine tyrosine protein kinase (SlEpk1), silencing of which led to delayed PCD (Pombo et al., 2014). However large scale proteomics analysis of differences and similarities between PTI and ETIs induced by different R-gene – effector combinations has not been performed in any patho-system.

In this study we perform proteomic investigations of protein fractions obtained from PTI and two different ETI models in Potato. Protein samples for investigating PTI were obtained from Agrobacterium infiltrated leaflets (Manuscript VI Figure 1). Protein samples for investigating ETI interactions were obtained from two different Potato – *P. infestans* R gene-Avr gene combinations. One set of ETI samples were obtained from infiltration of

Agrobacterium transformed with IpiO into leaflets of Rpi-blb1 resistance gene containing Desirée plants (Manuscript VI Figure 1), while the other set of ETI samples were generated by infiltration of Agrobacterium transformed with Avr2 into leaflets of Desirée containing an R2 type resistance gene.

Agrobacterium is known to possess several PAMPs such as Ef-Tu, peptidoglycans and muropeptides, all of which have the potential to induce PTI responses (Erbs et al., 2008). A study in Arabidopsis has shown that elf26 (well characterized PAMP) and agrobacterium induce expression of a similar set of genes. However, information about the full spectrum of agrobacterium PAMPs that induce PTI remains unknown (Rico et al., 2010).

Molecular information with regards to IpiO-Rpi-Blb1 mediated ETI interaction is also limited. In the *Phytophthora brassicae* interaction with Arabidopsis, it has shown that IpiO can bind to a receptor like kinase (RLK) LecRK-I.9, which is a cell wall-plasma membrane adhesion protein (Gouget et al., 2006). Arabidopsis mutants overexpressing LecRK-I.9 show callose deposition and resistance to *Phytophthora brassicae* (Bouwmeester et al., 2011). These results suggest that the role of IpiO is to alter host cell wall-membrane continuum and callose deposition in order to establish infection. In Potato plants expressing Rpi-blb1 resistance gene, IpiO has been shown to act as an avirulence factor since this interaction has been shown to lead to HR (Vleeshouwers et al., 2008). Information regarding downstream molecular signalling due to Avr2-R2 interaction is also limited. However, it has been shown that host BSU-LIKE PROTEIN 1 (BSL1) that is involved in brassinosteroid mediated signal transduction is essential for interaction between AVR2 and host R2 resistance gene (Saunders et al., 2012).

All the infiltrated leaflets were phenotyped, 3 days post infiltration. A strong and even cell death was observed that coincided with infiltrated area in both the ETI interactions (Manuscript VI Figure 1). In contrast, Agrobacterium infiltrated leaflets (PTI) showed only occasionally small areas of cell necrosis in the infiltrated zone (Manuscript VI Figure 1) while leaflets infiltrated with infiltration media did not shown any type of cell death symptoms (Manuscript VI Figure 1). The cell death observed in the ETI interaction can be associated to an HR reaction.

In order to increase protein identification and coverage, and also to make an attempt to understand subcellular regulation of PTI and ETI, a modified protocol that enables extraction of proteins from various subcellular fractions

was employed (see Materials and Methods section of Manuscript VI for more details). The protocol is based on successive centrifugation steps wherein supernatant at each step is extracted into a different buffer leading to four different buffers containing four different protein fractions, namely - CEB, MEB, NEB, and CNEB fractions. A gel based analysis was performed to identify differences in the profiles of proteins present in each fraction. Banding pattern analysis revealed that the protein profiles were indeed different (Manuscript VI Figure 2), indicating that this protocol resulted in isolation of different protein fractions. For instance, a band associated with histones was identified in the CNEB fraction indicating that proteins associated with chromatin were successfully extracted in the CNEB fraction (Manuscript VI Figure 2).

Considerably higher amount of protein concentration in the membrane fraction (MEB; average of 150 ug), and the growing evidence of the importance of membrane associated proteins (especially plasma membrane proteins) in plant pathogen interactions (Elmore et al., 2012) led us to further analyse this fraction in detail. In comparison to the MEB fraction, the NEB fraction yielded lower amounts of protein. However, gel based analysis indicated that this fraction had a relatively distinct protein profile in comparison to other fractions, suggesting that NEB fraction, contains potentially interesting proteins. In addition, nuclear proteins have also been implicated to play an important role in plant defence signalling (Motion et al., 2015). Therefore, in addition to the MEB fraction, the NEB fraction was also chosen for further investigation.

Comparative analysis of protein abundances in the MEB fraction revealed that there was almost 50% overlap in proteins that significantly changed in abundance between the PTI and ETI interactions (Manuscript VI Figure 3). There was also a large overlap in proteins that significantly changed in abundance between the two different ETI interactions (Manuscript VI Figure 3). A comparative analysis of proteins mediated by two different ETI interactions has not been performed previously, and this data indicates that similar signalling components are upregulated and downregulated by different R-gene - Avr interactions. Further analysis of MEB proteins regulated in the PTI interaction revealed upregulation of an LRR like receptor protein kinase (Manuscript VI Table 1) was also upregulated in ETI. A recent report has suggested that the expression of this kinase in Arabidopsis correlates with auxin (Wu et al., 2015), this particular kinase could also be an interesting target for sustainable resistance in the future as it upregulated in both PTI and ETI. In

addition, a translationally-controlled tumor protein homolog (Manuscript VI Table 1) and TAO1 (Manuscript VI Table 1) proteins were also upregulated, both of which have been shown to be induced in response to effectors produced by the gram negative bacterium *Pseudomonas syringae* in Arabidopsis (Jones et al., 2006, Eitas et al., 2008). A protein annotated as glycolate oxidase (Manuscript VI Table 1) was specifically upregulated in the PTI interaction. Leaf glycolate oxidase has been previously associated with increased production of hydrogen peroxide in response to stress (Mhamdi and Noctor, 2015). Further evidence of hydrogen peroxide induced molecular signalling is indicated by the increased abundance of a peroxidase protein (Manuscript VI Table 1) during PTI and ETI interactions. Plant peroxidases use hydrogen peroxide as a substrate to catalyse the oxidation of a number of different substances (Almagro et al., 2009). Another protein annotated as ABC-transporter like (Manuscript VI Table 1) increased in abundance specifically in PTI interaction, in Arabidopsis a homologue of this transporter has been shown to be induced in response to oxidative stress (Manara et al., 2014). A MAR binding filament 1 (Manuscript VI Table 1) protein was also upregulated specifically in the PTI interaction. This protein has been shown to be induced in response to COS-OGA elicitor treatment in tomato; furthermore COS-OGA is also induces hydrogen peroxide burst in Arabidopsis cell cultures and suspensions (Ledoux et al., 2014). Upregulation of all the above mentioned proteins suggests induction of oxidative burst that has been shown to be one of the processes associated with PTI. Analysis of the 47 proteins that were downregulated in the MEB fraction during PTI interaction (Manuscript VI Figure 4) revealed presence of several (26 proteins) photosynthesis related proteins, which is in coherence with previous reports that have suggested repression of photosynthesis during pathogen infection (Attaran et al., 2014).

Analysis of MEB proteins regulated specifically in the ETI interaction revealed significant increase in abundance of two superoxide dismutases (Manuscript VI Table 3). In plants, superoxide dismutases protect organelles and metabolic processes from destruction by the reactive oxygen species (ROS) produced in response to stress (Alscher et al., 2002). Further indication of active protection to ROS generation specifically during ETI is indicated by upregulation of a chloroplastic lipocalin (Manuscript VI Table 3) that has previously been shown to provide ROS tolerance (Charron et al., 2008). While ROS production seems to characterize PTI, tolerance to ROS production seems to be specifically induced in ETI.

Two proteins associated with guanosine triphosphate (GTP) binding activity (Manuscript VI Table 3) were upregulated only during ETI interactions. Although a significant role of heterotrimeric G proteins in plant innate immunity has been proposed (Trusov and Botella, 2012), these results indicate that they are specifically involved in the ETI component of immunity in Potato. Although we identified considerable overlap between proteins regulated by different R-gene- Avr interactions in the MEB fraction, there were also proteins that were regulated specifically in each ETI interaction. Phospholipase A1 (Manuscript VI Table 3) was specifically upregulated in the ETI-Avr2 interaction. This protein belongs to a class of DAD (Defective in Anther Dehiscence) like proteins that are involved in JA synthesis (Canonne et al., 2011), indicating a probable role of JA in this ETI interaction. Another protein annotated as a Heat shock protein 70-3 (Manuscript VI Table 3) was also specifically regulated by ETI-Avr2 interaction. This is a cGMP dependent protein that is upregulated in response to stress induced hydrogen peroxide production (Marondedze et al., 2013), again underpinning the role of G-protein signalling in ETI. Specific proteins regulated by the ETI-IpiO interaction were fewer in comparison to the ETI-Avr2 interaction (Manuscript VI Figure 4). One of them was a serine /threonine protein kinase (Manuscript VI Table 3) that was specifically identified in IpiO induced ETI. The closest Arabidopsis homolog is a plastid localised STN7 protein kinase that is known to regulate ROS signalling by maintaining redox balance and photosynthetic activity during stress (Mittler et al., 2011).

Analysis of the NEB fraction also revealed that there was a large overlap in proteins that were significantly regulated in PTI and ETI interaction; in addition there was also a large overlap between proteins regulated by ETI mediated by two different R gene-Avr interactions (Manuscript VI Figure 4). A Proteinase inhibitor 1 (Manuscript VI Table 2) was upregulated in the PTI interaction; it also increased in abundance in both the ETI interactions too. Proteinase inhibitors are induced in response to a variety of bacterial and fungal pathogens (Loon et al., 2006), indicating that it constitutes general antimicrobial defence response in the plant. Further evidence of activation of proteins associated with general antimicrobial defence is exemplified by Snakin-2 (Manuscript VI Table 2) upregulation, that has also been shown to possess antimicrobial activity (Balaji and Smart, 2012). Surprisingly, a large number of proteins related to cell wall degradation such as Beta-galactosidase (Manuscript VI Table 2), Alpha-glucosidase (Manuscript VI Table 2), Pectinesterase (Manuscript VI Table 2) and Expansin like protein (Manuscript VI Table 2) were also upregulated in the PTI interaction. A protein annotated

as DUF26 domain-containing protein 1 (Manuscript VI Table 2) was specifically induced in the PTI interaction. DUF26 domain containing proteins have been shown to be induced in response to various defence signals. Specifically *HvCRK1*,a DUF26 domain containing protein has been shown to regulate basal resistance, but not R gene dependent programmed cell death in Barley (Rayapuram et al., 2012), which is in coherence with our observation of DUF26 domain containing protein only in the PTI interaction.

A large number of proteins in the NEB fraction that were regulated in ETI were also identified in the PTI interaction (Manuscript VI Figure 4). However, we also identified several proteins that were specifically regulated in the ETI interaction but not in PTI. Heat shock protein 70-3 (Manuscript VI Table 4) was upregulated only in the ETI interaction. Homologs to this protein have been previously implicated to interact with different NB-LRR R proteins (Lukasik and Takken, 2009), and this is in line with our observation only in the ETI interaction. A putative multiprotein bridging factor 1 (Manuscript VI Table 4) was also specifically induced in ETI interactions. It has been hypothesized that multiprotein bridging factor is a hub that links ROS signalling and pathogen stress response (Miller et al., 2008). The influence of multiprotein bridging factor protein-complex on lipid metabolism has been shown (Miller et al., 2008). In coherence with this, proteins related to lipid metabolism (Manuscript VI Table 4) annotated as a lipase and esterase respectively were also specifically regulated in the ETI interactions. Both lipases and esterases have also been previously identified to be associated with R - gene mediated defence response (Shah, 2005). Increased abundance of lipase also correlates with the phenotypic observation of hypersensitive response in the ETI interaction (Manuscript VI, Figure 1), and this role of lipases has also been previously shown (Lam, 2004). SBT4E (Manuscript VI Table 4), a protein with subtilisin like protease activity was also specifically induced in ETI interactions. Subtilisin like proteases are known to contribute to stress induced hypersensitive response molecular signalling (Dickman and Fluhr, 2013). Proteins (Manuscript VI Table 4), with RNA binding activity were also specifically induced in the ETI interaction. The role of RNA binding proteins in regulating plant immune processes have been identified previously in numerous studies (Woloshen et al., 2011). One of these proteins is AKIP1. Recently it was shown that transient expression of one of the Potato transcripts that encodes an AKIP in tobacco leaves leads to HR cell death phenotype (Na et al., 2015). Our observation of AKIP1 and SBT4E specifically in ETI interaction is in line with HR cell death development observed in the ETI interaction 3 dpi (Manuscript VI, Figure 1). A protein annotated as Fiber

protein Fb19 (Manuscript VI Table 4) containing a universal stress domain protein was also specifically regulated in the ETI interaction, proteins containing universal stress domain have been previously implicated in protecting cells from ROS produced during stress (Loukehaich et al., 2012). In addition to this protein, a catalase (Manuscript VI Table 4), another protein involved in oxidative stress protection was also specifically regulated in the ETI interaction. In combination, these results further indicate processes linked to tolerance to oxidative stress are specifically regulated in ETI.

Nineteen proteins in ETI interaction mediated by IpiO and 32 proteins in Avr2 mediated ETI were found to be uniquely upregulated, respectively (Manuscript VI, Figure 4). A protease inhibitor related protein (Manuscript VI Table 4), a peptidase with an antifungal activity increased in abundance in IpiO induced ETI. A Bel1 like homeodomain transcription factor (Manuscript VI Table 4) that has also been previously implicated in plant defence responses (Sharma et al., 2014) was specifically upregulated in the IpiO mediated ETI. In Arabidopsis, this transcription factor has been shown to be a substrate for MPK3/6 dependent phosphorylation (Hoehenwarter et al., 2012). MPK3/6 dependent molecular signalling is also a crucial component of pathogen induced MAP kinase signalling pathway, but mainly associated with PTI (Meng and Zhang, 2013). In contrast, EMB1507 (Manuscript VI Table 4) was specifically upregulated in the Avr2 induced ETI. This protein is a ribonucleoprotein, the Arabidopsis homolog of this protein has been shown to be associated with the MOS4 complex to mediate R gene dependent defence responses (Monaghan et al., 2009). EMB1507 has an RNA helicase activity two other proteins specifically identified in Avr2 induced ETI have RNA binding activity; identification of these three proteins, in addition to another upregulated RNA helicase protein (P400015859; Manuscript VI Table 4) suggests a more prominent role of RNA dependent regulation of immune responses in Avr2 induced ETI.

5 Conclusions

5.1 BABA mediated induced resistance

Lab-based pathogen infection assays show that at 10 mM, BABA effectively protects Potato plants against late blight disease caused by *Phytophthora infestans*. Transcriptomic and proteomic analysis of leaflets sampled 48 hours after BABA treatment revealed that 10 mM BABA regulates more transcripts in comparison to 1 mM BABA, which is in line with the observed protection against *P. infestans.* Approximately 5000 transcripts alter in response to 10 mM BABA; these transcripts are associated with a range of cellular functions that include stress responses and primary metabolism. Proteomic analysis of the secreted protein fraction from leaflets sampled from BABA treated plants at both 1 mM and 10 mM concentration revealed increased abundance of protein markers that signify activation of plant defence, one such example is PR-1. In addition, there were other proteins like the citrate binding protein that increased in the apoplast in response to 10 mM BABA treatment, whose annotation does not indicate direct relation to plant defence.

5.2 Phi mediated induced resistance

At the concentration tested, Proalexin (Potassium phosphite; Phi) renders Potato plants resistant to infection by *P. infestans* as observed from detached leaflet assay. Phi mediated resistance to *P. infestans* is durable as plants infected 5 days after Phi spray also stay protected. We observed that Phi is mobile and rapidly translocates systemically; however systemically translocated Phi does not seem to induce protection to *P. infestans*. Phi mediated induced resistance cannot be attributed to interference with phosphate metabolism as phosphate levels did not change in Phi treated plants. Phi has a

rapid and transient effect on the transcriptome, Phi induced transcriptional activity was observed at 3, 6 and 11 hours post treatment, while no significant transcriptional activity was observed at later time-points. In similarity to BABA, Phi induced transcripts that were related to a myriad of plant processes, specifically higher number of immune and defence related transcripts were induced rapidly (3 hours) after Phi treatment. Transcripts related to cell wall metabolism were repressed 11 hours after Phi treatment. Phi treatment also induced the expression of a transcript related to citrate binding protein 6 hours after treatment; this protein was also observed in BABA treated plants. In fact, a 50% overlap in transcripts regulated by 10 mM BABA and transcripts regulated by Phi 3 hours after application was observed, this overlap went down to 30% between 10 mM BABA and Phi 11 hours after application. Regardless, transcripts that were regulated by both the inducers show high correlation in their expression levels. Although transcriptomic data indicates induction of transcripts and processes related to SA and JA, Phi gave protection in both SA deficient and *coi1* silenced JA insensitive plants to *Phytophthora infestans*.

5.3 Potato-*Dickeya solani* interactions

In order to perform molecular analysis of interactions between Potato and *D. solani* as well as to screen for blackleg disease responses from large plant populations, an assay based on *in vitro* grown Potato plants was developed. Potato genotypes tested with both the conventional greenhouse screening assay and *in vitro* assay responded similarly to *D. solani*. As proof of concept, this *in vitro* assay was used to screen disease development on a crossing population obtained from a cross between moderately resistant and susceptible genotypes. A continuum in disease development was observed suggesting multi-genic control of resistance to *D. solani* in the tested population. Further statistical analysis of the infection scores resulted in two groups, a moderately resistant and a susceptible group that differed significantly in their responses to *D. solani*. The transcriptome of each genotype from these populations was sequenced. A trait association analysis was performed to identify transcripts that significantly differ between the moderately resistant and susceptible groups. This analysis yielded close to 20 transcripts that were only induced in the genotypes that belonged to susceptible group while were not induced or absent in the moderately resistant group. Since these transcripts were induced only in the genotypes belonging to the susceptible group, they were considered as potential susceptibility factors, i.e. transcripts that potentially facilitate susceptibility. Analysis of these transcripts revealed that a majority of them

were related to transcriptional regulation. In order to functionally validate these potential susceptibility transcripts, we developed an assay to screen disease response of *Nicotiana benthamiana* plants to *D. solani*. We propose a method, which involves identification of homologs of "susceptibility factors" in *N. benthamiana*, silencing the "susceptibility factors" using virus induced gene silencing (VIGS) and screening for *D. solani* response of the VIGS and control plants. We also used the *in vitro* assay to study molecular aspects of Potato–*D. solani* interactions by screening blackleg disease response of salicylic acid deficient *NahG* and jasmonic insensitive *coi1* Potato plants. Analysis of these results revealed that both salicylic acid and jasmonic acid pathways are necessary for mediating resistance to *D. solani* in Potato. A similar result was observed with regards to soft rot disease of tubers caused by *D. solani*.

5.4 Potato-*Alternaria solani* interactions

The role of SA and COI1 in Potato defence to *A. solani* was previously not known; to address this question we used a combination of pathogenicity assays, pathogen biomass and hormone level quantifications as well as a time-series transcriptome analysis. Pathogenicity assays revealed that SA is necessary for defence to *A. solani* as salicylic acid deficient *NahG* plants had larger lesions in comparison to *coi1* and wildtype cv. Désirée plants 10 days post inoculation. SA was also induced in inoculated *coi1* and wildtype cv. Désirée plants. However, pathogen biomass quantification at an earlier time point, 5 days post inoculation revealed that there was significantly higher pathogen biomass in inoculated *coi1* and *NahG* plants in comparison to wildtype cv. Désirée plants. Furthermore JA was induced in all inoculated plants. Therefore, while SA is necessary for symptom development and pathogen growth restriction, COI1 seems to be only involved in early pathogen growth restriction. Transcriptomic analysis of inoculated and control plants indicates that wildtype cv. Désirée plants induce transcripts related to plant defence rapidly i.e. 24 hours after inoculation while the same transcripts are not observed in inoculated hormone related transgenic plants, indicating that many transcripts activated at early time points are SA or COI1 dependent. At later time points (72 and 120 hours post inoculation), transcripts related to cyclopentenone response and repression of auxin signalling were observed only in inoculated *coi1* and *NahG* plants. Since we found that SA had an important role in defence response to necrotrophic pathogen *A. solani* which is in contrast to the documented role of salicylic acid in pathogen defence, we further investigated transcripts induced by pathogen inoculation but at the same

time repressed by SA. One of the most prominent transcripts was annotated as citrate binding protein (CBP); this transcript was highly induced in inoculated *NahG* plants in comparison to mock inoculated plants during later stages of infection, with no significant differences in induction in inoculated and mock-inoculated wild type and *coi1* plants. We hypothesized that this transcript facilitates susceptibility to *A. solani*. In order to test this hypothesis, transgenic Potato plants that either overexpress CBP or are silenced (RNAi) in CBP expression were generated. *A. solani* inoculation of the transgenic plants revealed that CBP overexpressing lines were significantly more susceptible in comparison to CBP silenced and wildtype cv. Désirée plants confirming the role of CBP as a susceptibility factor.

5.5 Comparative proteomics of PAMP (PTI) and Effector triggered immunity (ETI) in Potato

Information about proteins that regulate PTI and ETI responses in Potato is limited. In this regards, proteomic analysis from Agrobacterium-infiltrated Potato leaflets that served as a PTI model was performed. Identified proteins were compared with those that were obtained from investigation of Agro-infiltration based ETI interactions mediated by 2 different R-gene - Avr gene interactions, namely Rpi-blb1 interaction with the avirulence effector IpIO and R2 gene interaction with the corresponding avirulence effector Avr2. Phenotypic analysis of the infiltrated leaflets revealed consistent cell death in the entire infiltration zone in the ETI interactions. A considerable overlap among significantly regulated proteins mediating PTI and ETI interactions was observed. Furthermore, this large overlap was also observed among proteins regulated by 2 different R-gene–Avr interactions. Analysis revealed that proteins related to oxidative stress, general antimicrobial defence, cell wall metabolism were upregulated in the PTI interaction. Analysis of the ETI interaction indicated that there was increased abundance in proteins related to oxidative stress tolerance. In addition, proteins with GTP binding activity were also specifically upregulated in the ETI interactions, significant upregulation of proteins with RNA binding activity and lipid metabolism was also observed specifically in the ETI interactions. Specific differences in protein regulation between ETI mediated by IpiO and AVR2 were also identified. Transcription factors STN7 and Bel1, chloroplastic lipocalin and a protease inhibitor related protein were specifically upregulated in IpiO mediated ETI, while a phospholipase, heat shock protein 70-3 and several RNA binding proteins including EMB1507 were specifically upregulated in AVR2 mediated ETI.

6 Future Perspectives

- Recent studies have identified *impaired in BABA-induced Immunity 1* (*IBI1*) gene, a post-transcriptionally regulated aspartyl-tRNA synthetase (AspRS) as the receptor that perceives and mediates BABA dependent priming in Arabidopsis. How is BABA perceived in Potato? To check if it is indeed a functional *IBI1* homolog in Potato, one should assay cellular levels of free aspartic acid in BABA treated plants as it has been shown that BABA blocks AspRS activity resulting in increased aspartic acid levels. If it is *IBI1* homolog in Potato, then these studies can be complemented with transgenic silencing and investigating its effect on BABA dependent induced resistance.

- In order to get a better understanding of transcriptional status associated with the "induced state" of Potato defence, it would be worthwhile to perform a time course BABA transcriptomic experiment similar to the one we performed using Phi. This could also help in identifying if BABA has a transient transcriptomic effect like Phi, if yes then is quick transient effects on transcriptome a feature of induced resistance?

- It will also be interesting to test if significantly higher protection in the field can be obtained by combining Phosphite and BABA with or without reduced doses of fungicides.

- Our studies with regards to Phi have shown that Phi activates genes related to defence and induces protection in Potato to *Phytophthora infestans*. However, it is also know that Phi is metabolically inert *in planta*, the question is what precisely links Phi molecule´s presence to

defence activation? Is phosphate starvation response (PSR) the link? Although we do not observe changes in phosphate levels of Phi treated plants, we did identify induction of transcripts related to PSR. Hammond et al. (2011) identified 200 transcripts that characterize phosphate stress in Potato plants, 90 transcripts regulated by Phi were also found in this list of 200 transcripts. Therefore, Phi could induce transient PSR that leads to induction of resistance. In order to test this, *P. infestans* response of phosphate starved (gradient in starvation) and optimally fertilized Potato plants should be performed. If phosphate starved plants show improved resistance in comparison to *P. infestans*, then further experiments need to be performed to identify the link between Phi and PSR.

- Further experiments can also be performed with regards to the role of plant hormones in mediating Phi dependent induced resistance in Potato. Present knowledge indicates that hormonal regulation in response to Phi depends on the patho-system. Salicylic acid (SA) regulates Phi dependent induced resistance in Arabidopsis against *Hyaloperonospora arabidopsidis* while auxin and abscisic acid partly regulate the same in Arabidopsis response to *Phytophthora cinnamomi*. Our results in Potato indicate that at the Phi concentration tested, SA and COI1 signalling might act in a compensatory manner. However, this can be further explored by treating SA deficient and *coi1* silenced plants with lower Phi concentration and assaying disease response of hormone transgenic plants.

- Can specific transcripts from the transcriptome data in relation to resistance induction be used as markers (maybe translated to DNA markers) to identify genotypes that can better respond to induced resistance? Can breeding programs select for “enhanced inducibility”?

- The aim with the *in vitro* based blackleg disease screening assay is to test large plant populations. Although the assay has shown promise in this regards, there is opportunities in improving its throughput capacity and in exploring options that could automate phenotyping/scoring. Another aspect of the *in vitro* assay that has the potential to be explored is the association between symptoms and pathogen inoculum. Does the level of pathogen biomass correlate with the symptoms observed? If the pathogen inoculum correlates with the

phenotype, then the *in vitro* assay can be combined with a PCR based method to simplify disease scoring.

- Associating plant phenotypic traits such as architecture with *Dickeya solani* response is an area of research that can be further investigated. Several traits relating to stem architecture of genotypes that were tested for *D. solani* response were also measured. Preliminary analysis indicates that infection score is correlated to stem width (Unpublished data). This means that plants with wider stems show a higher probability of susceptibility to *D. solani*. In this regards, the first step is to identify the connection between putative blackleg disease susceptibility genes identified in our analysis and plant architecture/ development. Further investigations by analysing the effect of silencing these genes on plant phenotype and *D. solani* response could be an approach to elucidate this connection between stem width and *D. solani* susceptibility in greater detail.

- In the experiments focused on studying molecular aspects of interaction between *Alternaria solani* and Potato, we observed that both salicylic acid (SA) and COI1 (that is dependent on jasmonic acid) are necessary for mediating resistance. It is also known that resistance to *Alternaria solani* is dependent on maturity and age; early maturing genotypes are susceptible while late maturing ones are moderately resistant, and therefore does salicylic acid and COI1 dependent defence connect ageing and resistance to *A. solani*? Investigation of transcriptomic/proteomic differences in SA and COI1 dependent defence responses before and after *A. solani* infection in early and late maturing genotypes could provide answers to the link between maturity, SA/COI1 defence and *A . solani* resistance.

- We show that citrate binding protein (CBP) susceptibility factor to *Alternaria solani*. Is it really repressed by SA? The only known role of homologs to this protein is its ability to bind citrate, citrate has been shown as a nutrient source by several other bacterial pathogens during infection, is that also the case with *Alternaria solani*? If it is, then does salicylic acid regulate this process by inhibiting citrate availability to the pathogen, which is indeed observed from lower pathogen growth in wildtype plants in comparison to inoculated salicylic acid deficient *NahG* plants. An experiment to test this hypothesis could be to measure citrate levels before and after infection in *NahG*, CBP

overexpressor´s, CBP RNAi knockout and wildtype plants and correlate this to pathogen growth.

- The prospects of testing if Phi and BABA induce resistance to blackleg and early blight disease can also be further explored.

- Proteomic investigation of IpiO mediated Effector triggered immunity (ETI) revealed specific downregulation of a chitin binding lectin. Cell walls of oomycetes like *Phytophthora infestans* are composed of cellulose unlike fungi whose cell walls are made of chitin. It is maybe surprising to identify downregulation of this lectin only in a specific interaction between a *P. infestans* effector IpiO and corresponding R-gene (Rpi-blb1). One hypothesis that can be tested is that this protein acts as an effector target. It has been previously shown that Avr4 effector from the fungus *Cladosporum fulvum* is a lectin with a chitin binding domain, and during infection, Avr4 produced by *C. fulvum* is coated on the cell walls to protect them from plant chitinases and escape detection from the immune system (van Esse et al., 2007). Does the *P. infestans* effector IpiO recruit this chitin binding lectin? If so, does this R-gene function by inhibiting this recruiting process? A first step to test this hypothesis can be to compare the expression of this lectin in a susceptible and a resistance (Rpi-blb1 - IpiO) *P. infestans* interaction. If they are regulated in the opposite direction, wherein this lectin is highly induced in the susceptible interaction while is repressed in the resistant interaction, then further tests like a transient expression of the effector and this lectin and subsequent *P. infestans* infection in *Nicotiana benthamiana* system or *P. infestans* response of knockout/overexpressors in Potato can be performed.

- Studies aimed at addressing the interaction between late blight, early blight and blackleg disease can be performed. Under field conditions in Sweden, there is probability of occurrence of each of these diseases; therefore it will be interesting to study if the interaction between pathogens causing these diseases is cooperative or antagonistic. Modelling this interaction in relation to plant and soil microbiome can help in developing improved disease management programs.

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