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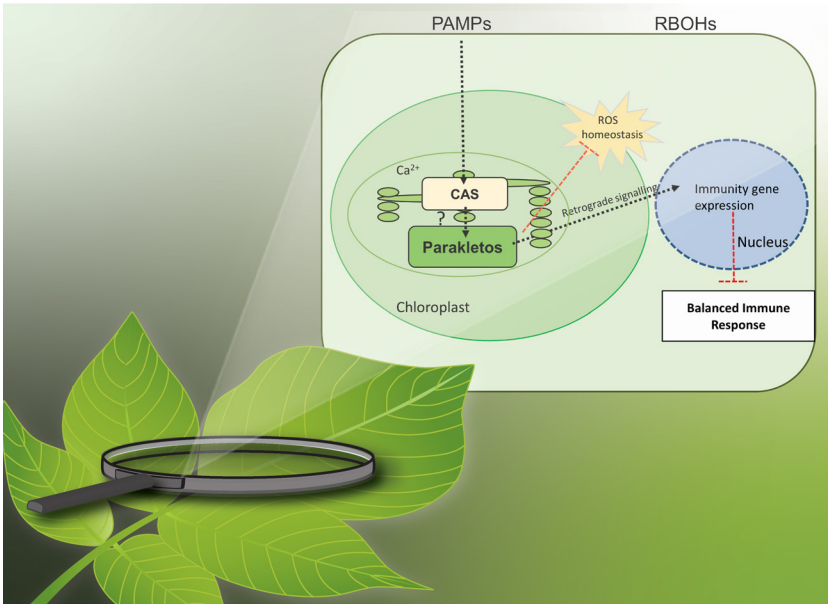
Insights into potato plant immunity reveals Parakletos as a novel ROS suppressor

Muhammad Awais Zahid



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Cover: A potato leaf and a schematic diagram illustrating: suggested role of Parakletos after flg22 perception in wild type plant (Illustration of leaf by Susanne Johansson and schematic diagram by Muhammad Awais Zahid)

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Abstract

Potato (*Solanum tuberosum L.*) is the world's third most commonly grown food crop with very high yield potential. However, its production is hampered by several pathogens, with consequent yield losses. Existing control methods include frequent, costly fungicide applications, and classical breeding is complicated in potato. One way to develop new long-term solutions is to improve understanding of plant molecular immunity and factors capable of providing broad-spectrum resistance. To assist such efforts, comparative proteomic techniques were applied to enhance understanding of changes in protein abundance during the immune responses of potato. These focused on a PTI (Pattern-triggered immunity) model and two ETI (Effector-triggered immunity) models (both related to resistance genes to *Phytophthora infestans*, the causal agent of late blight) in potato to enhance understanding of changes in protein abundance during its immune responses. Numerous proteins increased in abundance in all observed immune responses, including one identified as sterol transporter protein 2, which is intriguing because sterol content plays an important role in plant immunity, and oomycete pathogens like *P. infestans* rely on their hosts for sterols. The abundance of RNA binding proteins also changed in different immune reactions. A few proteins changed in abundance in only one of the ETI models, e.g., histones were downregulated in one (ETI responses to *P. infestans* effector Avr2), whereas a putative multiprotein bridging factor was upregulated in the other (ETI responses to *P. infestans* effector IpiO). Intriguingly, the proteomic differences between the two ETI models were of similar magnitude to those between the ETI models and PTI. The next step was to study the general PTI-related defence potato proteome by two-step fractionation and new bioinformatics analyses. Five candidates with potential importance in our proteomic dataset were selected for functional validation studies. To facilitate validation studies, a luminol-based assay was developed to study biphasic reactive oxygen species (ROS) bursts in potato leaves induced by flg22 and *P. infestans* as well as an easy, cost-effective method using near-IR scanning to quantify leaf wounding and disease lesion areas without damaging leaves. Over-expression and silencing of one identified protein, named Parakletos, respectively increased and reduced *P. infestans* infection in *Nicotiana benthamiana*. Moreover, its overexpression suppressed the ROS burst response to flg22, while its silencing increased it. Transcript analyses showed upregulation of defence-related genes (e.g., *ICS1*, *PR1*, *PTI5*, and *RBOHB*) in response to flg22 in *Parakletos*-silenced plants. Expression of light-harvesting complex B6 (*LHCB6*) was also enhanced in plants overexpressing *Parakletos*. It was found that Parakletos co-localized with the Calcium Sensing Receptor (CAS) in chloroplasts, and that it is functionally dependent on CAS. Furthermore, CRISPR/Cas9-mediated knock-out (KO) of *parakletos* in potato enhanced broad-spectrum resistance to late and early blight in controlled condition. It also reduced infection to *Pseudomonas syringae* in *N. benthamiana*. Moreover, during field trials the *parakletos*-KO lines showed enhanced resistance to *P. infestans*. These findings contribute to our understanding of plant immunity and provide a new susceptibility-gene based strategy for building broad-spectrum disease resistance in crops.

Keywords: Potato, plant immunity, PTI, ETI, ROS, *Phytophthora infestans*, Parakletos

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Dedication

I dedicate this thesis to my parents for their love, support, and encouragement.

Contents

List of publications

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

I. Resjö, S[*]., **Zahid, M.A**[*]., Burra, D.D., Lenman, M., Levander, F. and Andreasson, E[a]., 2019. Proteomics of PTI and two ETI immune reactions in potato leaves. International journal of molecular sciences, 20(19), p.4726.

II. **Zahid, M.A**., Lenman, M., Resjö, S., Carlsen, F.M., Konakalla, N.C., Petersen, B.L., Vetukuri, R., Kieu, N.R., Andreasson E., 2021. Parakletos: A novel ROS suppressor involved in broad spectrum pathogen resistance, and CRISPR/Cas9 gene knock out enhanced *Phytophthora* resistance in field grown potatoes. (Manuscript)

III. **Zahid, M.A**[a]., Vetukuri, R.R, Andreasson E., 2021. A quantitative luminol-based assay for ROS burst detection in potato leaves in response to biotic stimuli. Methods in Molecular Biology, In Press.

IV. **Zahid, M.A**[a]., Sandroni, M., Vetukuri, R.R. and Andreasson, E., 2021. A fast, nondestructive method for the detection of disease-related lesions and wounded leaves. BioTechniques, 71(2), pp.425-430.

[*] Equally contributing authors

[a] Corresponding author

The contribution of Muhammad Awais Zahid to the papers included in this thesis was as follows:

I. Contributed to parts of the study, performed the laboratory experiments, and wrote the manuscript together with co-authors.

II. Designed the study together with co-authors. Planned and performed the experimental work with co-authors. Wrote the manuscript with input of the co-authors.

III. Designed the study together with co-authors. Planned and performed the experimental work. Wrote the manuscript with input of the co-authors.

IV. Designed the study together with co-authors. Planned and performed the experimental work. Wrote the manuscript with input of the co-authors.

Muhammad Awais Zahid also contributed to studies presented in the following articles that are not included in this thesis.

I. Iqbal, M., Jamshaid, M., **Zahid, M.A**., Andreasson, E., Vetukuri, R.R. and Stenberg, J.A., 2021. Biological control of strawberry crown rot, root rot and grey mould by the beneficial fungus *Aureobasidium pullulans*. BioControl, pp.1-11.

II. Kalyandurg, P.B., Sundararajan, P., Dubey, M., Ghadamgahi, F., **Zahid, M.A**., Whisson, S. and Vetukuri, R.R., 2021. Spray-induced gene silencing as a potential tool to control potato late blight disease. Phytopathology, 02-21-0054-SC.

Abbreviations

cROS	Chloroplastic ROS
CAS	Calcium-sensing receptor
Ca^{+}	Calcium
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
HR	Hypersensitive response
NOX	NADPH oxidase
PR	Pathogenesis-related
PRR	PAMP recognition receptor
PTI	PAMP-triggered immunity
PSI	Photosystem I
PSII	Photosystem II
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species

R-gene	Resistance gene
RBOH	Respiratory bust oxidase homolog protein
SA	Salicylic acid
VIGS	Virus-Induced Gene Silencing

1. Introduction

Potato is the third most consumed food crop globally, but it is severely affected by various pests and pathogens, resulting in significant crop losses (FAO 2014). Pests and pathogens are responsible for approximately 17% of yield losses in potato production worldwide (Savary *et al.* 2019). To feed the world's increasing population while maintaining the same land area for crop production, new sustainable ways must be developed and applied to control plant diseases. In Sweden, about 21% of all fungicides used are applied to potato crops, which are grown on less than 1% of the country's farming land (Eriksson *et al.* 2016). This heavy use of fungicide on potato crops is mainly to counter late blight disease caused by *Phytophthora infestans*.

To control late potato blight, farmers usually rely on intensive use of fungicides and, to a lesser extent, resistant cultivars. However, the use of fungicides poses environmental concerns and could lead to emergence of fungicide-resistant pathogen strains. Resistant cultivars are developed through introduction of resistance (R) genes, but virulent races of *P. infestans* have rapidly evolved that can overcome all major R genes, and classical breeding of potato is time-consuming and complex, partly because it is tetraploid (Fry 2008). Therefore, it is essential to develop alternative strategies to control late blight. A promising approach for developing new long-term solutions is to acquire deeper understanding of plant molecular immunity and seek factors that may confer broad-spectrum resistance.

This thesis is based on studies designed to enhance understanding of plant immunity through proteomic analyses of potato plants' PAMP-triggered immunity (PTI) responses, related to general plant immune responses, and

two effector-triggered immunity (ETI) responses specific to potato-*P. infestans* interactions (Resjö *et al.* 2019, Paper I). The studies also included functional characterization of candidate proteins identified in the proteomics analyses in the model plant *Nicotiana benthamiana*. To assist functional validation, the studies included development of a luminol-based assay for detecting bi-phasic ROS bursts (Zahid *et al.* 2022, Paper III) and a method for detecting disease-related lesions (Zahid *et al.* 2021, Paper IV). Screening of candidates resulted in discovery of a novel protein that was called 'Parakletos' (Ancient Greek for helper) and found to be negatively involved in plant immunity (Paper II).

In further studies, moving from the model plant back to the focal crop, *parakletos* knock-out (KO) mutant potato lines were generated using CRIPR/Cas9 technology. The resulting lines were studied in controlled conditions and their resistance to *P. infestans* and *Alternaria solani* was tested. Moreover, *parakletos*-KO lines were further tested in genetically modified organism (GMO) field trails.

2. Background

2.1 Potato

Potato (*Solanum tuberosum* L.) is the third most important food crop in the world after wheat and rice (FAO, 2014). Potatoes are grown in over 100 countries with diverse (tropical, subtropical, and temperate) climatic conditions. Potato has high nutritional value due to its high contents of carbohydrates, minerals, vitamins, and antioxidants (Lutaladio & Castaldi 2009). Because of its high yields and nutritional contents potato is an important food-security crop (Devaux *et al*. 2020).

The potatoes cultivated today originated from the Andean region of Peru, and wild relatives are still present there (Spooner et al. 2005). In 1532, potato was brought to Europe following an invasion of Peru led by Francisco Pizarro (Hawkes & Francisco-Ortega 1993). However, potato cultivation was not recorded until 1567, in the Canary Islands, and it subsequently spread throughout Europe, and then the rest of the world. Initially, potatoes were mostly grown as ornamental plants in botanical gardens, and it took more than a century for potato to become a major food crop. In Sweden, it was first planted in 1655 by Olof Rudbeck at the botanical garden in Uppsala. There was no documented cultivation of potato for the next 70 years anywhere else in Sweden, until Jonas Alströmer started to grow it on his farm in Alingsås, with material probably imported from England. This was the first serious attempt to grow potatoes for food in Sweden. Alströmer convinced Swedish farmers of the potential of potato cultivation (Bodensten 2021) and people in Sweden started to appreciate potatoes widely after the

Pomeranian war (1757-1762), when returning soldiers spread the word about potato, thereby increasing production. Another reason for the increase in potato production was the discovery by Eva de la Gardie (1748) that potatoes were excellent for making spirits, which led her to become the first female member of the Royal Swedish Academy of Sciences.

However, potato crops are vulnerable to various biotic and abiotic stressors. *Inter alia*, crop yields can be reduced by early blight, blackleg disease, and diseases caused by viruses (e.g., potato virus Y), yellow potato cyst nematode, and potato tuber moth. However, late blight causes unrivalled damage to potato crops.

2.2 Potato late blight

The devastating potato disease late blight was first reported on the American east coast in 1843, and it quickly spread not only in America but also elsewhere, including all over Europe. During the 19th century potato cultivation increased so substantially in Ireland that much of the Irish population became totally dependent on it. In 1845, late blight came to Ireland and caused one of the worst famines in European history, called the Irish Potato Famine. This led to the death of more than 1 million people, and migration of almost 1.2 million people to the USA and other countries. The Irish population has still not returned to levels before the ravages of late blight at this time. Late blight hit almost all of Europe, but it had the most devastating effect on Ireland, as potato was the staple food there (Andrivon 1996). At the beginning of 1860, Anton de Bary explained the life cycle of the pathogen, which he classified as a fungus that he called *Phytophthora infestans* ('plant destroyer' in Greek). Now *P. infestans* is classified as an oomycete rather than a fungus for several reasons. For example, like other oomycetes its cell walls consist of cellulose and other glucans rather than chitin as in true fungi. *Phytophthora infestans* is a hemibiotrophic pathogen. During a biotrophic phase it takes nutrients from a host plant and forms a specified feeding structure. After this phase it enters a necrotrophic phase in which it kills host tissues (Grünwald & Flier 2005). It's been 170 years since the first, and worst, epidemic of late blight in Europe, but farmers still expend a lot of energy in controlling it, and scientists have also been trying to

understand it for many years. In efforts to control late blight, farmers in relatively high-income countries usually rely on fungicides. Estimated annual global financial losses due to late blight amount to 7 billion euros (Haverkort *et al.* 2008; Haverkort *et al.* 2016). In Sweden, potato cultivation accounts for ca. 1% of the total area of cultivated agricultural land, but ca. 21% of the fungicide used in Swedish agriculture. This high use of fungicide on just 1% of total cultivated land is mainly to counter late blight disease. To avoid long-term risks to human health and the environment from the excessive use of synthetic chemicals, current research efforts are focused on developing sustainable crop protection methods, including developing resistant cultivars, biological control and spray induced gene silencing (Kalyandurg *et al.* 2021). For any sustainable methods to work efficiently it is also very important to understand plant immunity.

2.3 Plant innate immunity

In nature, plants are prime targets for microbial pathogens as they are rich sources of energy and nutrients. Microbial pathogens, such as viruses, bacteria, fungi, and oomycetes attack plants by employing diverse strategies that affect their growth. Plants, unlike animals, lack an adaptive immune system, but they have evolved two inter-connected innate immune systems (Jones & Dangl 2006; Zhou & Zhang 2020; Yuan *et al.* 2021b), described below, that perceive and respond to attacking pathogens.

2.3.1 Two tiers of plant immunity

The first line of plant defence is activated upon recognition of pathogen-associated molecular patterns (PAMPs) through membrane-bound pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI) responses (Jones & Dangl 2006; Couto & Zipfel 2016). All PRRs identified to date are members of receptor-like kinase (RLK) or receptor-like protein (RLP) families (Boutrot & Zipfel 2017). One of the well-characterized PRRs in Arabidopsis is FLAGELLIN-SENSING 2 (FLS2), which recognizes epitopes of the bacterial peptide flg22 from flagellin. Another is the EF-TU receptor (EFR), which recognizes part (elf18) of the bacterial elongation factor Tu (Bigeard *et al.* 2015). For functionality, both EFR and FLS2 require

co-receptor BRI1-ASSOCIATED RECEPTOR-LIKE KINASE/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3), which is essential for downstream PTI signalling (Tang *et al.* 2017). Activation of PTI results in reactive oxygen species (ROS) bursts, calcium influx, callose deposition, mitogen-activated protein kinase (MAPK) cascades and defence-related transcriptional programming (Couto & Zipfel 2016).

Although PTI is sufficient to inhibit growth of diverse microorganisms, effective pathogens often overcome host plants' resistance by secreting virulence molecules called effectors into host cells that can suppress PTI responses, resulting in effector-triggered susceptibility (ETS) (Jones & Dangl 2006; Nicaise *et al.* 2009). However, due to plant-pathogen arms races, resistant plants are equipped with a second tier of defences, involving resistance (R) genes that respond to pathogen effectors by initiating effector-triggered immunity (ETI). Most plant R genes contain nucleotide binding-site leucine-rich repeats (NLRs, also known as NBS-LRRs) and the corresponding proteins, which are found inside cells (McHale *et al.* 2006). Successful recognition of effector proteins by R genes results in a hypersensitive response (HR) that inhibits pathogen propagation locally by inducing programmed cell death at the infection site, sustained ROS production and induction of defence-related genes (Cui *et al.* 2015). For example, through interaction with the E3 Ubiquitin Ligase CMPG1, *P. infestans* effector Avr3a suppresses PTI-related INF1-mediated immune responses (Bos *et al.* 2010). However, Avr3a's suppressive impact can be reversed if it is identified by host plants that have a corresponding R3a resistance gene, which then results in HR. Another *P. infestans* effector, Avr2, induces HR in plants carrying a resistance gene of the R2 family (Gilroy *et al.* 2011). Avr2 also reportedly interacts with BSU-like protein 1 (BSL1), which is putatively involved in brassinosteroid-associated signal transduction (Saunders *et al.* 2012). It was recently shown that PTI and ETI responses are not exclusive, instead they synergistically enhance resistance (Ngou *et al.* 2021; Yuan *et al.* 2021a). One of the important signalling component in both tiers of plant immunity is ROS production.

2.4 Reactive Oxygen Species (ROS)

ROS have important functions as signalling molecules in regulation of many biological processes in plants, including growth, development, and responses to biotic and abiotic stimuli (Waszczak *et al.* 2018). ROS generation, both as a byproduct of cellular metabolism and active ROS production in various subcellular localizations, is strictly regulated (Castro *et al.* 2021). The term ROS refers to reactive forms of oxygen that include hydrogen peroxide (H_2O_2), hydroxyl radical (HO), superoxide anion ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) (Mittler 2017). H_2O_2 is the most stable, has a longer half-life than the other ROS, and is often believed to act as an intercellular and intracellular signal that initiates downstream actions (Quan *et al.* 2008). ROS can be generated in various subcellular compartments, including apoplast, peroxisome, mitochondrion and chloroplast (Mignolet-Spruyt *et al.* 2016). However, apoplast and chloroplast are considered to be vital sources of ROS production (Shapiguzov *et al.* 2012; Sierla *et al.* 2012). Plant NADPH oxidases (NOXs), also known as RBOHs (respiratory burst oxidase homologs), are found in the plasma membrane and play a pivotal role in ROS production during plant immunity responses (Qu *et al.* 2017). AtRBOHD is one of 10 RBOHs that play an important role in ROS production during immunity responses of Arabidopsis plants (Wang *et al.* 2020). NbRBOHB, a homolog of AtRBOHD, is required for ROS production induced by flg22 or *P. infestans* in *N. benthamiana* (Yoshioka *et al.* 2003; Yoshioka *et al.* 2016). RBOHs' N-termini include various regulatory sites, such as phosphorylation sites and EF-hand motifs (Oda *et al.* 2010). Like ROS, calcium (Ca^{2+}) is a secondary messenger that helps to propagate intracellular signals, and it is essential for PAMP-induced ROS production (Stael *et al.* 2015; Marcec *et al.* 2019). PAMP perception results in increases in cytosolic Ca^{2+} levels that are perceived by calcium-dependent protein kinases (CDPKs or CPKs) that phosphorylate RBOHD, which raises ROS generation in combination with action of the PRR-associated kinase BIK1 and calcium-sensing receptor (CAS) (Seybold *et al.* 2014).

Perception of PAMPs results in biphasic transient ROS accumulation, often called oxidative bursts (Sierla *et al.* 2012). The first ROS burst is one of the first responses, occurring in the apoplast within a few minutes of PAMP perception (Shapiguzov *et al.* 2012). NOXs and class III peroxidases are

mainly responsible for the first ROS burst, while the second is mainly due to strong chloroplast contributions and is called the cROS (de Torres Zabala *et al.* 2015; Camejo *et al.* 2016). The cROS is important for effective plant immunity, because chloroplasts are major sites of ROS production and play important roles in both redox homeostasis and retrograde signalling (Kachroo *et al.* 2021). It was recently shown that ETI boosts the second PTI-induced ROS burst, which occurs a few hours after pathogen recognition and levels of various PTI components increases related to NOX, including CPKs and receptor-like cytoplasmic kinases (RLCKs) (Ngou *et al.* 2021; Yuan *et al.* 2021a). Furthermore, PRR signalling (PTI) is necessary for maximum RBOHD phosphorylation during ETI, but NLR signalling increases RBOHD levels, highlighting the necessity of both PRR and NLR signalling for robust ROS generation during ETI (Yuan *et al.* 2021a).

2.5 Role of chloroplasts in plant immunity

Chloroplasts are essential not only for oxygenic photosynthesis and primary metabolism, but also as active environmental sensors that integrate cellular responses to stress (Chan *et al.* 2016). They are involved in biosynthesis of phytohormones, such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) (Sowden *et al.* 2017). Further, they have major roles in ROS and calcium bursts, as well as redox homeostasis and retrograde signalling, which affects nuclear gene expression (Stael *et al.* 2015). Chloroplasts are important sites of ROS production through oxygen reduction during electron transport (Asada 2006). cROS bursts are mainly generated by PSI, but PSII contributes to the total amount of ROS in the thylakoid membrane under stress conditions (Leister 2017). $O2^{\bullet-}$ can be formed on the reducing side of PSII, whereas H_2O_2 can be produced on the electron donor side of PSII, and then converted to $OH^{\bullet}$ via Haber-Weiss reactions (Pospíšil 2009; Pospíšil 2016). cROS has known association with PTI-related immune responses. For example, a *P. syringae* strain (DC3000hrPA) with effector secretion deficiency rapidly induces H_2O_2 production in Arabidopsis (de Torres Zabala *et al.* 2015). Another study showed that co-inoculation of DC3000hrpA with DCMU (which blocks electron transport between PSII and plastoquinone), resulted in abolishment of cROS, indicating that cROS bursts are generated

downstream of PSII production of superoxide/H_2O_2 (Mubarakshina *et al.* 2010). $^1O_2$, a highly reactive oxygen species generated in PSII when excited chlorophyll in its triplet state reacts with oxygen, is putatively involved in retrograde signalling and participates in ETI-induced HR by lipid peroxidation (Zoeller *et al.* 2012; Leister 2017).

Several components and signalling cascades are involved in plant immune signalling, with known and unknown functions. One component, CAS, is a thylakoid membrane-spanning protein with the C-terminus facing the stroma, and a key regulator of plant immune responses in chloroplasts (Nomura *et al.* 2012; Medina-Puche *et al.* 2020). In Arabidopsis *cas* mutants, treatment with flg22 largely abolishes calcium flow in the chloroplast stroma, indicating that CAS is the main calcium receptor in chloroplasts (Nomura *et al.* 2012). Further, *cas* deletion reportedly compromises immunity responses to both virulent and avirulent *Pseudomonas* strains (Nomura *et al.* 2012). Additionally, Wang et al. (2012) found that cROS bursts induced by Ca^{2+} were decreased in *cas* mutant lines, indicating Ca^{2+} dependency of CAS action and suggesting that calcium signalling may link extracellular PAMP sensing and chloroplast responses, although the exact function of CAS is unknown. Further, it has been suggested that CAS uses $^1O_2$ for signaling to nucleus for transcriptional reprogramming (Stael *et al.* 2015). Recently, a plant defence pathway linking plasma membrane and chloroplast has been discovered, which can be co-opted by plant pathogens during host-pathogen co-evolution, leading to promotion of virulence through suppression of SA responses (Medina-Puche *et al.* 2020). Upon perception of an elicitor associated with biotic stress, such as flg22, CPK16 localization changes from plasma membrane to chloroplast, inducing PTI-related chloroplast defences. However, it has a proposed role as a modulator of plant defences, although its exact function in chloroplasts is unclear. In a similar co-opted pathway with immunity-suppressing effects, geminivirus C4 protein translocated from the plasma membrane to the chloroplast interacts with CAS, reducing calcium bursts, callose deposition, and resistance to *P. syringae*.

Light-harvesting complex II protein LHCB5 is one of three highly conserved minor chlorophyll a/b-binding proteins associated with PSII (the others being LHCB4 and LHCB6). Liu *et al.* (2019) found that LHCB5 is phosphorylated

light-dependently during infection by the rice blast fungus *Magnaporthe oryzae*, and that resistance is connected to variations in ROS generation, with an overexpressing line producing more ROS than controls.

3. Aims and objectives

The overall aim of the project this thesis was based upon was to improve understanding of plant immunity in potato plants and identify novel factors that may confer broad-spectrum resistance and could be used for developing sustainable disease control strategies. We aimed to employ proteomic techniques to study immune responses to PAMP-triggered immunity (PTI), related to general plant immune responses, and effector-triggered immunity (ETI) responses specific to potato-*P. infestans* interaction. Further objectives were to study functions of candidate proteins identified in the proteomics study and develop both an easy, cost-effective method to quantify disease lesion areas to help in phenotyping and a quantitative luminol-based assay for detecting ROS bursts in potato leaves in responses to biotic stimuli.

4. Summaries of appended papers

Paper I: Proteomics of PTI and Two ETI Immune Reactions in Potato Leaves

The study reported in this paper was designed to improve understanding of potato plant immunity through quantitative proteomic analysis of PTI and two ETI models. To study PTI-related responses, we infiltrated disarmed *Agrobacterium* into potato (var. Desirée) leaflets. To investigate ETI interactions, we analysed samples of Desirée leaflets containing the R2 resistance gene infiltrated with *Agrobacterium* transformed with *P. infestans* effector Avr2, and leaflets containing the Rpi-blb1 resistance gene infiltrated with *Agrobacterium* transformed with *P. infestans* effector IpiO (Burra *et al.* 2018).

In total, 869 proteins were identified and quantified. Changes in the abundance of 243 of these proteins significantly differed ($p < 0.05$) in at least one of the immune responses to those in controls (Supplementary Material 1, Paper I). In all three immune responses, around a third of quantified proteins overlapped and the difference between the two ETIs was similar to the differences between the PTI and ETIs sets (Figure 1, Paper I). During PTI interaction the abundance of germin, proteases, and a CASP-like protein increased, in accordance with expectations during early phases of plant immunity responses (Davidson *et al.* 2009; Roppolo *et al.* 2011; Thomas & Van der Hoorn 2018). Further we found that sterol carrier protein 2 increased in abundance in all three immune reactions, which is interesting as sterol content plays an important role in plant immunity and oomycete pathogens such as *P. infestans* rely on their hosts for sterols. The abundance of several proteins with RNA-binding activity also increased in ETI responses,

supporting the growing body of evidence that RNA-related proteins play a role in regulating plant immunity (Staiger *et al.* 2013). We found that the abundance of four histones was only upregulated in the ETI-Avr2 interaction, and a putative multiprotein bridging factor only upregulated in the ETI-IpiO interaction. The study also included the first proteomic investigation of protein methylation in potatoes, which revealed a previously unknown methylation site in histone H3 as well as increased methylation of serine hydroxymethyltransferase during the ETI interaction.

A proteomic analysis of another protein fraction that we previously conducted showed that specific protein differences between different ETIs are in a similar range to the differences between PTI and ETI in downstream signalling, prior to the initiation of HR (Burra *et al.* 2018). Findings presented in Paper I support the notion that downstream signalling differs amongst ETIs to the same extent as ETI differs from PTI.

Paper II: Parakletos: A novel ROS suppressor involved in broad-spectrum pathogen resistance, and CRISPR/Cas9 gene knock-out enhanced *Phytophthora* resistance in field-grown potatoes

The second and major objective of the studies was to elucidate key aspects of general plant immunity by studying PTI-related immune responses and functionally characterize potentially important candidate proteins. Quantitative proteomic analysis of potato leave identified 264 proteins (Paper II, Figure 1) whose expression was significantly affected by challenge with disarmed *Agrobacterium* and thus are potentially involved in general plant immune responses.

Screening of candidate proteins identified a chloroplastic protein that we called Parakletos. Functional validation studies showed that over-expression of *Parakletos* in *N. benthamiana* increased *P. infestans* growth, while its silencing enhanced resistance (Figure 1). Results showed that these traits were associated with ROS burst levels and Parakletos is a negative regulator of resistance to *P. infestans* (Paper II, Figure 3). *Parakletos* overexpression suppresses the flg22-induced first ROS burst and increases susceptibility to *P. infestans* infection, while silencing resulted in increased ROS burst and *P. infestans* resistance. One of the gene that was more strongly expressed in the *Parakletos*-silenced plants than in controls was Nb*RBHOB*, indicating how Parakletos may affect the plasma membrane-related ROS burst. Further, we found that *Parakletos* silencing enhanced both the flg22-induced first ROS burst as well as the second burst, which is associated with cROS.

Parakletos silencing also increased transcription of several defence-related genes in flg22-induced stress responses. We found that expression of the SA biosynthesis-related gene *ICS1* and SA signalling pathway marker gene *PR1* increased in response to flg22 in *Parakletos*-silenced plants. Accordingly, flg22 treatment increased expression of the PTI marker gene *PTI5*, which putatively influences SA responses indirectly via interactions with other transcription factors (Gu *et al.* 2002). Furthermore, elevated SA levels have been shown to promote ROS accumulation, in accordance with our detection of elevated levels of transcripts of both ROS- and SA-related genes in *Parakletos*-silenced plants following biotic stress (Shirasu *et al.* 1997; Liu *et al.* 2017).

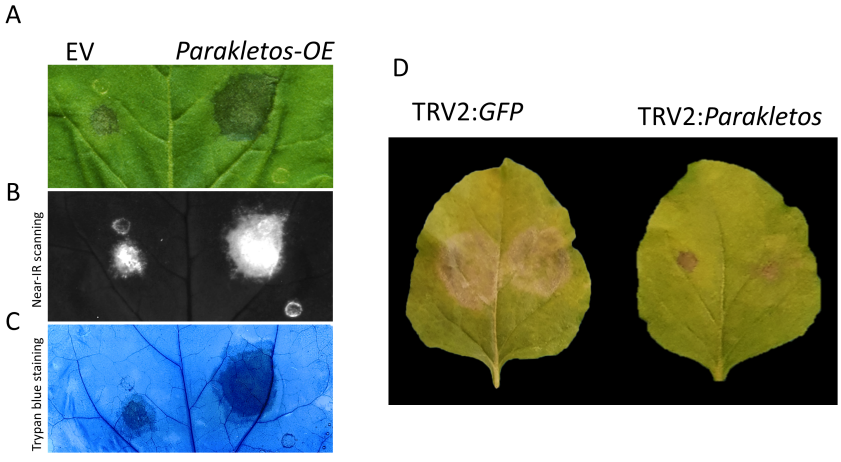

Figure 1 Parakletos negatively regulates resistance to *Phytophthora infestans*.(A) Image of *Nicotiana benthamiana* leaf 7 days post-infection with *P. infestans* a day after infiltration with disarmed *Agrobacterium* carrying a *Parakletos*-OE vector on one side and empty vector (EV) on the other. (B-C) Same leaf scanned with Bio-Rad near-IR scanning and further trypan blue stained image of same leaf. (D) Image of *N. benthamiana* leaves in which *Parakletos* was silenced with VIGS by inoculation with TRV2:*Parakletos* and control plant inoculated with TRV2:*GFP* constructs, challenged with *P. infestans* three weeks later, and photographed 6 days post-inoculation (dpi).

Interestingly, when *Parakletos* was overexpressed in *N. benthamiana* we observed a considerable increase in *LHCB6* expression (Paper II, Figure 4A). Our findings reveal a possible link between *LHCB6* expression and Parakletos in the control of ROS homeostasis in chloroplasts at PSII, or a disassembly process mediated by LHCB5 phosphorylation after pathogen attack reportedly observed in rice (Liu et al., 2019).

CAS, located in thylakoid membranes, is a positive regulator of plant immunity. We found that Parakletos and CAS co-localize in chloroplasts, and Parakletos is functionally dependent on CAS since the effect of silencing *Parakletos* is reversed by silencing both of them in *N. benthamiana*. These results suggest that Parakletos acts downstream in the CAS pathway in chloroplasts (Paper II, Figure 9). It has been suggested that pathogen effectors directly or indirectly interact with CAS in chloroplasts and suppress plant immune responses. Thus, since Parakletos is also present in chloroplasts it might act downstream in the CAS response pathway and pathogens' effectors may interactively influence Parakletos with CAS.

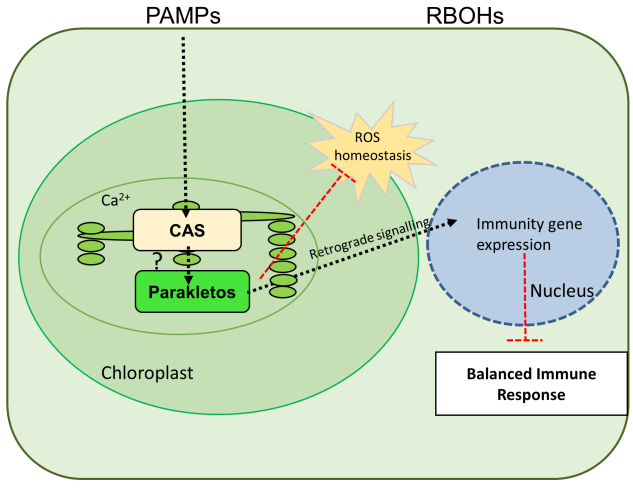

Figure 2 Schematic diagrams illustrating: suggested role of Parakletos suppression after flg22 perception in wild type plants.

In addition, we showed that CRISPR/Cas9-mediated KO of *parakletos* in potatoes enhances broad-spectrum resistance to *P. infestans* and *Alternaria. solani* in controlled conditions and reduced infection to the bacterial pathogen *Pseudomonas syringae* in *N. benthamiana* (Paper II, Figure 7). Deletion of *parakletos* had no apparent effects on the plants' growth, so we hypothesized that it only functions in stress conditions (Paper II, Figure 9), making it an interesting candidate for use in agricultural contexts. We further suggested role of Parakletos after flg22 perception in wild type plants (Figure 2). Moreover, field trials showed that CRISPR/Cas9-mediated *parakletos*-KO potato plants were significantly more resistant to late blight disease in field conditions than controls and we observed no growth defects in them (Paper II, Figure 8). As *P. infestans* severely reduces potato yields and requires intensive fungicide treatments, our studies on the new ROS repressor plant protein Parakletos may provide valuable new approaches for sustainable agriculture, especially when integrated with other measures.

Papers III & IV: Development of methods for detecting ROS bursts in responses to biotic stimuli and quantifying disease-related lesions

During the studies we also developed methods for detecting ROS in potato using a luminol-based assay and quantifying disease-related lesions in infected plants. ROS are important signalling agents in plants and animals, and the importance of detecting biphasic ROS bursts in responses to pathogens is increasingly clear (Mittler 2017; Li *et al.* 2021; Wang *et al.* 2022). In important crops like potato it could help screening of germplasm and mutants generated by, for example CRISPR/Cas9 technology and identifying signalling pathways involved in ROS bursts and pathogen defence. In this study a detailed protocol for quantifying ROS bursts induced in potato leaf discs in response to the bacterial PAMP flg22 and *P. infestans* was developed (Paper III).

A fast, non-destructive method using near-infrared scanning with standard lab equipment (a Bio-Rad Scanner) was developed to analyze disease-related lesions. This method has strong advantages over classical trypan blue staining, which is time-consuming and involves use of toxic chemicals. Pathogen-inoculated and wounded leaves from potato, tomato, spinach, strawberry, and Arabidopsis plants were used for proof of concept (Paper IV). The results showed that the new near-infrared scanning-based protocol gave similar results to trypan blue staining. Furthermore, a macro in FIJI was developed to quantify damaged areas of leaves.

5. Concluding remarks

This thesis presents a lab to field study designed to improve understanding of plant immunity responses involving proteomic analyses of potato, analyses of ROS bursts in potato, and detection of disease-related lesions. We also functionally characterized candidate proteins that may play important roles in potato immunity in the model plant *N. benthamiana*. Moreover, the effects of an identified candidate protein (Parakletos, helper in Ancient Greek) were tested on potato in both lab and field conditions. Thus, the thesis presents both crop-to-model and model-to-crop studies.

Parakletos appears to be a potential Achilles heel in potato immunity against multiple diseases, as its deletion or silencing can reduce infection of plants, while its over-expression increases infection. In lab conditions, knock out of this gene significantly increases potato resistance to late and early blight. It also reduced infection to the bacterial pathogen *Pseudomonas syringae* in Patakletos silenced *N. benthamiana*. Parakletos plays a role in immunity-related ROS production as its overexpression suppressed the ROS burst response to flg22, while its silencing increased it. Moreover, CRISPR-Cas9 mediated loss of *parakletos* gene function reduced severity of late blight disease in a field trial. In summary, Parakletos is a promising S-gene without any negative growth consequences and could be used for breeding new disease-resistant cultivars.

6. Future prospects

- Our research shows that Parakletos localizes in the chloroplast (using mcherry fusion proteins, Paper II). However, we have not observed Parakletos under confocal microscopy after flg22 stress, and it would be interesting to observe effects of flg22 on Parakletos during stress responses, as we believe that its main role is in stress conditions. Short-term flg22 treatment may suppress Parakletos, help it to move to another location, or have no direct effect.

- The Parakletos sequence includes a predicted acetylation site. Several studies have found that non-histone protein acetylation has a function in plant immunity (Song & Walley 2016). For example, pathogen effectors have been shown to directly acetylate NLR protein RPM1 in suppression of plant immune responses (Lee *et al.* 2015). Further experiments could include tests of hypotheses that acetylation may be involved in the putative role of Parakletos in stress responses, or suppression of immune responses through generation of acetylated mimic and dead versions of Parakletos.

- Experiments to elucidate the connections between roles of ROS and Parakletos in immunity responses are also warranted. In the studies presented here we used a luminol-based assay for ROS detection, but it would be interesting to use other available tools such as DAB, H2Dcfda and NBT stains, and ROS inhibitors, to elucidate their functional relationship with Parakletos.

- Experiments to examine protein complexes in plant thylakoid membrane using blue native polyacrylamide gel electrophoresis are also warranted. As Parakletos is present in chloroplasts and has a link with *LHCB6* it would be interesting to examine the protein complexes after expressing or silencing Parakletos, with and without biotic stress. Recent studies have shown that also as LHCB5 and LHCB3 participate in plant immunity (Liu *et al.* 2019; Qiu *et al.* 2021). It would also be interesting to test the functional relations between other LHCBs and Parakletos in *in vivo* interaction studies.

- We have shown that Parakletos is functionally dependent on the calcium-sensing receptor (CAS). However, it would be interesting to further probe the interaction between CAS and Parakletos by co-immunoprecipitation and bimolecular fluorescence complementation assays. Further, it would be interesting to monitor Ca^{+} signalling in responses to biotic stresses using luminol-based assays.

- We found that *parakletos*-KO only has functional effects when plants are stressed, so it would be interesting to test its effects on responses to abiotic stressors such as salt and drought.

- Our field trails were restricted to a single year at one location. It would be interesting to extend the trials to different potato-growing locations and seasons.

- We found that *Parakletos*-KO provides broad-spectrum resistance against *Pseudomonas*, early blight and late blight. However, it would be interesting to test the possibility of using it to reduce fungicide applications or other measures, and thus improving agricultural sustainability in integrated pest management strategies, since changing its expression seems to have the clearest effects early in the season.

- A luminol-based ROS assay was developed to monitor changes in their levels in potato plants in responses to PAMPs such as flg22 and

the plant pathogen *P. infestans*. Although the assay works, adjustment is required for longer term ROS detection, to avoid drying of the reaction mixture. We used the current assay to detect biphasic ROS bursts induced by *P. infestans* sporangia. However, cultural filtrates, hyphal cell walls, or other elicitors could be used, and it would be interesting to evaluate biphasic ROS bursts in responses to resistant and non-resistant potato cultivars.

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Popular science summary

Potato is the third most important food crop globally, after wheat and rice. However, various pests and pathogens can cause severe yield losses, especially the fungus-like pathogen *Phytophthora infestans*, which causes late blight disease. The pathogen was responsible for the Irish potato famine in the mid-19th century, and plant breeders have struggled ever since to rein it in. Late blight disease can cause losses of up the whole potato crop, and a common way to control it is through application of chemical fungicides. Today, 20-30% of the fungicides used in Swedish agriculture are applied to potato crops, even though potato is grown on just over 1% of cultivated land in Sweden. Those chemical applications are costly, environmentally harmful, and pose risks of *P. infestans* developing resistance to pesticides. Thus, in order to combat late blight disease new strategies need to be developed, and for this a detailed understanding of plants' defensive responses to *P. infestans* is needed. To assist efforts to acquire such understanding, we investigated the potato plants' molecular-level immune system in the studies that form the basis of this thesis. First, potato proteins that changed in abundance following activation of immune responses were examined, and a protein "Parakletos" that is potentially important for invading pathogens and that may promote infection was identified. The deletion of Parakletos in potato plants not only resulted in enhanced disease resistance to late blight but also in enhanced resistance to another major disease, early blight. In this study, we also performed field trials that showed that there is less late blight disease on Parakletos-deleted plants. This research could help improve potato varieties' resistance and integrate these approaches in effective, sustainable control strategies. Assays for detecting Reactive Oxygen Species (which are also involved in plant immunity responses) and quantifying disease lesions on leaves were also developed

Populärvetenskaplig sammanfattning

Potatis är den tredje viktigaste livsmedelsgrödan i världen, efter vete och ris. Olika växtskadegörare kan orsaka allvarliga skördeförluster, särskilt algsvampen *Phytophthora infestans*, som orsakar potatisbladmögel. Algsvampen utlöste den irländska potatissvälten i mitten av 1800-talet, och växtförädlare har kämpat sedan dess för att tygla den. Sjukdomen kan i värsta fall orsaka förlust av hela potatisskörden, och ett vanligt sätt att kontrollera den är genom kemisk bekämpning. Idag används 20–30 % av de svampmedel som förbrukas i svenskt jordbruk på potatisodlingar. Potatis odlas dock bara på drygt 1 % av den odlade marken i Sverige. Dessa kemiska bekämpningar är dyra, miljöskadliga och utgör risker för att *P. infestans* utvecklar resistens mot bekämpningsmedel. Man behöver alltså utveckla nya strategier för att bekämpa potatisbladmögel, och för detta behövs en detaljerad förståelse av växternas försvar mot *P. infestans*. För att få sådan förståelse undersöktes i denna avhandling potatisens immunsystem på molekylär nivå. Först undersöktes proteiner hos potatis som ökar i mängd efter aktivering av immunförsvaret, och ett protein som är potentiellt viktigt för invaderande skadegörare och som kan främja infektion identifierades. Detta protein gavs namnet "Parakletos". Genetiskt modifierad potatis utan Parakletos hade inte bara ökad sjukdomsresistens mot potatisbladmögel utan också ökad resistens mot en annan allvarlig sjukdom, torrfläcksjuka. Dessutom utförde vi fältförsök som visade mindre potatisbladmögel på potatisar utan Parakletos. Denna forskning kan bidra till utvecklingen av potatis med förbättrad resistens mot potatisbladmögel, som kan integreras i effektiva och hållbara kontrollstrategier. Förutom potatisförsöken utvecklades också metoder för att detektera reaktiva syreradikaler (som också är involverade i växtimmunitet) och metoder för kvantifiering av sjukdomsskador på blad.

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This thesis is based on studies designed to improve understanding of plant immunity by proteomic analysis of potato leaves and functional characterization of proteins that changed in abundance during immunity responses. This resulted in discovery of a novel protein, 'Parakletos' and subsequent demonstration that it is negatively involved in plant immunity. Assays to detect Reactive Oxygen Species and quantify disease lesions on leaves were also developed.

Muhammad Awais Zahid received his doctoral education at the Department of Plant Protection Biology, SLU, Alnarp; and obtained his MSc in Crop Sciences from the University of Hohenheim, Germany.

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