Plant pathogen defense: Signalling, resistance and cell death

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Akademisk avhandling för filosofie doktorsexamen i Naturvetenskap med inriktning Biologi, som med tillstånd från Naturvetenskapliga fakulteten kommer att offentligt försvaras fredagen den 24:e april 2015 kl. 10.00 i Hörsalen, Institutionen för biologi och miljövetenskap, Carl Skottsbergs gata 22B, Göteborg.

Examinator: Professor Adrian Clarke, Institutionen för biologi och miljövetenskap, Göteborgs Universitet

Fakultetsopponent: Professor Dr. Jürgen Zeier, Molekulare Ökophysiologie der Pflanzen, Heinrich-Heine-Universität, Düsseldorf

ISBN: 978-91-85529-78-0

ISBN: 978-91-85529-78-0
Tryck: Kompendiet
Digital source of thesis: http://hdl.handle.net/2077/38369

Till Sofia

”Hooray! I’m useful. I’m having a wonderful time!”

J.A. Zoidberg

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ABSTRACT

Pathogenic microorganisms are present everywhere in nature and infect both animals and plants. Phytopathogenic microorganisms cause diseases on plants, and are responsible for crop loss amounting in the order billions of dollars annually. Plants have however co-evolved with these organisms and have consecutively been forced to develop mechanisms that prevent disease. The plant immune system unlike that of animals lack adaptive cells and rely on the innate immunity of each plant cell. There is however no doubt in the effectiveness of the plant immune system as most plants are healthy most of the time.

The plant immune system consists of two main tiers of defense responses; the MAMP triggered immunity (MTI) and the Effector triggered immunity (ETI). MTI is triggered by recognition of microbe associated molecular patterns MAMPs. MTI strengthens the cell by producing antimicrobial substances, proteins and by fortifying the cell wall. This stops the majority of non-adapted microbes. A subset of microbes have adapted to these measures and evolved effector proteins that subdue the MTI responses. Again, plants have responded, by evolving resistance (R) proteins that recognize effector activity and mount the swift responses that are ETI. The plant responses during ETI are commonly termed the hypersensitive response (HR) and culminate in programmed cell death of the infected and sometimes surrounding cells.

The thesis has approached the plant disease resistance response in four ways. The first focused on improving methods for quantifying the programmed cell death response during ETI (**Paper I**) and lipid analysis by chromatography (**Paper II**). These methods are then used in the following pappers. The second part focused on signalling during the HR. Signalling on gene regulation level (**Paper III**) and various parts of lipid metabolism (**Paper IV, V** and **VI**) during the HR was pursued. The main results from these studies include the high redundancy identified among *Arabidopsis thaliana* phospholipase D isoforms in producing the lipid phosphatidic acid, the identification and initial characterization of the enzyme (AGAP1) that is responsible for producing head group acylation of lipids in *A. thaliana* and the reported involvement of a chloroplast localized 13-lipoxygenase in initiating the HR related programmed cell death in *A. thaliana.*

The third part of the thesis proposes a role in the HR in *A.thaliana* for two reactive molecules; indole acetonitrile (**Paper VII**) and sulforaphane (**Paper VIII**). Both compounds induce cell death when infiltrated into leaves and studies using mutants suggest that absence of these compounds result in a reduced cell death response. A redox related mechanism for these compounds is suggested. The fourth and final part of the thesis aimed to investigate if novel components could be identified in post penetration response against powdery mildew funguses. Much less is known on the relative dependence of MTI and ETI of this system, the results from **Paper IX** suggest that besides the known involvement of the protein EDS1, additional components are present.

In conclusion, this thesis contributes with insight into different aspects of how lipid-, redox- and hormone signalling contributes to resistance and cell death in plants.

TABLE OF CONTENTS

ABBREVIATIONS

Arabidopsis	*Arabidopsis thaliana*
Bgh	*Blumeria graminis* pathovar (pv) *hordei*
DAMP	Danger-associated molecular patterns
DGDG	Digalactosyl diacylglycerol
dnOPDA	Dinor-oxo-phytodienoic acid
Ep	*Erysiphe pisi*
ETI	Effector-triggered immunity
ET	Ethylene
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Hpa	*Hyaloperonospora arabidopsidis* pv *arabidopsis*
HR	Hypersensitive response
IAN	Indole acetonitrile
JA	Jasmonic acid
LOX	Lipoxygenase
LRR	Leucin-rich repeat
MAMP	Microbe-associated molecular pattern
MGDG	Monogalactosyl diacylglycerol
MTI	MAMP-triggered immunity
NB-LRR	Nucleotide binding-leucine rich repeat
OPDA	12-oxo-phytodienoic acid
PA	Phosphatidic acid
PC	Phosphatidylcholine
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PLC	Phospholipase C
PLD	Phospholipase D
PRRs	Pattern recognition receptors
Pst	*Pseudomonas syringae* pv *tomato*
R-proteins	Resistance proteins
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
T3SS	Type III secretion system

Note on nomenclature

The nomenclature of this thesis follows the TAIR (www.arabidopsis.org) recommendation for gene, mutant and protein names. Wild type alleles of genes are capitalized and italicised (ex. *PEN1*), whereas mutant alleles are lowercase and italicised (ex. *pen1-1*). Protein gene products are capitalized (ex. PEN1). Bacteria expressing a specific effector protein (using Avr prefixes) are reffered to by colon separation (ex. *Pst*:AvrRpm1).

PAPERS

This thesis is based on the following papers, referred to by their respective roman numerals throughout the thesis.

(Paper I)

Oskar N Johansson[1], Anders K. Nilsson[1], Mikael B. Gustavsson, Thomas Backhaus, Mats X Andersson and Mats Ellerström. (2015). A quick and robust method for quantification of the hypersensitive response in plants

Resubmitted to Molecular Plant Microbe Interactions

This paper describes the development of a vacuum infiltration procedure for inoculating plant tissue with bacterial suspensions in order to measure the hypersensitive response of plants. By using this technique evidence that both bacterial pre-cultivation conditions and inoculum titer affect the outcome of the leaked electrolytes during the hypersensitive cell death response is provided. This method was used in **Paper III, IV, V, VI VII, VIII** and **IX**.

◆

(Paper II)

Anders K. Nilsson, **Oskar N. Johansson**, Per Fahlberg, Feray Steinhart, B. Mikael Gustavsson, Mats Ellerström, and Mats X. Andersson. (2014) Formation of oxidized phosphatidylinositol and 12-oxo-phytodienoic acid containing acylated phosphatidylglycerol during the hypersensitive response in Arabidopsis

* Phytochemistry - 101, Pages 65–75

Herein a method for analyzing plant lipid extracts with LC-MS for both targeted and non-targeted quantification was developed. Using this new technique it was possible to separate lipid species with similar masses. Additionally several previously undescribed phospholipid species produced during the hypersensitive response, elicited by recognition of the bacterial effector AvrRpm1 were identified. The lipid profiling method is used in **Paper IV, V,** and **VI**.

◆

(Paper III)

Oskar N. Johansson[1] Olga Kourtchenko[1], Anders K. Nilsson, Erik Kristiansson, Andreas Czihal, David Mackey, Helmut Bäumlein, Mats X. Andersson, Mats Ellerström. Early transcriptional changes in Arabidopsis in response to the *Pseudomonas syringae* effector AvrRpm1.

Manuscript

A custom transcription factor cDNA array was used to identify early transcriptional changes upon transgenic expression of the bacterial effector AvrRpm1 in *Arabidopsis thaliana*. This paper provides new insights into transcriptional behavior of the plant as early as 15 minutes after elicitation. Additionally, two transcription factors involved in initiating cell death were identified by transiently silencing them with antisense oligonucleotides.

◆

(Paper IV)

Oskar N. Johansson, Per Fahlberg, Elham Karimi, Anders K. Nilsson, Mats Ellerström, Mats X. Andersson. (2014) Redundancy among phospholipase D isoforms in resistance triggered by recognition of the *Pseudomonas syringae* effector AvrRpm1 in *Arabidopsis thaliana*.

Frontiers in Plant Science – 5: 639

The complete set of phospholipase D (PLD) encoding genes in *Arabidopsis thaliana* was investigated for involvement in both MAMP- and effector triggered immunity against *Erysiphe Pisi* and *Pseduomonas syringae* respectively. The data show that only the PLDδ isoform are involved in MAMP triggered immunity against *E. pisi* whereas several isoforms contributes to the hypersensitive response during effector triggered immunity against *P. syringae.*

[1] Both authors contributed equally

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Anders K. Nilsson, **Oskar N. Johansson,** Per Fahlberg, Murali Kommuri, Mats Töpel, Lovisa Bodin, Per Sikora, Masoomeh Modarres, Sophia Ekengren, Chi-Tam Nguyen, Edward Farmer, Olof Olsson, Mats Ellerström and Mats X. Andersson. Acylated monogalactosyl diacylglycerol: Prevalence in the plant kingdom and identification of an enzyme catalyzing galactolipid head group acylation in *Arabidopsis thaliana*.

(Paper V)

Manuscript

This paper describes the identification and characterization of the enzyme AGAP1 responsible for galactolipid head group acylation. T-DNA insertion mutants in AGAP1 were produced only neglible amounts of acylated lipids. Electrolyte leakage assays suggest an involvement in disease resistance signalling, as a minor increase in cell death response is reported whereas no loss of resistance against *Pseudomonas syringae* expressing AvrRpm1 was found. Additionally, the presence of head group acylated galactolipids and oxophytodienoic acid (OPDA) containing lipids throughout the plant kingdom are investigated. Evidence suggests that these acylated galactolipids are formed both upon wounding and pathogen elicitation

◆

Oskar N. Johansson, Anders K. Nilsson, Per Fahlberg, Mikael B. Gustavsson, Lovisa Bodin, Björn Lundin, Mats X. Andersson. 13-lipoxygenase activity is involved in early effector triggered immune responses in *Arabidopsis thaliana.*

(Paper VI)

Manuscript

This paper characterized the importance of 13-lipoxygenase activity in the lipidome of the hypersensitive response and ascribed 13-LOX activity an extended role from that of producing precursors to OPDA and JA. Presumably LOX2 actively promotes hypersensitive response induction by production of lipid hydroperoxides that in turn can affect cellular redox and thus cell death.

◆

Oskar N. Johansson, Elena Fantozzi, Per Fahlberg, Anders K. Nilsson, Nathalie Buhot, Mahmut Tör, Mats X. Andersson. (2014) Role of the penetration resistance genes *PEN1*, *PEN2* and *PEN3* in hypersensitive response and race specific resistance in *Arabidopsis thaliana.*

(Paper VII)

The Plant Journal – 79: 466-476 **

Microarray data show that the *PEN* genes are induced not only by fungal MAMPs as previously reported, but also upon recognition of bacterial effectors. The *pen*-mutants display reduced hypersensitive cell death against avirulent *Pseudomonas syringae* and reduced resistance against *Hyaloperonospora arabidopsidis.* Presumably this effect is partly due to reduced amounts of indole glucosinolate breakdown products in the mutants. Hence, evidence is provided that these types of compounds are involved in hypersensitive response signalling.

◆

Mats X. Andersson[1], Anders K. Nilsson[1], **Oskar N. Johansson**, Gülin Boztaş, Lisa E. Adolfsson, Francesco Pinosa, Christel Garciav Petit, Henrik Aronsson, Mahmut Tör, Mats Hamberg and Mats Ellerström. (2015) Involvement of the Electrophilic Isothiocyanate Sulforaphane in Arabidopsis Local Defense Responses

(Paper VIII)

Plant Physiology – 167: 251-261 ***

Here the isolation of the compound sulforaphane that is released from plant tissue upon bacterial effector recognition is described. Sulforaphane initiate cell death upon exposure to plant tissue. Evidence suggests that sulforaphane acts as a redox signal in plant cells during the hypersensitive response and cell death against both oomycete and bacterial pathogens.

[1] Both authors contributed equally
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(Paper IX)

Oskar N. Johansson, Ellham Karimi, Megiel van Sloten, Nathalie Buhot, Mats X. Andersson.
Post penetration defense against non-host powdery mildews in *Arabidopsis thaliana*

Manuscript

In **paper IX**, components of post penetration resistance of powdery mildews are investigated. FMO1 is identified as an important component of post penetration resistance against *Erysiphe pisi* and through a forward genetic screen of an EMS mutagenized *pen1 eds1* population. Several additional putative mutations in genes that contribute to pathogen resistance are reported.

INTRODUCTION

1

Plants and their diseases are an important part of human history 1.1

Utilization of plants have played a major role throughout the evolution of humans, and shaped the development of human society in profound ways. Plants provide essential nutritional value, act as medicinal resources as well as being used for recreational purposes and as building material. Since the rise of agriculture as part of human food production, outbreaks of plant disease epidemics have had severe consequences when harvests have been lost. Such losses have historically been attributed to the wrath, anger or plain mischief of gods and deities, punishing humans for lack of sacrifice or obedience (Stakman 1957). We find traces of this in all major religions today, from the monotheistic Christian, Muslim and Jewish God to the gods of polytheistic Hinduism and Shintoism. Several passages in scripture describe the anger of the gods by giving the plants diseases, such as the book of Haggai of the Old Testament, "I smote you and every work of your hands with blasting wind, mildew and hail; yet you did not come back to Me,' declares the Lord" (Haggai 520BC).

Being a divine punishment, the idea of prevention was to please the gods in various ways, by performing rituals or sacrificing tribute. One of the major events of the 6th century was the extreme weather phenomenon of 535-536. Most of the northern hemisphere was covered with a haze of ash and dust, likely from a volcanic eruption. The veil blocked out much of the sunlight and preceded a harsh winter, and several consecutive cold years (Larsen et al. 2008). This was naturally attributed to the wrath of gods and the succeeding famine caused a significant population loss, as documented in both eastern- and western historical documents (Graslund and Price 2012, Richardson 2001). In Scandinavia at the time, where Viking culture was in its infancy, an excavation has revealed a large deposit of several kilograms of gold dating back to the period, suggested to have been a divine offering to please the gods after the cataclysmic event (Axboe 1999). In Norse pagan mythology, predominating in pre-Viking and Viking culture in Scandinavia at the time, such offering would likely have been used for paying tribute to the æsir Freyr, vowing for a better harvest that would not be lost due to disease or drought. Would the harvest ever fail, as in the case of the year 536, the farmer could rest assured that Freyr would pay the ultimate price during the times of Ragnarök, being slain by the fire giant Surtr (Sturluson 1220). Taken together this illustrates the profound measures humans have been willing to take in order to ensure good harvests.

Understanding how loss of plants occurs due to herbivores such as insects or mollusks has historically been straightforward. The nature of plant diseases however, remained elusive until the development of microscope in the 1600s. Until then, the presence of the microbial world was largely unknown and likely fueled the interpretation of disease outbreak as divine intervention or spontaneous generation. The Greek philosopher Theophrastus, father of botany, is one of the earliest contributors to the study of plant pathology and described how rusts, smuts and other diseases struck roman crops and how measures were taken to prevent these (Howard 1996). The devastating effects of fungal rust caused by *Puccinia* Sp. culminated in the creation of a supernatural deity that ruled over disease outbreaks. As a result, Romans performed annual rituals in the end of April known as "Robigalia", to please the god of rust, Robiga (Zadoks 1985).

Acknowledgement of the microbial world by development and refinement of the microscope and lenses by Robert Hooke (1635-1703) and Antonius van Leeuvenhook (1632-1723) in combination with emerging philosophical ideas during the enlightenment shifted the paradigm from divine interpretations to science based explanations and relinquished the need for religious sacrifice (Agrios 2005). However it took another century before the independent nature of disease-causing microbes found on diseased plants were identified independently by Micheli and du Monceau in the early 18th century (Micheli 1729, Monceau 1728). Until then, spores were believed to be part of- or produced by the plant itself. Though it took another two decades before the French scientist Tillet convinced the scientific community that these spores were the origin of plant disease (Tillet 1755). Scientific endeavor during the succeeding centuries described several causes of plant diseases and ascribed them to microbial pathogens.

As intercontinental trade- and commerce expanded, new plants, their fruit and their diseases traveled fast across the globe, a concept known as the Grand/Columbian Exchange (Crosby 2003). Bringing back

diseases to Europe from trade routes originating in South America proved catastrophic when the oomycete *Phytophthora infestans* was introduced in the 1840s (Andrivon 1996). Given the monumental importance of potato in common peoples´ diets throughout central and northern Europe, the blight initiated by the oomycete caused the death of more than one million people in Ireland alone, and the emigration of several millions across Europe to America (Goodwin et al. 1994).

1.2 Disease prevention

Strategies to prevent crop loss have been employed during the course of human history, in parallel to religious sacrifice. Important milestones in this struggle have been the development of crop rotation and introduction of pesticides. Selective breeding for enhanced resistance and higher yield throughout history have dramatically changed the genetic makeup of domesticated plants (Bai and Lindhout 2007, Jiao et al. 2014). The gene as mediator of traits was until the last century unknown. Resistance breeding was in many cases successful, however increased yield and resistance have not always gone hand in hand. Thus there has been a desire for tools to prevent crop loss due to pests.

Development of pesticides to combat insects and disease causing pathogens has been the weapon of choice during the last centuries, besides manually removing pests and washing plants. Farmers in early societies depended on very crude pesticides such as sulfur and later arsenic and mercury, methods even referred to in Homers renowned Odyssey (Shankar 2012, Torgeson 1967). Attempts to control pests by usage of toxic plants have been employed as early as 2000 BC, with various results, until more sophisticated alternatives such as tobacco extracts were developed during the 16th and 17th century (Thacker 2002). During the last century, development of synthetic pesticides and their usage on crop plants have provided ample restriction of pest proliferation but have resulted in devastating effects to the environment. The effects of DDT usage and that of other pesticides in nature throughout the 20th century are beginning to be realized. Pesticide usage has been linked not only to environmental effects but exposure upon distribution is associated with increased risk of developing diseases such as cancer (Alavanja et al. 2003), respiratory problems (Hoppin et al. 2006) and neural ailments (Mostafalou and Abdollahi 2013).

Diseases cause severe losses throughout the world

Plant pests still pose a very real threat. The prevalence and outbreak of disease vary with environmental conditions and distribution of infectious agents. Predominance of large scale monocultures that lack genetic variance renders many cultivated plants potentially even more susceptible to disease outbreak. As of today, the yield loss of crop plants due to pathogens, herbivores and weeds are high. Direct loss as a consequence of these threats is estimated to average in the 20-40% range annually (Savary et al. 2012). Contribution of the respective categories vary somewhat, pathogenic microbes reduces crop yield in the range 10-20% over all, whereas slightly higher losses are attributed to herbivores. However, these numbers do not reflect the dramatic loss in some species, for instance, as much as 50-80% of cotton production can be lost due to pest damage (Oerke 2006). These numbers do not take into account post-harvest losses, which would increase the amount additionally. Plant diseases thus result in a substantial economic damage. Estimates for soy bean demonstrate that 250 million USD worth of produce is lost annually in USA due to disease outbreaks (Wrather and Koenning 2006). Parasitic plants, particularly *Striga* sp. (Witchweeds) are also a cause of great concern worldwide. *Striga* sp. affects as much as 40% of cereal crops, causing losses of more than 10 billion USD in Africa alone (Scholes and Press 2008, Westwood et al. 2010). Currently, crop losses of cereal plants including barley, rye, oats and wheat in Scandinavia are predominately caused by fungal pathogens. More specifically, outbreaks of head blight and leaf blotch caused by *Fusarium* sp. and powdery mildew caused by *Blumeria* sp. are the main cause of the problems in Scandinavia (Savary, et al. 2012). In addition to direct crop loss, many fungi, including *Fusarium* sp. produces a multitude of mycotoxins that make the surviving plants unsuitable for human- or animal consumption (Desjardins and Proctor 2007).

Increased levels of atmospheric carbon dioxide during the forthcoming century will likely lead to an elevated temperature and more extreme weather conditions worldwide (Stocker 2013). Global warming will have consequences on crop production and on the interactions of plant with their respective pathogens.

IPCC-models estimate that an elevated temperature of four degrees will be the consequence of the expected increase in carbon dioxide levels in Scandinavia. It is believed that the effects of global warming are in favor of the plant pathogens and not plants themselves, although a slight increase in yield in healthy plant is also expected (Roos et al. 2011, Savary, et al. 2012). The result of globally increased temperature is expected to lead to a worldwide crop loss of over 15% (Oerke 2006).

Strategies to reduce crop losses in addition to dispersing pesticides have been used with some efficiency (Lin 2011). Interestingly, just increasing diversity by co-cultivating multiple varieties of the same species has been shown to be successful in many instances, reducing crop loss to rice blast outbreak by over 80% (Zhu et al. 2000). Co-cultivation of plant species from one or two different families can also be used to reduce spread of disease. Either by mixing directly to reduce dispersal of diseases or by surrounding fields with non-crop plants that attracts predatory insects (Mitchell et al. 2002, Rea et al. 2002, Thomas et al. 1992).

To increase yield and reduce use of pesticides, understanding plant resistance mechanisms will ultimately lead to strategies that can reduce crop- and timber loss due to pathogens. The results herein consist mainly of fundamental research and have further progressed the understanding of interactions between organisms in general and plant and their pathogens in particular. In addition, the thesis describes the refinement of several methods for studying plant pathogen interactions.

PLANT DISEASES

2

2.1 Plants pathogen co-evolution

Unicellular organisms were the principal form of life for a very long time. Even then, organisms competed for nutrients and living space. The process, known as natural selection, makes competitive organisms more likely to pass on their genes to subsequent generations (Darwin 1859). Organisms less well adapted are ruthlessly overthrown by more competitive organisms. Modern endosymbiontic theory describes how ancestral prokaryotic cells engulfed other prokaryotic cells to give rise to the first eukaryotic common ancestor 1.5-2 billion years ago (Mast et al. 2014). Thus, the concepts of cooperation, hostility and varying degrees of interactions between cells are as old as the history of life itself.

Plants are descendants of green algae, the first eukaryotic photosynthetic organisms, originating from the engulfment of a prokaryotic cyanobacterial ancestor, giving rise to the chloroplast (Keeling 2010). The plant life niche has since given them a unique position as the main producers of oxygen and biomass on land. This makes them essential for all higher organisms and liable to attack from a wide variety of entities. Thus, organisms across both the eukaryote and bacterial domain of life utilize varying ways of parasitizing on plants in order to gain carbohydrates produced in photosynthesis.

Insects and larger herbivores and insects feed on plants to retrieve nutrients. Many plants rely on production of secondary metabolites that discourage the myriad of herbivores and pathogens that hope to feed of them. Such metabolites can be directly toxic, even lethal, taste foul or cause enough problems to deter from eating. These molecules are produced either beforehand or upon attack and are not normally required for plant life. Secondary metabolites not only target assailants directly, several examples of plants releasing volatile organic compounds that attracts predators of the attacking herbivores have been discovered (Halitschke et al. 2008). Phytochemicals can affect almost all known animal organ systems, including muscle- and lung tissue (Lee et al. 2014). Compounds, like digitoxin can cause cardiac arrest even in small doses (Yang et al. 2012). Psychoactive compounds including mescaline, cocaine and nicotine interfere with the chemistry of the brain and nervous systems (Danielson et al. 2014, Heien et al. 2005, Kyzar et al. 2012). The molecular specificity of many of these secondary metabolites makes them useful for both medical- and recreational use or as potent poisons.

Plants have evolved defenses

Microbial pathogenic assailants lack the forceful measures of herbivores and instead invade plant tissue to proliferate on or within, causing disease. To combat attacks from the diversity of pathogenic microorganism, plants must use somewhat different strategies than those used against herbivores.

Microbial pathogens use these diverse strategies of entrance, infection and dispersal and are usually divided into groups depending on their lifestyle. The crudest way of attack means killing the plant and feed of dead plant tissue. This lifestyle is termed necrotrophy and is a major source of post-harvest crop loss (Laluk and Mengiste 2010). These organisms release cell wall degrading enzymes, toxic metabolites and have been known to hijack the plant host´s cell death machinery to overcome plant defenses and kill host cells (Govrin and Levine 2000, Mengiste 2012). Mechanistically this is performed in many different ways, for instance, the necrotrophic fungi *Sclerotinia sclerotiorum* perturb the oxalic acid homeostasis to initiate autophagy (Kabbage et al. 2013), other organisms secrete RNAs that tamper with plant transcription activity (Weiberg et al. 2013).

The opposite strategy is represented by biotrophic pathogens, which are dependent on living hosts for sustained life. Biotrophic pathogens do not kill their plant host under the infection process and are dependent on living plant cells to be able to utilize its nutrients. Hemi-biotrophs start their infection process as biotrophs but over time turn into necrotrophy as the plant cells die, as a result of strain caused by the infection. Biotrophs and hemi-biotrophs therefore have evolved sophisticated methods to circumvent detection, suppress plant defenses and reprogram the gene expression of the host (Koeck et al. 2011).

The interaction of plant and microbial organisms is highly complex. Not only does it include pure parasitism in the case of pathogens, but also various degrees of mutualism. Mutualistic relationships between organisms have evolved several times throughout history and denote a successful way of increasing fitness

for both organisms. Organisms living on (ectophytes) and within plants (endophytes) without causing disease and eliciting defense responses are an important component of both developmental- and biotic interaction processes (Reinhold-Hurek and Hurek 2011). Several such favorable interactions are known to exist in plants, and the bacterial population on the leaf surface is in the order of millions of bacteria per gram leaf (Lindow and Brandl 2003). Endophytic actinobacteria can induce defense responses that prevent several fungal strains from causing disease (Conn et al. 2008). Hence, these functions resemble the microbiota of the gut- and skin of humans that helps fend of pathogens. Even highly pathogenic *Pseudomonas* sp. are known to proliferate as ectophytes for extended periods until beneficial conditions of increased moisture or open wounds appear, and pathogenesis is initiated (Hirano and Upper 2000, Lindow and Brandl 2003). Favorable conditions often also require that a sufficient bacterial concentration has been reached; activating quorum sensing mediated potentiation of virulence factors (Chatterjee et al. 2007).

Bacterial pathogens enter plants either via wounds or through natural openings, such as stomata. *Pseudomonas* sp. actively contributes to wounding by expressing *INAZ* genes that promotes formation of ice crystals that damages cells (Baertlein et al. 1992).

Stomatal aperture is varied in the plant by altering osmotic potential in guard cells surrounding the pore. Regulation of stomata aperture is a tightly controlled procedure influenced by light, carbon dioxide and abscisic acid (ABA) (Shimazaki et al. 2007). Recognition of bacterial pathogens encourages rapid stomatal closure to prevent entry. Adapted phytopathogenic bacteria have evolved toxins that force stomata to open and allow them access to the plant tissue. The polyketide coronatine, a jasmonic acid-isoleucine analog, is used by *Pseudomonas* sp. to overcome stomata closure mechanisms and promote virulence (Geng et al. 2012, Melotto et al. 2006). Coronatine also reduces the production of indole glucosinolates by targeting the expression of the transcription factor *MYB51* involved in regulation of their biosynthesis (Geng, et al. 2012, Millet et al. 2010). Interestingly MYB51 was one of the transcription factors identified in **Paper III** to be down regulated in Arabidopsis thaliana by the bacterial effector protein AvrRpm1, and breakdown products of indole glucosinolates were suggested to be involved in plant defense signalling (**Paper VII**).

To travel between plants, some bacteria attach to aerosols and are dispersed by wind and rain while others use biological vectors (Lindemann and Upper 1985). Biological transmission presents yet another route, since transmission can occur also upon contact between vectors (Mann et al. 2011). Some bacteria infect both plants and their insect vectors and cause disease in both organisms (Nadarasah and Stavrinides 2011). Insects that harbor plant pathogenic bacteria performed *pro bono*, the insects use these microbes to suppress defenses elicited by feeding (Chung et al. 2013).

Most bacterial pathogens also produces other toxins that subdue plant cells and promote infection, including syringomycin, tabtoxin and phaseolotoxin from *Pseudomonas* sp. and albicidin from *Xantomonas* sp. (Tarkowski and Vereecke 2014). In addition to toxins, many pathogens secrete enzymes that enhance virulence by detoxifying secondary metabolites or that are able to interfere with plant cell signalling (Duca et al. 2014, Fan et al. 2011). In **Paper VIII** the compound **sulforaphane** was found to be released from *A. thaliana* tissue during defense responses. Adapted *Pseudomonas* sp. have been shown to harbor *SAX* (Survival in Arabidopsis Extracts) genes, which detoxify released compounds (Fan, et al. 2011), including sulforaphane. This promotes virulence, since sulforaphane have direct antimicrobial properties against bacteria (Tierens et al. 2001). **Paper VIII**. Furthermore *Pseudomonas* sp. can convert indole acetonitrile (**IAN**), studied in **Paper VII**, into indole-3-acetic acid (Kiziak et al. 2005). Reduced levels of indole acetonitrile impede activation of plant cell death and instead promote bacterial growth.

Fungal- and oomycete pathogens are the causal agents of many important diseases worldwide. Spores from families of rust- and mildew causing fungi, including *Blumeria* sp. and *Puccinia* sp., are wind dispersed across continents to invade new terrain (Brown and Hovmoller 2002). Other fungi proliferate in soil and infect plant roots or seeds. Pathogenic fungi and oomycetes have evolved mechanisms for entering plant cells and are not always dependent on wounds or natural openings. Once landed, the fungal spore germinates and the process of penetrating the plant cell wall ensues.

Plant viruses are not nearly as well understood as those infecting humans. In general, plant viruses are

small , in the 200-500 nm range, and rod-shaped (Agrios 2005). Most plant viruses depend on passive transmission via insect- or nematode vectors. Other parasites are found even within members of the plant kingdom. Parasitic plants use invasive means of acquiring nutrients and water from other plants. Most parasitic plants do not photosynthesize themselves but instead utilizes a specialized root structure, also known as a haustorium, which attaches to- and protrudes into the target plant vascular systems for retrieval of water and nutrients (Yoder and Scholes 2010). Consequently, parasitic plants present photosynthetic plants with yet another threat that they have to be able to cope with.

A shortage of mobility options makes plants disinclined to relocate in response to threats. Neither do plants possess the circulatory systems that mammals do. Hence, the possibility to have specialized immune cells is not an option, but has instead forced plants to rely on defensive capabilities within all individual cells.

Plants may appear pleasing to our eyes, they do however harbor an inhospitable micro- and macro environment to deny other organisms access. Perennial plants often produce thick bark supplemented with secondary metabolites. Its constituents comprise, but are not limited to cellulose, hemicellulose, lignin, tannins and suberin. These structures prevent outside access to living tissue and provide support for vertical growth (Alfredsen et al. 2008). Some plants produce thorns and spikes that are part of the herbivoral defense by deter from feeding. Most plants produce a thick waxy surface on the leaf, the cuticle. The hydrophobic nature of the cuticle makes it inhospitable to microbes. To further deter pathogens, the cuticle is sequestered with molecules that impede microbes (Bednarek and Osbourn 2009). Additionally plants regulate the cuticle ectobiota, either by varying the levels of polyphenols that chelate iron ions, reducing the availability of the much needed Fe^{3+} (Karamanoli et al. 2011) or by harboring non-virulent ectophytic bacteria that interfere with pathogenic quorum sensing (Dulla et al. 2010).

Adapted pathogens overcome defenses

Co-evolution of plant and their microbial enemies over the course of time has resulted in adaption of pathogens to overcome plant defenses. Once microbes successfully adapts to plant defenses, the selective pressure shifts to the plant population to refine its methods of defense. Selective pressure has shifted back and forth between the assailant and the defending plant as an ever raging evolutionary battle. This is described in the zigzag model in which progressively stronger and more adaptive responses have been evolved in both plant and pathogens (Jones and Dangl 2006).

Secreted proteinaceous effectors that interfere with defense signalling have been the weapon of choice for most microbial pathogens. Plants have in turn evolved means of monitoring effector activity by evolving resistance (R) proteins. The idea of one pathogenic component being recognized by one plant resistance component, known as the gene-for-gene concept, was introduced in the 1940s by Flor and associates (Flor 1942). Though, the genetic relationship was elucidated later on. Harboring a large set of effectors that effectively overcome plant defenses might seem advantageous for the pathogen, however there is a flipside. Recognition of microbial effectors initiates a second wave of defenses, stronger than those elicited by mere recognition of microbes. Increasing the number of effectors result in a larger number of plant species evolving mechanisms to recognize them and has resulted in some assailants gradually becoming more adapted to a specific plant species (specialists) and harmless to the majority of other plants. On the other hand, some pathogens lack the specialized tools to overcome certain plants defenses but are instead able to attack a larger set of plant species (generalists).

The concept is known as host range and is most easily exemplified by two well-known herbivores, koala bears (*Phascolarctos cinereus*) that eat mainly *Eucalyptus* sp. (low host range) and goats (*Capra aegagrus*) that are known to eat more than thousand different plants (high host range) (Barrett and Heil 2012). By adapting to a low host range, but becoming more specialized in overcoming a certain plants´ defenses, pathogens and herbivores increase their competitiveness on available host plants, as most others will fail attacking it.

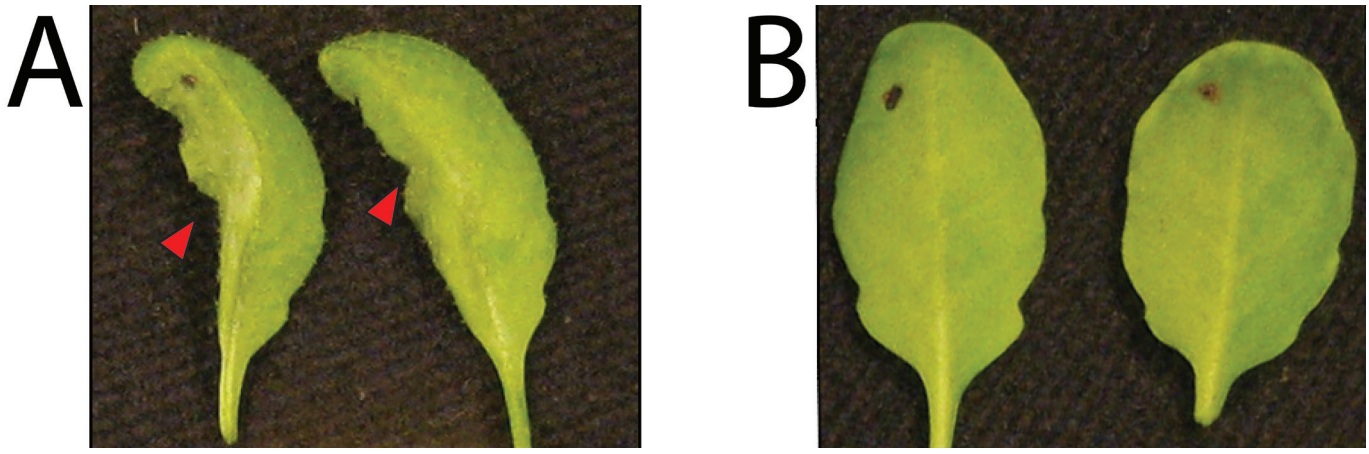

Figure 1. Collapse of leaf tissue as a response to bacterial infection. Left side of the leaves (**Black dot**) are inoculated with a *P. syringae* suspension. Upon recognition (**A**) the entire left side of the leaf collapses (**Red arrows**), whereas plants lacking the cognate resistance protein do not trigger cell death and do not collapse (**B**). This enables bacterial growth that gives the leaves a slightly yellow hue.

The hypersensitive response 2.2

The term hypersensitiveness was coined by Stakman in 1915 and it refers to the apparent overreaction plants upon inoculation with non-adapted pathogens (Stakman 1915). The term hypersensitive response (**HR**) has since been used as a term to describe the multitude of phenomena associated with the stronger defense reaction elicited by recognition of microbial effectors. The most drastic feature of the HR, as a consequence of effector recognition, is the execution of genetically programmed cell death (**PCD**), which causes the macroscopic collapse of whole leaves (**Figure 1**, Stakman 1915). Sacrifice of individual cells and an intricate cell death program exists in most organisms and likely arose before the emergence of multicellular organisms, as a defense against viral pathogens (Engelberg-Kulka et al. 2006).

PCD was originally described in the 1960s, in tadpole developmental processes (Tata 1966) and implementation of cell death programs are an important part of many developmental and stress related responses in multicellular organisms. Plants are no exception, sacrifice of a single and in some cases the surrounding cells is the result of a minute, controlled response from the plant. Like mammalian cells, plant cells excrete molecules that promote cell survival. If not present in high enough concentration, cells will initiate cell death (McCabe et al. 1997). Perturbation of this homeostasis by pathogen interaction or other stimuli can initiate PCD. Discovery of lesion mimetic mutants that are hypersensitive to such stimuli has been a useful tool for elucidating programmed cell death routes in plant cells. Many such mutants exhibit enhanced resistance towards and activate defenses at a lower microbial inoculum (Lorrain et al. 2003). Local PCD will trap the feeding structures of obligate biotrophs in the dead cell causing the fungus, oomycete or parasitical plant to eventually run out of energy and die, saving the rest of the plant from colonization. In some infections, cell death per se does not seem to affect the proliferation of pathogens (Shapiro and Zhang 2001, **Paper VII, VIII**), possibly as a consequence of the pathogens hemi-biotrophic lifestyle. Transport of signals to remote plant tissues are reduced when cell death is lacking, even if resistance locally is not affected, and hence spread of resistance to neighboring cells and remote tissue is reduced in the absence of PCD (Shapiro and Zhang 2001). Several bacterial effectors are known to actively inhibit cell death (Jamir et al. 2004), including the hemi-biotrophic bacteria *Pseudomonas syringae*. Hence, these bacteria likely prefer living cells that they can actively retrieve nutrients from.

In plants, PCD is classified into two categories depending on the morphological changes that transpire during cell death progression. For a long time there was a quest for plant apoptosis like mechanisms, similar to those of mammalian cells. Following animal apoptosis, phagocytes engulf cellular remains of cells. The latter is clearly not present in plants. Similarly, plants lack the specific cysteine proteases, caspases, which orchestrate cell death in mammalian cells. Thus, this search has not borne fruit, and it is generally presu-

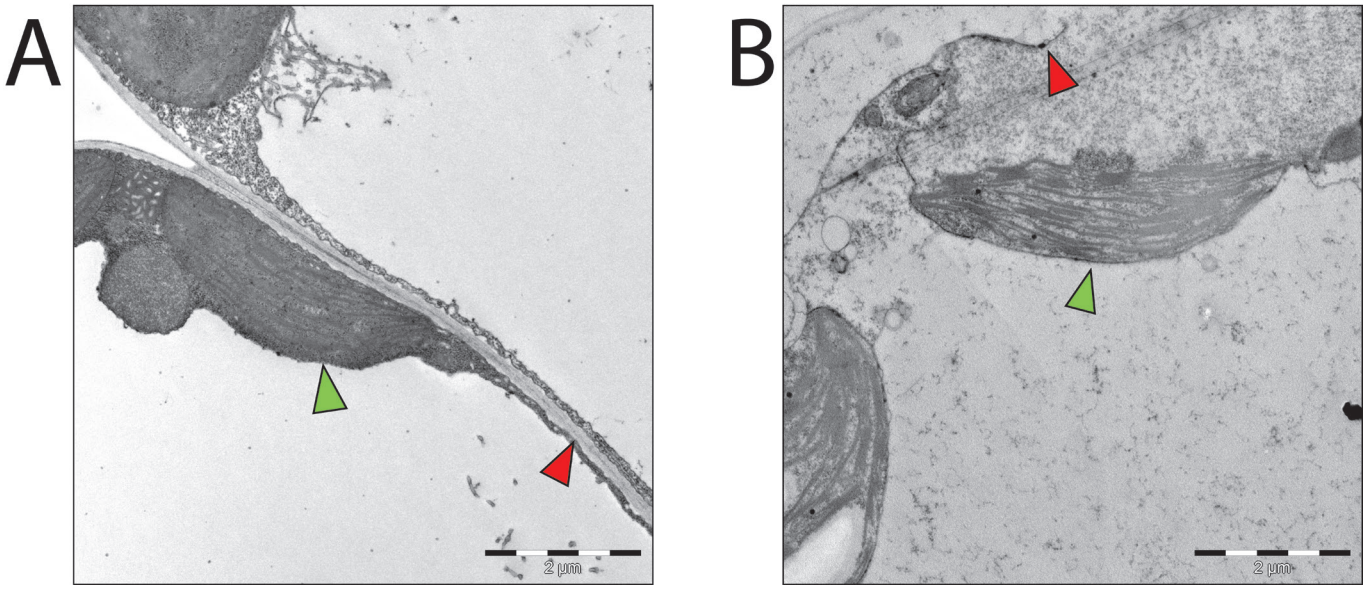

Figure 2. Ultrastructure of plant cells before (**A**) and two hours after (**B**) infection with *P. syringae* expressing the effector AvrRpm1 that is recognized by the plant. Recognition induces programmed cell death of the plant cell. Characterizing features of this include degradation of organelles such as the chloroplast (**Green arrow**) and plasma membrane release from cell wall (**Red arrows**). Additionally plasma membrane rupture and release of cellular content is clearly visible (**B-Red arrow**).

med that plants lack apoptosis mechanisms. The two classes of PCD differ in one central aspect, that being whether or not the vacuolar membrane, the tonoplast, ruptures and releases cell degrading enzymes (van Doorn 2011). PCD displaying tonoplast rupture is referred to as autolytic cell death. Autolytic cell death resembles what transpires in mammalian- and yeast cells during cell death with autophagic features. There are a number of events directly foregoing autolysis; the number of small lytic vacuoles in the cytosol are increased, many cellular organelles are degraded, and the chromatin within the nucleus is condensed (**Figure 2**) (van Doorn et al. 2011).

The hypersensitive response related programmed cell death (**HR-PCD**) presents a unique case. It shares some features with PCD types associated with animal cells, including apoptosis and additionally retains unique features (Coll et al. 2011). This is likely a consequence of the sturdy cell walls that surround plant cells and intercellular compartments such as chloroplasts and vacuole.

Non-autolytic cell death is the other type of cell death mechanism in plants and it resembles mammalian necrosis. This necrotic like cell death is not to be misinterpreted as accidental damage. In contrast, non-autolytic cell death is preceded by an increase in cellular ROS, swelling of mitochondria, shrinkage of cell volume and rupture of the plasma membrane (van Doorn, et al. 2011).

2.3 Disease resistance is increased throughout the plant

Once defenses are mounted at the local level, resistance is increased also throughout the plant (systemic acquired resistance, SAR). This enhances the chance of surviving recurring pathogen exposures. Primed basal resistance shift the focus of plant responses during the defense from triggering cell death to actual resistance, since the plant will have additional time to produce antimicrobial proteins and low molecular weight compounds (Fu and Dong 2013).

Epigenetic covalent modifications of the DNA molecule, such as methylation of cytosine residues and indirect modification of histone proteins that alter DNA folding and chromatin structure have been shown to extend the enhanced resistance across generations (Chinnusamy and Zhu 2009, Dowen et al. 2012) making the activation swifter on succeeding infections (Slaughter et al. 2012). Additionally, it has been shown that the genetic recombination activity increases after viral infection, thus increasing variability as well as resistance in subsequent generations (Kathiria et al. 2010)

Even though the SAR phenomenon has been known for more than a century, the quest for the mobile signal that mediates SAR is still ongoing. Salicylic acid (**SA**) was among the first proposed and exogenous application of SA mimics the response during SAR (Vanloon and Antoniw 1982). However, grafting experiments using plants expressing *NAHG*, a bacterial protein able to degrade SA, suggested that while SA is required both locally and systemically it is not the mobile signal that activates SAR (Gaffney et al. 1993, Vernooij et al. 1994). Also the SA derivative Methyl Salicylate (Me-SA) and the phytohormone jasmonic acid (**JA**) was suggested to act as mobile signals (Park et al. 2007). Later studies showed that Me-SA is dispensable for SAR induction, and while JA can induce SAR, it is not the mobile signal (Attaran et al. 2009, Truman et al. 2007). Two other compounds that have been proposed to be involved in SAR signalling are azelaic acid (AZA) and glycerol-3-phosphate (G3P). These compounds were initially believed to be mobile signals that mediated SAR as they are translocated through the petiole (Chanda et al. 2011, Jung et al. 2009). Though, G3P cannot initiate SAR on its own when applied exogenously, and later studies showed that while it is not the mobile signal, G3P is required for SAR activation (Mandal et al. 2011, Yang et al. 2013). AZA on the other hand can stimulate SAR when sprayed onto leaves, however, it primes the plant by promoting G3P production and is not the mobile signal (Yu et al. 2013). More recently, the hormone auxin (Truman et al. 2010), pipecolic acid (Navarova et al. 2012) and the diterpenoid dehydroabietinal (Chaturvedi et al. 2012) was suggested to be involved in SAR signalling. Hence, the pursuit of the signal is not ended, and it is becoming more apparent that it is not as simple as one single component being translocated and perceived.

Recently, other, more exotic stimuli has been proposed to induce resistance. For instance, mycorrhizal symbiosis prime defenses against pathogens. Plants perceive signals from their mycorrhizal symbionts, activating gene expression, initiate production of secondary metabolites and activate other parts of resistance signalling (Cameron et al. 2013, Veresoglou and Rillig 2012, Zamioudis and Pieterse 2012). Other stimuli that induce resistance include mechanical stimulus and possibly the sound of being chewed (Appel and Cocroft 2014, Gus-Mayer et al. 1998, Jayaraman et al. 2014). Many plants alert not only distal parts of itself but also neighboring plants, both within the same species and that of others. Tomato plants for instance have when subjected to attack been shown to alert neighboring tomato plants connected to a common mycorrhizal network (Babikova et al. 2013, Song et al. 2014).

METHODS AND MODELS IN PLANT PATHOLOGY

3

Since it is impossible to study every plant pathogen interaction, plant pathologists have turned to model systems that have allowed deeper study of a few interactions in detail. Based on these models, investigation of- and generalization to other plants can be made. Hence, models might not reflect reality in each and every aspect but provide a framework to start from. To be able to investigate plant pathogen interactions, methods that allow researchers to assess the levels of infection and defense signalling are required. Described below are the models and methods used in this thesis.

3.1 Model systems

The model plant Arabidopsis thaliana

A key model within the plant molecular biology community since several decades is a small weed-like plant in the Brassicaceae family, the thale cress, *Arabidopsis thaliana* (hereafter Arabidopsis). Arabidopsis has several advantages as a model over other plants and was the first plant to get its genome sequenced, revealing about 27000 protein coding loci (Arabidopsis Genome 2000, Swarbreck et al. 2008). Among the advantages in addition to being fully sequenced is that it is diploid, has a short generation time, self-pollinates, is easy to manually pollinate, produces plenty of seeds and requires a small amount of seed storage- and plant growth space. During the first decade of the 2000s several initiatives to generate knock out lines of the genome have resulted in a very high degree of coverage. These lines can now easily be obtained online from stock centers (Scholl et al. 2000). Most of these knock out lines are based on insertions of transfer DNA (T-DNA) into the genes by a modified version of the bacterial pathogen *Agrobacterium tumefacienas*. Depending on where in the gene the T-DNA is inserted, transcript level and/or activity of the gene product may vary considerably. Optimally T-DNA insertions terminate the gene encoded trait by producing a transcript without function.

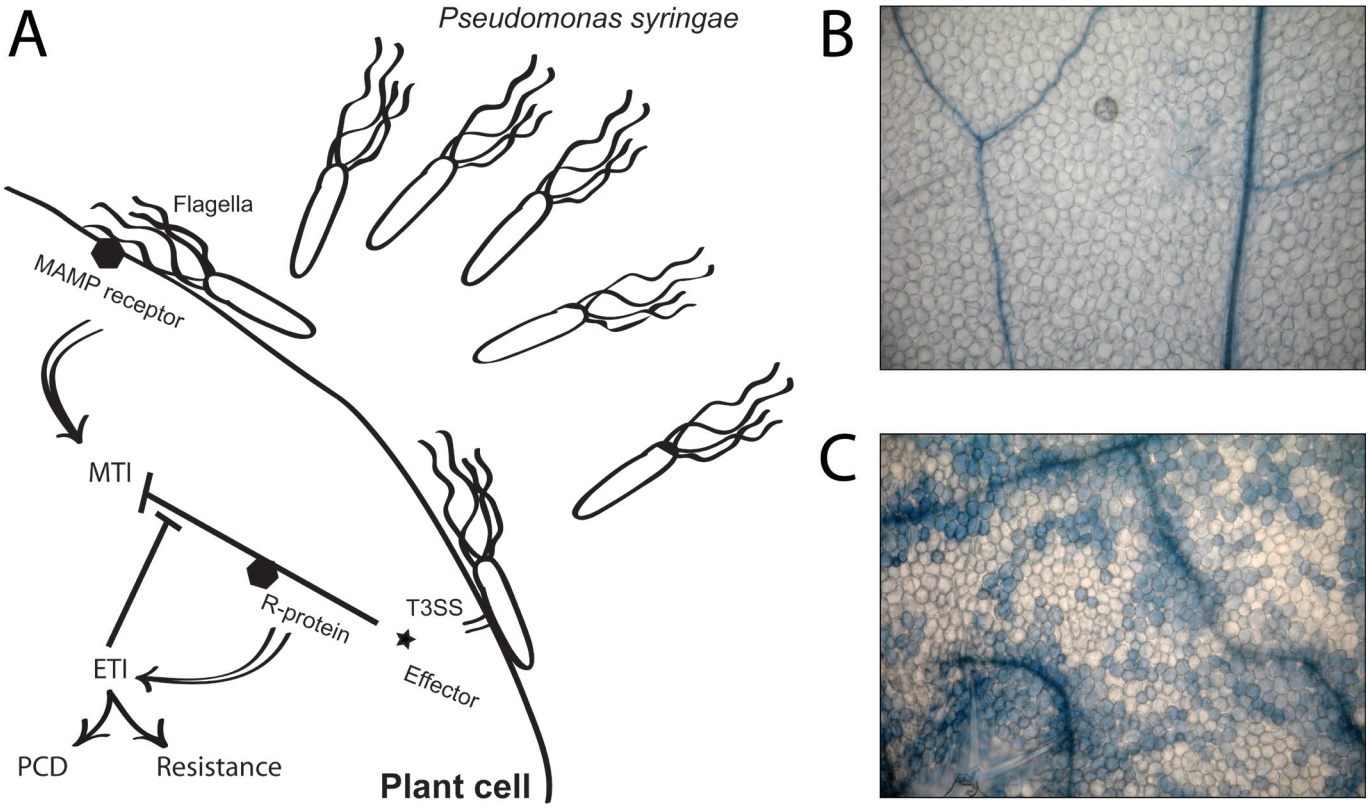

Figure 3. Model of *Pst* infection (**A**). Bacteria are recognized by membrane bound receptors and trigger defense responses (MTI). Adapted bacteria inject effector proteins through the type three secretion system (T3SS) that interfere with defense responses. Plants have in turn evolved resistance (R-Proteins) that monitor the integrity of the defense responses. If perturbations is detected a stronger defense response is initiated (ETI), that leads to programmed cell death and resistance. Infected mesophyll cells of Arabidopsis prior (**B**) and 4 hours after (**C**) infection. Cells are stained with trypan blue that selectively stains dead cells.

This is not always the case, sometimes T-DNA insertion cause knockdown of transcript level or results in no effect at all (Wang 2008). T-DNA insertion mutants are used in **Paper IV, VI, VII** and **VIII**.

Different climatic conditions throughout the world have given rise to ecotypes of plants from the same species, adapted to local environments. This is true also for Arabidopsis, and has contributed to a large genetic diversity. Two of these ecotypes commonly used, also in these studies, are Wassilewskija (Ws-0) from Belarus and Coloumbia (Col-0) from Germany.

Several model systems for plant pathogen interaction studies have been developed and can now be considered established models. Four such pathogens; one hemi-biotrophic bacteria, two biotrophic fungi, one biotrophic oomycete (*Pseudomonas syringae* pv *tomato* (*Pst*), *Blumeria graminis* pv *Hordei* (*Bgh*), *Erysiphe pisi* pv Pisi (*Ep*), *Hyaloperonospora arabidopsidis* pv *arabidopsis* (*Hpa*)) have been used as models in combination with Arabidopsis in this thesis.

The *Pst* bacterium is small, rod shaped, Gram negative, similar in size to the human gut bacteria *Escherichia coli* (*E. coli*) and is readily cultivated on nutrient plates. *Pst* causes leaf spots on its host plants and each pathovar are known to infect in the order of 50 plant species (Katagiri et al. 2002). The pathovar used in this thesis originates from tomato (*Solanum lycopersicum*) but readily infects Arabidopsis.

Upon entry to the plant, *Pseudomonas* sp. make their way to cells by whipping motions of their polar flagella. As water is the medium of motility, apoplastic water potential plays a significant role once bacteria have entered the plant. Hence, adapted strains of *Pseudomonas* sp. alter their cell wall glycosylation pattern and increase their excretion of polysaccharides to retain water by forcing hydration of these molecules (Beattie 2011, Wright and Beattie 2004). (Wright and Beattie 2004). Once a bacterium is close to target cells, attachment structures are produced that anchor the bacterial cell to the plant cell wall and facilitates injection of effectors (Duque et al. 2013), (**Figure 3**).

Some of the better studied effectors-R-protein pairs include the *Pst* effectors AvrRpm1 and AvrRps4 and their respective R-proteins RPM1 and RPS4. As such, plant responses following recognition of AvrRpm1 have been the chief focus of this thesis (**Paper I-VIII**). RPM1 together with at least one other R-protein, RPS2 monitor the integrity of the plasma membrane resident protein RIN4 (RPM1 interacting protein 4), involved in regulating defense responses and targeted by at least four *Pst* effectors (AvrRpm1, AvrB, AvrRpt2 and HopF2) (Axtell and Staskawicz 2003, Liu et al. 2009, Mackey et al. 2003, Mackey et al. 2002, Wilton et al. 2010). In addition, another type of *Pst* effector, AvrRps4, was included in some experiments (**Paper I, VI** and **VII**) as a contrasting effector to investigate a broader set of signalling transduction events, as AvrRps4 targets EDS1 (Enhanced Disease Susceptibility 1) (Bhattacharjee et al. 2011). Loss of either RPM1 or EDS1 results in several orders of magnitude increased *in planta* growth of *Pst* expressing the effectors (*Pst*:AvrRpm1 and *Pst*:AvrRps4 respectively) (**Paper VII**).

One approach to study the effect of an effector-R protein interaction independently of the pathogen is to use a transgenic, dexamethasone-inducible system (**DEX**). The system harbors the bacterial effector AvrRpm1 transformed into the plant genome under a dexamethasone inducible promoter DEX:AvrRpm1 (Mackey, et al. 2002). The system expresses AvrRpm1 in all cells that come in contact with dexamethasone and thus produces a slightly stronger response from the plant as to that of the whole bacteria, the DEX system is used in **Paper II, III** and **VIII**. The system removes any responses triggered by the bacterium itself or other effectors that are expressed.

If plant tissue is left to float in a body of water after bacterial inoculation, ions released during HR readily leak into the apoplast and will diffuse into the water. Changes to the electrolyte quantity can readily be quantified using an electrode that measures electric conductance. This readout has been used with much success since its development, to assess the ability of plants to mount defense reactions (Mackey, et al. 2002). Mode of *Pst* inoculation typically entails delivery of a bacterial suspension through the stomata by syringe-, vacuum- or spray inoculation. Incremental adaptions and advances to the procedure have been developed ever since. There is always room for developing new as well as refining- and perfecting existing methods to obtain additional, faster or more consistent information. The development of a vacuum based delivery method described in **Paper I**. Various aspects affecting the outcome of the released electrolytes were in-

vestigated by establishing a vacuum based method of bacterial infiltration. This method proved to be both faster and more consistent than tried and true syringe inoculation. Using the developed method, evidence is provided for a shift in onset and amplitude of HR related release of electrolytes by altering either the temperature of *Pst* culturing, composition of cultivating material or inoculum titer. Additionally the bacterial titer effect on HR kinetics was modelled. The kinetics of the HR was successfully fitted to a weibull-box cox function to describe this.

The highly synchronous infection process to study HR induced by *Pst* described in **Paper I** have proven fast and convenient throughout the studies presented herein and is used in **Paper III, IV, V, VI VII** and **VIII**. As it is possible to infiltrate large quantities of plant material, it is also conceivable that downstream isolation and quantification of molecules with low prevalence can be performed. Additionally, it is possible to pre-treat the leaf material with toxic substances without manual handling of a syringe, as is exemplified by radioactive labeling in **Paper I**.

The effect of bacterial pre-culturing conditions presents an interesting finding. However, it is not immediately apparent why *Pst* optimal growth condition is different from that of optimal virulence. This is possibly due to *Pst* reaching stationary growth phase earlier when cultivated favorably. The other important find is that the inoculum titer not only has an effect on the amplitude of released electrolytes, but also affects the temporal aspect of cell death initiation, and thus likely the infection process. Therefore, components such as culturing temperature, culturing media and bacterial titer are all important aspects to consider when assaying mutants for capability of initiating HR.

To study plant defenses against non-adapted obligate biotrophic fungi, the barley (*Hordeum vulgare*) pathogen *Bgh* is commonly used in combination with Arabidopsis. *Bgh* belongs to the fungi responsible for causing powdery mildew on plants, characterized by a white powder appearing on leaf surfaces, consisting of fungal hyphae and conidia. The *Bgh* genome was recently sequenced and analysis of the genome structure revealed an estimated size in the range of 140 MBp, encoding more than 200 predicted effector proteins (Spanu et al. 2010).

In contrast to most bacteria, biotrophic powdery mildews reside on the outside of the plant. Once fungal spores land on the plant epidermis they produce a germination tube that assesses physical- and molecular ques including hardness, chemical composition and hydrophobicity of the leaf surface (Glawe 2008). Fungal spores secrete lipases that use plant epidermal wax constituents to produce a set of aliphates that mediate adherence of the fungal spore to the plant cuticle (Carver et al. 1999, Feng et al. 2009). The degraded products of the cuticular waxes serve as germination ques for nearby spores and promote advancement of the fungal virulence process (Carver, et al. 1999, Hansjakob et al. 2010). Once attached, many fungal spores produce a penetration structure, an appressorium, which uses brute force to push through the plant cell wall (Howard et al. 1991), others grow through the stomata or use a combination thereof. Penetration is followed by invagination of the plant plasma membrane and construction of a feeding structure, haustoria (**Figure 4**) (Glawe 2008). From haustorial structures, fungal spores are able to obtain energy and construct secondary hyphae and later asexual conidiospores. *Bgh* lifestyle is complex and involves both asexual reproduction on leaf surfaces and sexual reproduction on leaf surfaces or during dispersion (Glawe 2008).

Bgh is readily cultivated on susceptible varieties barley. Traditional plant breeding has produced barley varieties with various degree of resistance to *Bgh*. In these studies the Barbro variety was used except for the study where spore quality was assayed. Powdery mildew spores are wind-dispersed in nature and can easily be released from the plant by shaking or blowing. Thus, by blowing/shaking infected plants over neighboring plants, fungal spores are readily transferred onto new plants. The infection of Arabidopsis is performed by putting the plants close to each other on the ground in a random pattern. A large cardboard box with an opening in the top is placed over the plants. Once in place, infected barley plants are shook above the hole in the box. Shaking releases a cloud of fungal spores that slowly reaches the plants, mimicking wind dispersal. This contraption is known as the settling tower and is used in **Paper VI, VII** and **IX**.

The other pathogenic powdery mildew that has been used in these studies is *Erysiphe pisi, Ep*. This fungus

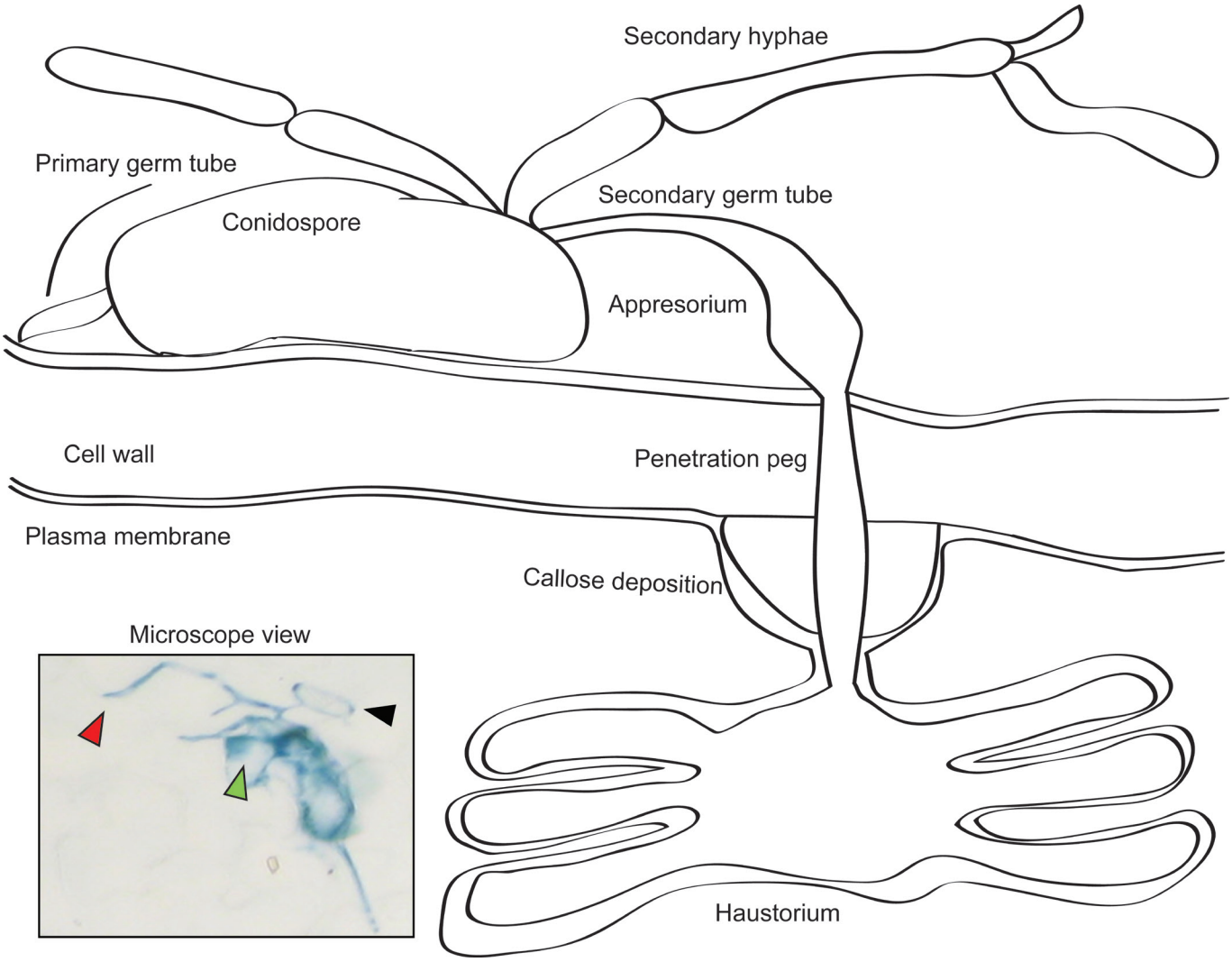

Figure 4. Model of powdery mildew infection. Spores landed on plant epidermal surfaces (**black arrow**) produces primary germ tubes that asses the surface. The appresorium forms and tries to penetrate the plant cell wall. Plant defenses are mounted, hallmarked by deposition of callose at the site of the penetration attempt. Failure to stop the penetration results in fungal invagination of the plasma membrane and construction of a haustorial feeding body. Nutrients are translocated from the plant into the haustorium and into the fungal spore. This results in advancement of the fungal hyphal network (**red arrow**) and finally construction of asexual conidiospores. Successful penetration attempts can be stopped by the plant by activation of programmed cell death (**green arrow**)

is cultivated on its host plant the green garden pea (*Pisum sativum*), the Kelvedon wonder variety. While the life cycle and infection strategy is similar, the morphology of *Ep* spores is somewhat different from *Bgh. Ep* spores are slightly larger, produce a different type of germination tube and a morphologically different haustorium (Falloon et al. 1989). Furthermore *Ep* spores are more firmly attached to the fungal mycelium on pea plants than *Bgh* is on barley. Thus, the settling tower method is unsuitable for infection of *Ep*. Instead, a soft brush is used to collect spores from the pea plants and then used to sprinkle them above each Arabidopsis plant to be infected (used in **Paper IV, VI** and **IX**). A similar mode of cultivation is used for the oomycete *Hyaloperonospora arabidopsidis, Hpa,* though spores are cultivated on susceptible lines of Arabidopsis and put in a water suspension instead of being blown during infection process (used in **Paper VII** and **VIII**).

Bgh spores are unable to penetrate Arabidopsis epidermal cell walls in all but 10-20% of the interactions. Spores that penetrate are efficiently stopped by post penetration defenses including PCD of the infected plant cell. *Ep* spores are to a higher degree able to overcome Arabidopsis penetration defenses but are eventually stopped by PCD and post penetration defenses before any new spores can be produced.

Since the culturing conditions of bacteria were found to have a large impact on the outcome of the HR

(**Paper I**) an investigation of fungal culturing, i.e. the plant cultivar was pursued in **Paper IX**. Three Swedish cultivars, Barbro, Mitja and Gustav with varying susceptibility to *Bgh* were selected for further investigation. Staining of barley leaves, show the dramatic difference between the varieties in terms of resistance. Hence, the Gustav variety, being the most resistant, also allowed production of the fewest conidial spores. However these spores were more virulent when exposed to the pen1 eds1 mutant that is impaired in both penetration and post-penetration resistance. Barbro being the least resistant of the three produced a throng of fungal spores that were significantly less virulent than both the Gustav and Mitja varieties.

Results from **Paper IX** suggest that the age of the spores is important for their ability to infect Arabidopsis. Spores would form more rapidly on a susceptible cultivar and thus be comparatively older than spores formed on a more resistant cultivar. This presents a significant find and warrant further investigation. If a subset of susceptible plants were to be co-cultivated with resistant varieties, the spores would possibly be less virulent on the majority of the field population and induce resistance before the more virulent spores from the resistant plant reach the leaf surface.

3.2 Visual-, genetic- and biochemical assessment of infection and defense response

The microscopic nature of plant pathogens demands use of microscopes to visually assess the progress of infection in many cases. A traditional technique for visualization is trypan blue staining, used in **Paper I, IV, VI, VII, VIII** and **IX**. Detached leaves or whole plants are submerged in an acidic phenol based trypan blue solution, boiled and consecutively de-stained in chloral hydrate (Keogh et al. 1980, Koch and Slusarenko 1990). Trypan blue selectively enters dead cells through ruptured membranes, providing an exclusion method of assaying the viability of cells. Additionally, the staining solution surrounds fungal structures and seeps into cavities of plant structures providing much needed shading and opaque to otherwise transparent cells. Subcellular structures have been visualized by electron microscopy as wavelengths of electrons are but a fraction of that of light. This gives significantly higher resolving power and has been used to visualize structural changes of chloroplasts during HR (**Figure 2** and **Paper VI**).

Fluorescent probes are commonly used in order to visualize small molecules. For instance, the sugar polymer callose, produced to enhance the cell wall, can be visualized by staining with aniline blue, **Figure 7**. Reactive oxygen species such as singlet oxygen can be visualized using the probe Sensor Green, (**Paper VI**). To investigate the localization of proteins a key technique is the use of fluorescently labeled tags. In **Paper V** such a reporter (green fluorescent protein, GFP) was fused to the acyl transferase studied to verify its localization to the cytosol.

Cellular activity can be measured and monitored in many different ways. The methods presented here are those used within the work presented. At the level of gene regulation, activity is routinely performed using reverse transcription quantitative polymerase chain reaction (qPCR, used in **Paper VI** and **Figure 9**) or arrays (used in **Paper III)**. Arrays can hybridize RNA or cDNA from transcribed genes and report transcript level of all of the genes on the array while qPCR specifically quantifies one gene at a time through amplification of transcripts of selected genes.

In order to quantify small organic molecules the method used throughout this thesis has been mass spectrometry coupled to either gas- or liquid chromatography (used in **Paper I, II, IV, V, VI VII** and **VIII**). Chromatography separates analytes by polarity and a mass selective detector quantifies ionized molecular species. As plants contain many different lipid species, many with nearly or exactly identical mass, it is imperative that the methods used for quantifying them are able to accurately separate different compounds with structural similarity.

A method for separating and quantifying polar lipids with close- or similar mass, including oxygenated lipids from plant tissue, based on gradient systems used in (Nilsson et al. 2012) was established (**Paper II**). While high resolution instruments are able to accurately perform this, this method uses a more affordable triple quadrupole mass detector. The method is based on liquid chromatography separation coupled to electro spray ionization and consecutive mass spectrometry.

As the improved separation successfully could identify several novel lipids as well as known isobaric lipids it was used to monitor changes to the lipidome during the plant defense reaction. Many strongly induced lipid species could be identified and characterized. Additionally, **Paper II** provide evidence that novel oxygenated SQDG, PG and PI species are formed during the hypersensitive defense reaction. The lipid-profiling method has been used in **Paper IV, V,** and **VI**.

PLANT DEFENSES

4

4.1 PART I - The plant is under attack

Initiation of defense responses is dependent on robust means of recognizing that plant cells are under attack. This is mainly achieved through proteins that recognize conserved molecular cues from microbes and herbivores. These cues are not only found on microbes that cause disease, but often also in many non-pathogenic organisms. In bacteria, such cues can comprise flagellin (Felix et al. 1999), peptidoglycan (Erbs et al. 2008, Gust et al. 2007), lipopolysaccharides (Newman et al. 1995), peptides (Oome et al. 2014) and non-methylated CpG DNA (Yakushiji et al. 2009). Viral coat proteins (Bendahmane et al. 1995), fungal- and oomycete cell wall fragments (Felix et al. 1993, Klarzynski et al. 2000, Pearce and Ride 1982, Sharp et al. 1984), various fatty acids, enzymes and molecular cues from eggs of insects (Hilker and Meiners 2006, Mattiacci et al. 1995) represent other molecular patterns that can be perceived by plant receptors and induce resistance. These molecular moieties fall into the common category of microbe associated molecular patterns (**MAMPs**) or herbivore associated molecular patterns (HAMPs). Another set of molecules not directly associated with pathogens or herbivores but by the activity of these are the DAMPs, danger associated molecular patterns (Boller and Felix 2009). DAMPs include for instance the plant´s own cell wall fragments and certain peptide fragments, as these suggests that a pathogen or herbivore is degrading the plant cells (Ryan and Pearce 2003). There are still several cues that are known to be recognized, but where either the receptor or downstream signal transduction are unknown (Felix and Boller 2003, Zipfel 2014).

MAMPs, HAMPs and DAMPs are readily recognized by a category of proteins termed pathogen recognition receptors (PRRs). Plants appear to rely solely on reception at the plasma membrane, in stark contrast to mammalian PRRs where both cytosolic and membrane bound PRRs have been identified (Zipfel 2014). Mammalian and plant receptors are not evolutionary related but a result of convergent evolution (Boller and Felix 2009). Two types of PRRs are commonly found in plants, receptor kinases (RK) and receptor like proteins (RLP). RKs have an exterior N-domain consisting of a ligand binding structure, often of the leucine rich repeat (LRR) type. The LRR structure is generally associated with protein-protein interactions, and thus suited for recognizing microbial molecular cues (Padmanabhan et al. 2009). RKs span the plasma membrane and the cytosolic C-terminal has the structure of a Ser/Thr kinase but often with a modified catalytic site (Dardick and Ronald 2006, Robatzek and Wirthmueller 2013). The other category of PRRs, the RLP, resemble RKs but lack the cytosolic kinase domain, therefore requiring additional components for further signal transduction (Zipfel 2014). PRRs have various structures, and signal transduction requirements, although several PRRs are known to form heteromeres with the receptor BAK1 (Shan et al. 2008). Recognition of MAMPs, HAMPS and DAMPs initiate what is known as MAMP triggered immunity, MTI.

The first described bacterial MAMP was a section of the bacterial flagella, consisting of 22 amino acid residues, the *flg22* epitope (Felix, et al. 1999). *Flg22* was shown to be recognized by the plant plasma membrane receptor kinase FLS2 (Sun et al. 2013, Zipfel et al. 2004). Mammalian innate immune system also recognizes flagellin. Though, there is variation as to what part of the flagella that is recognized, both between the animal- and plant kingdom and within the plant kingdom itself. Rice (*Oryza sativa*) recognize the *flg22* peptide, but trigger a stronger defense response when sensing the full length flagella (Boller and Felix 2009, Smith et al. 2003, Takai et al. 2008).

Flg22-FLS2 interaction and signal transduction is used as a model for threat perception in plants (**Figure 5**). Upon recognition, FLS2 interacts with two other receptors, BAK1 (also known as SERK3) and BKK1 (SERK4), a kinase (BIK1), BIK1 related proteins (PBS1, PBL1 and PBL2) and two U-Box E3 ubiquitin ligases (PUB12 and PUB13) to form a large complex (Lu et al. 2011, Lu et al. 2010, Roux et al. 2011). In unperturbed plants, FLS2 is associated with BIK1 in the plasma membrane. Association of FLS2-BIK1 with BAK1 occurs within seconds of flg22-FLS2 interaction, resulting in the release of FLS2-BIK1 association (Chinchilla et al. 2007, Lu, et al. 2010, Schulze et al. 2010). FLS2-BAK1 is then trans-phosphorylated and subsequent phosphorylation results in BIK1, and its homologues being phosphorylated. Phosphorylated BIK1 released from FLS2 is able to phosphorylate NADPH oxidases and facilitate early Ca^{2+} influx (Li et al.

2014), initiating the production of reactive oxygen species and defense signalling.

PART II - Defenses are initiated 4.2

Stress associated transport of cellular electrolytes was one of the earliest responses identified in plant pathogen interactions, established already in the first quarter of the 1900s (Atkinson et al. 1985). Since this was widely presumed to be due to dead cells losing their integrity and releasing their electrolytes, it was not until the late 1970s that it was discovered that this was a selective active transport for specific ions (Palta et al. 1977). Eight years later Atkinson and colleagues established that this process was used also for defense reactions triggered in tobacco cells inoculated with *Pseudomonas* sp. (Atkinson, et al. 1985). The electrolyte inflow following MAMP recognition consists primarily of calcium ions (Ca^{2+}) and protons whereas the outflow involve chiefly potassium ions (K^+) by ATP dependent K^+/H^+ exchange mechanisms (**Figure 5**). This acidifies the cytosol while leaving the apoplast slightly alkaline (Boller and Felix 2009). To compensate for the shift in electric potential, a considerable amount of anions, primarily chloride, is transported cross the membrane (Zimmermann et al. 1999).

Calcium signalling

Within seconds of a threat being identified by PRRs there is an influx of Ca^{2+} into the cytosol. This flow of Ca^{2+} appears to have varying strength and temporal duration in response to different MAMPs, resulting in slightly different cellular responses (Boudsocq et al. 2010). Initiation of Ca^{2+} signalling is believed to be dependent on PRR mediated activation of cyclic nucleotide gated channels (Ali et al. 2007). Though, the full array of Ca^{2+} channels involved is still poorly characterized.The biochemical-, hydration- and selective binding properties of Ca^{2+} render it vital for many cellular processes.

Increased cytosolic Ca^{2+} concentration is an ancestral conserved signal, from before the separation of plants and animals (Williams 2006). However, downstream responses to these concentration changes have diverged over the course of the millennia. Calcium-dependent protein kinases are responsible for a large portion of the transcriptional activation that is associated with the Ca^{2+} inflow in response to MAMPs (Boudsocq, et al. 2010). Modifications of the phosphorylation pattern of the proteome changes the spatial distribution of proteins and this in turn promotes further signal transduction. Another prominent type of proteins that reacts to calcium inflow is the Calmodulins. These acts as regulators and commonly work in association with members of transcription factor families including Calmodulin binding transcriptional activators, CAMTAs (Yang and Poovaiah 2002) and other Calmodulin binding proteins (Wang et al. 2009).

Reactive oxygen species signalling

Following ion fluxes over the plasma membrane, rapid production of reactive oxygen species (**ROS**) ensues, **Figure 5**. ROS are stronger oxidants than molecular O_2 and will damage cellular components if left unhandled. At least ten different processes in plant cells can produce ROS (Mittler 2002). During the oxidative burst the balance between processes that produce and detoxifies ROS shifts, hence the cellular redox is altered to an oxidative state. The ROS burst during MTI is primarily comprised of hydrogen peroxide (H_2O_2) and superoxide ions (O_2^-) produced by peroxidases and NADPH oxidases, respectively (Bindschedler et al. 2006, Daudi et al. 2012, O'Brien et al. 2012, Torres et al. 2002). Rose (*Rosa* sp.) and bean (*Phaseolus* sp.) cells appear to have different requirement for NADPH oxidases and peroxidases depending on the pathogen they are exposed to (Bolwell and Wojtaszek 1997). Thus, a preference of one over the other pathway for different plant-pathogen pairs appear to exist (Choi et al. 2007, Yun et al. 2012).

O_2^- can be dismutated into H_2O_2 either via enzymatic conversion by superoxide dismutases (SOD) or spontaneously (Karpinska et al. 2001). Alternatively, O_2^- can generate hydroxyl- ($HO^\cdot$) or hydroperoxyl radicals ($HOO^\cdot$) catalyzed by transition metal ions through the Fenton reaction (Asada 1999). The NADPH oxidases RbohD and RbohF are required for proper ROS production during MTI in Arabidopsis. Since

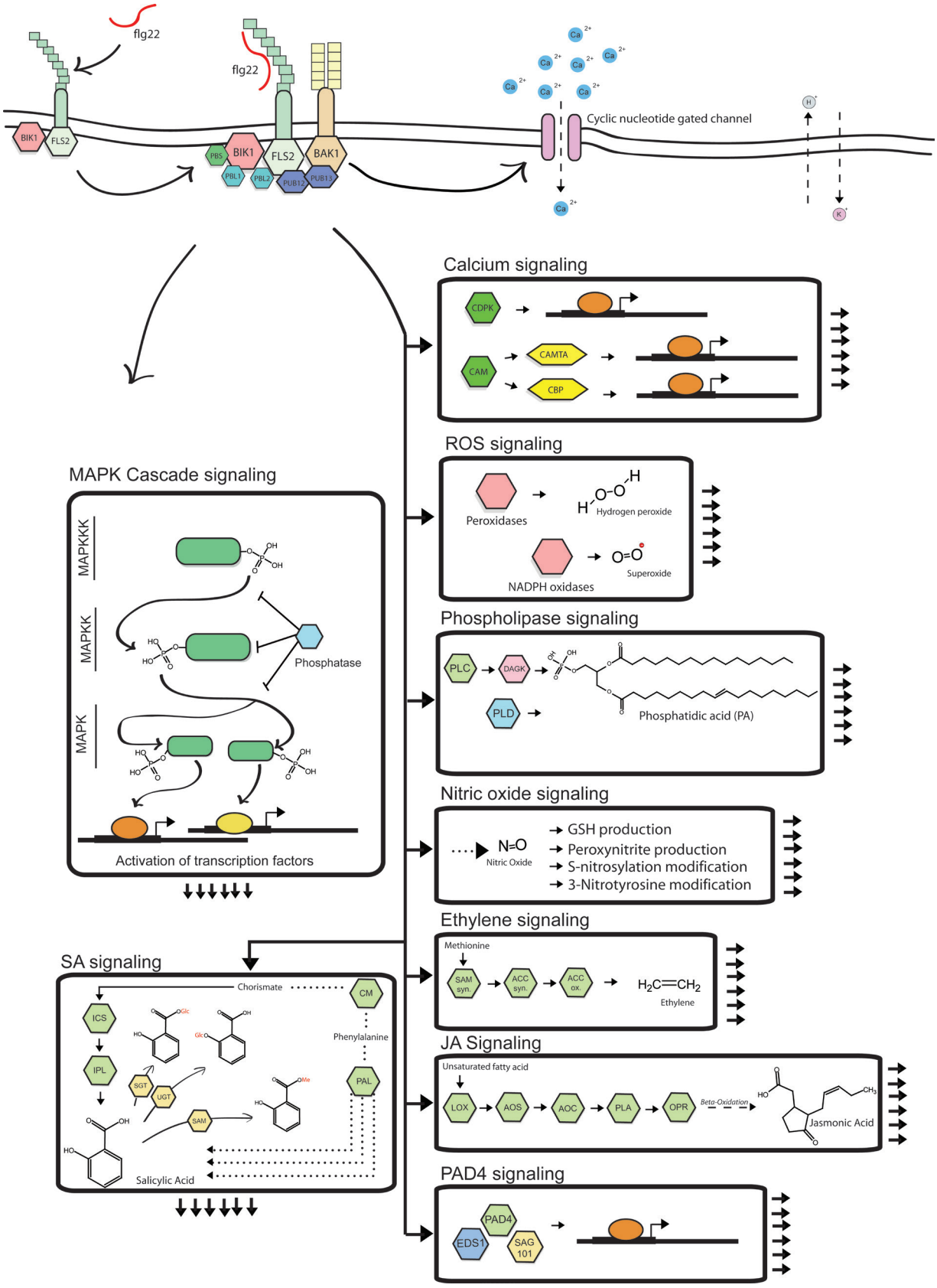

Figure 5. Model of defense responses triggered upon recognition of microbial molecular cues. Recognition initiates assembly of the PRR complex that in turn activates calcium channels, MAPK cascades, ROS, PA, NO, ET, JA, SA and PAD4 signalling. The responses are exist in an inrinsic network with many steps affecting the response of others.

mutants of RbohD and RbohF were only associated with a minor increase of bacterial growth in the case of RbohF, it is generally considered that ROS per se do not possess any major antimicrobial activity in the concentrations accumulated in the apoplast during MTI in Arabidopsis, or that pathogens are able to detoxify produced ROS (Chaouch et al. 2012).

Nitric oxide signalling

Nitric oxide (NO) is produced in response to MAMPs. The biosynthetic origin of NO in plants remains veiled, however, it is likely produced both in mitochondria and chloroplasts (Galatro et al. 2013, Vanlerberghe 2013). NO acts as a signalling compound in several kingdoms of life and its role in signal transduction has been extensively studied. The broad usage of NO reflects its attractiveness as a signalling molecule. NO has an almost non-existent dipole moment and no actual charge, hence it readily diffuses across biological membranes. NO forms metal-nitrosyl complexes with transition metals ions, primarily iron (Fe^{2+}/Fe^{3+}) ions, resulting in modification of proteins containing heme groups or 4Fe-4S clusters (Mur et al. 2006).

Apart from inorganic ions, NO reacts with S-nitrosoglutathione (**GSH**), producing GSNO, that serves as a stable NO pool. Reduction of GSNO into GSSG and NH_3 are in turn initiated by the enzyme GSNOR1 in Arabidopsis as yet another regulatory step (Feechan et al. 2005, Malik et al. 2011). Additionally, NO bind cysteine residues of proteins, producing a double bonded nitrosothiol group resulting in S-nitrosylation, a common post transcriptional modification of proteins in many organisms (Gonorazky 2014, Scheler et al. 2013). S-nitrosylation has been suggested to be part of the regulation of NADPH oxidase, ascorbate peroxidase and catalase activity (de Pinto et al. 2013, Yun et al. 2011b). Hence, NO can adjust ROS production (Fares et al. 2011, Maldonado-Alconada et al. 2011, Yun et al. 2011a). In parallel, the production of NO seems to be partly dependent on ROS production (Rasul et al. 2012). The chemistry of NO is complex as it readily reacts also with superoxide ions, resulting in the production of peroxynitrite ($ONOO^-$) that can react with tyrosine residues of proteins, converting them into 3-nitro-tyrosine, another common post transcriptional modification (Scheler, et al. 2013). Peroxynitrite itself is involved in triggering programmed cell death in plants in response to some pathogens (Alamillo and Garcia-Olmedo 2001). This taken together suggests that there are tight mutual control mechanisms (crosstalk) between NO and ROS.

Phospholipase signalling

Both phospholipase C (**PLC**s) and phospholipase D (**PLD**s) are activated almost immediately upon Ca^{2+} influx and produces the signalling lipid phosphatidic acid (**PA**) within seconds (Testerink and Munnik 2011). Hydrolysis of phosphatidylinositolphosphates, performed by PLCs results in production of diacylglycerol (DAG), that is phosphorylated into PA by diacylglycerol-kinase (DAGK), **Figure 6** (Walton 1995, van der Luit et al. 2000). PLDs on the other hand hydrolyses structural membrane phospholipids like PC and PE to directly produce PA (Gonorazky 2014). The two pathways present the plant with an opportunity to have two temporally separated pulses of PA production. Initial PA production peaks within minutes and represents a transient increase in PA catalyzed by both types of phospholipases (Yamaguchi et al. 2005). In contrast, the second burst initiates after the first hour, is stronger, longer lasting and is dependent solely on PLDs. PA is used not only in MTI signalling transduction but also in developmental processes, nutrient sensing and pollen tube growth, and serve various functions in other eukaryotes (Testerink and Munnik 2011).

Production of PA is dependent on NO production for some but not all MAMP responses (Laxalt et al. 2007, van der Luit, et al. 2000). PA is known to stimulate NADPH oxidase activity by directly binding to arginine residues, increasing ROS production (Zhang et al. 2009). Hence, there is crosstalk also between PA, ROS and NO. PLD dependent production of PA is inhibited by addition of primary alcohols such as n-butanol, as PLD prefer short chain primary alcohols , instead of water that is used to produce PA. Non primary alcohols are unable to do this (Ella et al. 1997). Thus, addition of *n*-butanol favors production of phosphatidylbutanol instead of PA (**Figure 6**). As previously shown, addition of *n*-butanol prior infection resulted in reduced penetration resistance against *Bgh* (Pinosa et al. 2013). Even though there are 12 isoforms of PLDs known in Arabidopsis, only PLDδ is involved in MTI-triggered cell wall based defenses against penetration

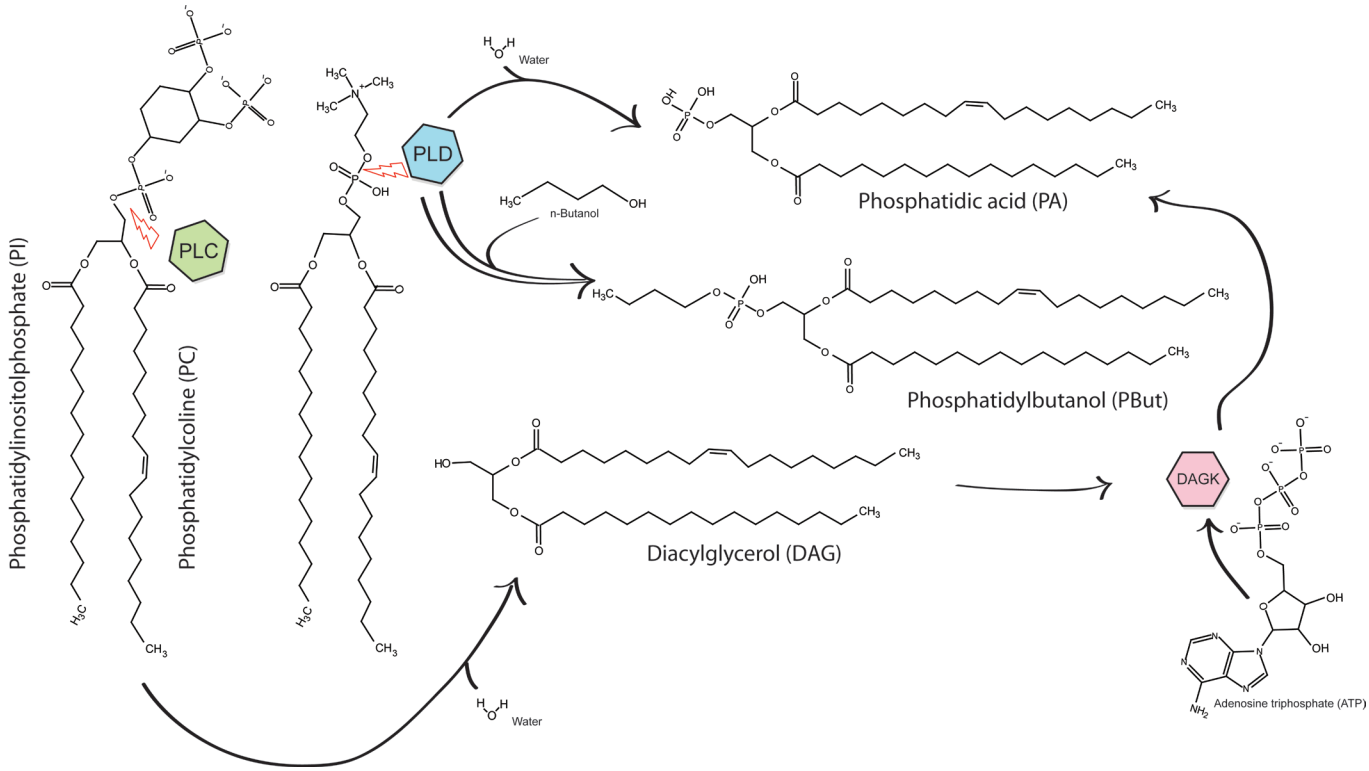

Figure 6. Model of PA production during defense responses in plants. Two pathways have been discovered. The first include PLD enzymes cleaving phospholipids like PC or PE to directly yield PA. The other produce PA through two consecutive steps from phosphatidylinositolphosphates. PLC cleaves the phosphate attached head group to yield diacylglycerol that is phosphorylated by DAGK into PA. PLD prefers primary alcohols to water and will in the presence of such yield phosphatidylalcohols like PBut.

attempts of *Bgh* (Pinosa, et al. 2013). Using a similar approach **Paper IV** provide evidence that the same is true also for another non-host powdery mildew, *Ep*. Thus, broadly activated PA signalling during cell wall based defenses against fungi appear to depend on the sole contributor PLDδ in Arabidopsis.

MAPK signalling

PRR recognition of their respective cues activates mitogen activated protein kinases (MAPKs, **Figure 5**) independent of ROS and NO production. MAPK phosphorylation cascades represent a common signalling theme among eukaryotes, and are involved in a variety of responses in plants. MAPK signalling commonly consists of three tiers of sequential phosphorylation events. This is initiated by phosphorylation of first level, the MAPKK kinases (MAP3K or MEKKK), by a receptor or receptor interacting protein. The MAP3Ks phosphorylates members of the second level, the MAPK kinases (MAP2K or MKK) which in turn phosphorylate MAPKs. Phosphorylation of MAPK stimulates its kinase activity and hence phosphorylation and thereby activation of transcription factors and other signalling nodes (Meng and Zhang 2013). This results in more than a thousand genes being either up or down-regulated as a response to MAMP elicitors (Zipfel, et al. 2004).

Two parallel MAPK cascades are triggered by FLS2 recognition of *flg22*, EFR recognition of elf18 and several other MAMP recognition events in Arabidopsis (Meng and Zhang 2013, Nicaise et al. 2009). The actual mechanics behind the initiation of the MAPK cascades by FLS2-BAK1 and other receptor complexes or their respective interaction partners is still unknown. The first MAPK cascade consists of a yet undescribed MAP3K, MKK4/MKK5 and finally MPK3/6. The activities of MKK4 and MKK5 are partly redundant, adding to the robustness of the signalling cascade. Phosphorylation of MPK3 and MPK6 activates transcription of a distinct set of genes, different from those activated by ROS and Ca^{2+} (Asai et al. 2002). The other MAPK cascade involves MEKK1, MKK1/2 ending with MPK4 (Huang et al. 2000, Matsuoka et al. 2002, Qiu et al. 2008).

Regulation of these phosphorylation events presents a control-mechanism that prevents MAMPs from non-pathogenic microbes from activating unnecessary defenses. If a sufficient threshold level is not reached by the signalling cascades, MAPK signalling are down-regulated within the hour (Tena et al. 2011). Plant phosphatases of Ser/Thr-, Tyr- and dual acting classes are all involved in the regulation of MAPK-signalling. Depending on the elicitor, the time before down regulation differs significantly (Meng and Zhang 2013).

Hormonal signalling

A set of hormonal signal realms are known to contribute to activation of MTI. MAMP activated defenses are dependent primarily on SA, JA and ethylene (**ET**) (Tsuda et al. 2009). In addition a fourth realm exists, embodied by defenses dependent on the protein PAD4 (Phytoalexin Deficient 4) (Glazebrook et al. 2003, Jirage et al. 1999). PAD4 forms a complex with EDS1 and SAG101 (Senescence Associated Gene 101) and requires these partners for initiating defenses (Wagner et al. 2013, Zhu et al. 2011). Though, there are examples of PAD4 mediated signalling by a yet unknown mechanism that does not require EDS1 or SAG101 (Pegadaraju et al. 2007). These four realms contribute to between 50% and 80% of MTI and exist in a complex inter-regulatory network that provides the robustness of the MTI (Kim et al. 2014, Tsuda, et al. 2009).

Brassinosteroids, gibberellins and cytokinins in general enhance resistance through activation of SA related defense while auxins have the opposite effect (Robert-Seilaniantz et al. 2011). Abscisic acid on the other hand appears to increase resistance against biotrophs and decrease it against necrotrophs.

SA is produced in the chloroplast both beforehand and *de novo* upon elicitation. The hormone is stored in the vacuole, conjugated to sugar groups. In plants, SA can be produced by two separate still not fully elucidated pathways, though, in plant disease signalling, the main route works through the protein ISOCHORISMATE SYNTHASE 1, ICS1/SID2 (**Figure 5**) (Vlot et al. 2009).

Several mechanisms have been proposed to explain the SA signalling. SA can for instance bind directly to catalase and ascorbate peroxidase, enzymes involved in degrading ROS, and also directly affect ROS production in the chloroplast (Mateo et al. 2006). The NPR (Non expressor of Pathogenesis Related proteins) proteins have been proposed as the major players in the downstream SA responses.

The cellular receptor of SA has been elusive, but both NPR3 and NPR4 directly bind SA itself as well as NPR1 (Fu et al. 2012, Moreau et al. 2012). NPR1 exists in the cytosol as an oligomer, upon monomerization it enters the nucleus and activates defense transcription in collaboration with TGA transcription factors (Johnson et al. 2003, Zhou et al. 2000). Oligomerization is accomplished by cysteine bridges and facilitated by GSNO modification (Tada 2009). These sulfur bridges can be broken by changes to the cellular redox facilitated by cellular ROS (Mou et al. 2003).

Several other proteins are known to be involved in modifying SA related signalling during the effector triggered signalling. For instance the proteins FMO1 (Flavin-dependent monooxygenase 1) and ALD1 (AGD2-like defense response protein 1). FMO1 primarily acts through regulation of EDS1 by a yet undiscovered mechanism and through regulation of *ALD1* transcription, whereas ALD1 is believed to act by producing pipecolic acid (Navarova, et al. 2012).

In addition to transcription factor activation, MAPK signalling stimulates production of the gaseous plant hormone ET via stimulation of ET biosynthesis enzymes (Li et al. 2012). ET in turn influences the levels of other defense hormones. The ETHYLENE INSENSITIVE PROTEIN 3 (EIN3) and its accomplice, EIN3-like 1 (EIL1), both transcription factors, repress the SA biosynthetic gene *ICS1* by binding its promoter (Chen et al. 2009). Thus, ET can significantly reduce the suppressive effect of SA on the JA pathway (Leon-Reyes et al. 2010). It is generally considered that SA is the main hormone required for defenses against biotrophs, while JA is used against necrotrophs. Thus, ET fine tune responses to be effective against necrotrophic pathogens rather than biotrophic, evidenced by the notion that virulent strains of *Pseudomonas* sp. display less severe virulence in ein2 mutants (Bent et al. 1992).

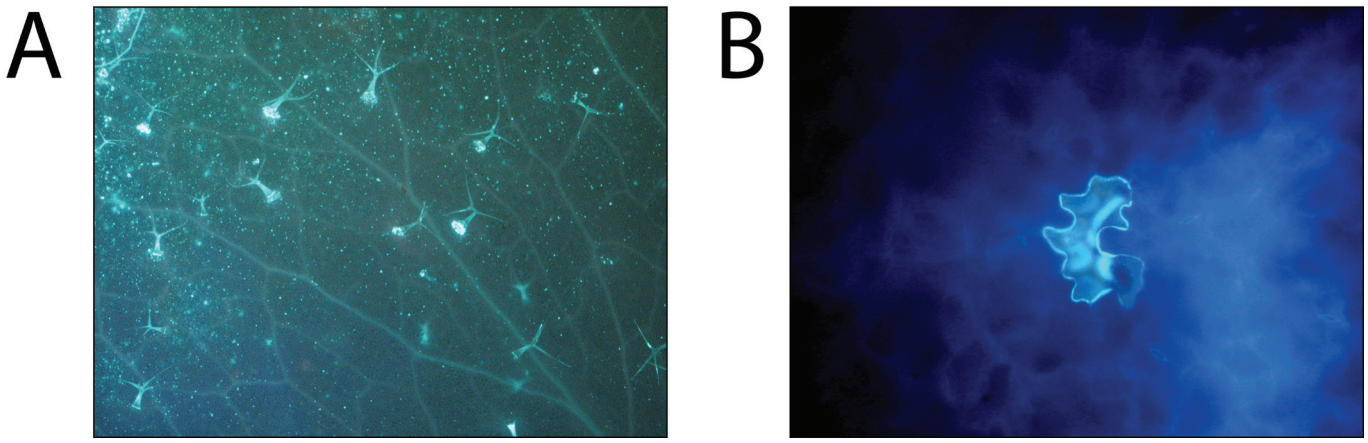

Figure 7. Deposition of callose in response to *Pst* (**A**) and *Bgh* (**B**) visualized by anniline blue staining and fluorescence microscopy.

4.3 PART III - Non-adapted pathogens are stopped by MAMP triggered immunity

Activation of MTI results in transcriptional reprogramming to enhance disease resistance long term. Strengthening- and mending compromised parts of the cell wall are an important part of MTI. Many higher plants synthesize and deposit the (1-3)-β-D-glucan callose. In Arabidopsis callose is produced primarily by the protein glucan synthase like 5 (GSL5 also known as PMR4) and to a lesser extent another still uncharacterized GSL (Jacobs et al. 2003). Deposition of callose to the cell wall is a hallmark of fungal- and oomycete penetration resistance, **Figure 7** (Bozkurt et al. 2012, Ellinger et al. 2013). Callose prevents spore penetration attempts, but callose is deposited in response to bacterial assailants as well (Ham et al. 2007, Hauck et al. 2003).

Upon pathogen challenge, such as that of *Bgh* and *Ep*, GSL5 is recruited to the site of infection. Callose and other toxic metabolites are believed to be transported via secretory vesicles in multi vesicular bodies to the site of attack (Bohlenius et al. 2010, Eggert et al. 2014, Ellinger, et al. 2013, Nielsen et al. 2012). Transport of these vesicles is assisted by syntaxins and exocyst proteins. Forward genetic screens identified the syntaxin SYP121 (PEN1) to be important for penetration resistance, whereas loss of the closely related homologue SYP122 has no effect on this (Assaad et al. 2004, Collins et al. 2003). This mechanism appears conserved between mono- and eudicot plants, as orthologues are found in both genera, evidenced by presence of the PEN1 homologue ROR1 in barley (Collins, et al. 2003). PEN1 ends up in the papillae structure consisting mainly of callose that is produced to prevent fungal spores from penetrating the cell wall. Vesicles from the trans-Golgi network export not only callose but also toxins and small RNAs that suppress pathogens through host induced gene silencing, HIGS (Nowara et al. 2010). PEN1 acts in close collaboration with the soluble N-ethylalemide sensitive factor adaptor protein SNAP33 and the two vesicle-associated membrane proteins VAMP721 and VAMP722 to form the ternary SNARE complex that facilitates vesicle fusion to the plasma membrane. At the membrane, exocyst (EXO70) proteins assist SNARE-membrane attachment (He and Guo 2009, Ostertag et al. 2013, Pecenkova et al. 2011).

Plant MLO (Mildew resistance locus O) proteins are required for penetration success of many powdery mildews (Huckelhoven and Panstruga 2011). These proteins are known to be involved also in bacterial- and oomycete triggered cell death and resistance, and have been proposed to be targeted by bacterial effectors (Kim and Hwang 2012, Lewis et al. 2012). MLO proteins likely act through calcium signalling as they tend to accumulate at the site of penetration attempts and interact with the calcium binding protein Calmodulin (Bhat et al. 2005, Kim et al. 2002). Mutation of *MLO* genes results in broad and durable resistance to powdery mildews also in field settings (Lyngkjaer et al. 2000). Loss of MLOs results in elevated transcriptional levels of indole glucosinolate biosynthesis genes as well as general defense genes (Consonni et al. 2010).

Thus, MLO proteins act as negative regulators of penetration defenses. The exact function of MLOs in healthy plants is elusive, but their occurrence in higher plants and even some algae vouch for an important yet unknown function (Devoto et al. 1999).

Callose deposition in Arabidopsis in response to *flg22* and other, but not all MAMPs, is dependent on the breakdown of the secondary metabolites indole-3-glucosinolate (I3G) and its derivative 4-methoxy-indole-3-glucosinolate (4M3IG) into their respective products (Clay et al. 2009) or production of SA and ROS (Luna et al. 2011, Nishimura et al. 2003). Involvement of glucosinolate breakdown products is emphasized by *flg22* initiating production of the transcription factor of MYB51, involved in regulating transcription of indole glucosinolate biosynthetic genes and consequently production of both I3G and its derivative 4M3IG (Clay, et al. 2009, Gigolashvili et al. 2007). Hydrolysis of the sugar group in indole glucosinolates is believed to be performed by the myrosinase PEN2 in Arabidopsis, originally identified to be involved in penetration resistance against non-adapted powdery mildews (Lipka et al. 2005). Glucosinolates have been presumed to be produced primarily as a defense mechanism against herbivores but recent evidence supports a broader role (Bednarek and Osbourn 2009). **Paper VII** show that one of the breakdown products of indole glucosinolates, in form of IAN can initiate cell death in plant tissue and **Paper VIII** shows that the same is true for one of the products of glucoraphanin degradation, the isothiocyanate sulforaphane.

Many of the PEN2 hydrolysis products are toxic to fungal pathogens and these are believed to translocate into the apoplast facilitated by the membrane bound ABC transporter PDR8 (PEN3). Removal of either PEN2 or PEN3 results in loss of penetration resistance in Arabidopsis towards non adapted fungi such as *Bgh* and *Ep.* The broad spectrum mildew resistance in the *mlo* mutants is dependent on both the PEN1 and PEN2/PEN3 pathways (Consonni et al. 2006). Combination of mutations in either *PEN2* or *PEN3* together with *PEN1* results in a slightly increased penetration rate compared to the single mutants. The triple *pen1 pen2 pen3* mutant did not confer any further loss of resistance when investigated but instead occasionally displayed runaway cell death and other phenotypes (**Paper VII**)

MTI also entails the transcription of pathogenesis related proteins (**PR**). PR proteins are found in all studied plants and consist of a diverse range of proteins with the common theme of being expressed in response to biotic stress. These include several chitinases (PR3, PR4, PR9, PR11) that breakdown fungal cell wall constituents, proteinases (PR7), nucleases (PR10) and several others proteins with defense related or yet unknown function (PR1, PR2, PR5, PR6, PR8, PR12-17) (Sels et al. 2008).

PART IV - Adapted pathogens overcome MAMP triggered immunity 4.4

Biotrophic plant pathogens must overcome MTI, and reprogram plants to surrender nutrients in order to proliferate within or in close proximity of plant cells. By evolving strong preformed- and inducible defenses, plants have put significant selective pressure on microbial pathogens to do this. This evolutionary pressure has resulted in several strategies to overcome and suppress MTI. Certain bacteria, including *Xantomonas* sp. produce biofilms consisting of polysaccharides that chelate Ca^{2+} ions, reducing the effectiveness of the ion flux, as less Ca^{2+} are able to enter the plant cell (Aslam et al. 2008). Others produce detoxifying enzymes that breakdown antimicrobial compounds or signalling molecules, **Paper VIII** (Fan, et al. 2011).

Pathogenic bacteria have evolved means of delivering protein molecules into host cells by exaption of the ancient flagella export system (Abby and Rocha 2012). The structure is known as the type III secretion system (**T3SS**) and is one of several secretion systems that exports proteins from the bacteria into the environment, but T3SS of phytopatogenic bacteria is used specifically for translocating bacterial proteins into the plant cytosol. The T3SS is anchored within, span both membranes of the gram negative bacterium and even extends a significant distance from the outer membrane (Galan and Wolf-Watz 2006). T3SS are encoded by a set of genes termed *HRC* in *Pseudomonas* sp. Disruption of any of these genes results in loss of bacterial effector translocation and virulence (Huang et al. 1993). Effector translocation through TTSS is initiated- and completed during the first ten minutes of bacterial attachment to cells (Mills et al. 2008). Thus, effectors delivery is fast enough to subdue plant MAMP recognition signalling.

Assembly of the FLS2-BAK1-BIK1-PUB12/13 complex is dependent of BAK1 phosphorylation of the BIK1 (Lu, et al. 2011). To overcome the MTI induced by recognition of *flg22* by the FLS2-complex, bacterial T3SS effectors from both *Pseudomonas* and *Xanthomonas* sp. have been shown to interfere with the phosphorylation of BAK1 (Shan, et al. 2008, Xiang et al. 2011) and kinase activity of BIK1 and RIPK, another kinase (Feng et al. 2012).

Numerous components of the plant defense system, in addition to PRR complexes are known to be targeted by pathogenic effectors, and many pathogens use several effectors for the same target. Indeed, P*seudomonas* sp. are known to target most MTI responses, including ET signalling (Cohn and Martin 2005), JA production (He et al. 2004), vesicle trafficking (Nomura et al. 2011), MAPK signalling (Cui et al. 2010) and many more.

Other microbial pathogens use different means of effector delivery, as their lifestyle differs from that of bacteria. Penetration of plant cell walls by fungal- and oomycete spores results in production of a haustorial feeding structure and presumed subsequent secretion of effectors into the colonized plant cell (Catanzariti et al. 2006, de Jonge et al. 2011). The number of putative effectors is staggering. Oomycete- and fungal pathogens carry several hundred predicted effector coding genes to manipulate and reduce plant defenses (Kamoun 2006, Spanu, et al. 2010)

While there is an assortment of potential effector targets within plants, it appears to be a high degree of overlap of cellular targets, also between pathogens that are evolutionary very divergent (Mukhtar et al. 2011). This reflects bottlenecks of plant MTI signalling, where a larger number of PRRs converge at certain nodes and activates similar sets of cellular responses. In contrast to the narrow range of effector targets, the structural and functional diversity to promote virulence is highly variable.

This is evidenced by the effectors used in this thesis. The effector AvrRpt2 from *Pst* is a cysteine protease that cleaves off the C-terminus of RIN4 (Axtell et al. 2003, Kim et al. 2005a) while AvrB and AvrRpm1 promotes phosphorylation of RIN4 via the kinase RIPK (Mackey, et al. 2002).

Once MTI is suppressed there is an active shift in plant transcriptional behavior, orchestrated by the pathogen. For instance, both bacterial and fungal pathogens initiate production of the plant SWEET type sugar transporters to retrieve carbohydrates from plant cells (Skibbe et al. 2010, Streubel et al. 2013). In **Paper III** at least 133 transcription factors, including WRKY family members, appears to be targeted by effector activity.

The aim of **Paper III** was to investigate early transcriptional changes in genes coding for transcription factors against the *Pst* effector AvrRpm1. To this end the transgenic DEX system that expresses AvrRpm1 *in planta* following dexamethasone treatment was used. Messenger RNA was isolated at 15, 30, 60 and 120 minutes after induction of AvrRpm1 in both Col-0 and *rpm1* backgrounds. A custom cDNA array was used to analyze changes in expression. Changes corresponding to either effector recognition or non-recognition were filtered and clustered to identify temporal patterns in the respective gene sets. 23 genes were shown to be differentially regulated between recognition and non-recognition of AvrRpm1 by RPM1. Among these genes were a large portion of WRKY transcription factors, usually associated with defense transcriptional activity (Eulgem 2006). Thus, effectors like AvrRpm1 actively suppress resistance genes as early as 15 minutes after introduction to plant cells in Arabidopsis.

Considering that the Arabidopsis genome contains more than 27000 genes (Swarbreck, et al. 2008) it is surprising that only a narrow range of transcription factor genes responds to recognition of AvrRpm1. **Paper III** suggests that only 76 transcription factor genes respond to recognition of AvrRpm1 by RPM1 within the first two hours. This corresponds to ≈0.3% of the genome and ≈7% of the array (1027 genes). In contrast, almost twice that number of transcription factors (133) is altered in the absence of RPM1. Some of these changes likely represent reprograming that promotes bacterial proliferation.

PART V - Plants recognize pathogenic effectors 4.5

Plants have devised a set of resistance proteins (**R-Proteins**) that recognizes effectors or effector activity and initiates a stronger defense response than that activated by MAMPs, the effector triggered immunity (**ETI**). A single effector-R-protein interaction pair is required for ETI to initiate. An interaction that is crucial for plants not to succumb to the efficiency of pathogenic effectors.

Most known R-proteins share a common LRR structure, similar to the PRRs consisting of a nucleotide binding domain (NB) and a variable N-terminal structure. NB-LRR receptors have one out of two types of N-terminal structures: either a coiled-coiled domain (CC-NB-LRR type) or a domain resembling animal Toll interleukin-1 receptors (TIR-NB-LRR type). Some evidence however points to the existence of other N-terminal structures (Meyers et al. 2003). CC-NB-LRR and TIR-NB-LRR vary in their respective downstream signalling component requirements. However, the exact mechanisms from effector reception to execution of the ETI is sparsely known. Examples of direct transcriptional activation by R-proteins do exist, but this is to date not considered the norm (Cui et al. 2014).

Plants do not possess an R-protein for every potential effector from every potential pathogen. This would be both evolutionary costly and crowd the cytosol with R-proteins. Likewise, it makes little sense for plants to produce a molecular memory like that of mammals, since infected cells would never encounter new infectious agents. This line of reasoning gave rise to the so called guard theory (Jones and Dangl 2006). The theory states that it is not always the effector protein per se that is detected by the plant but the disruptions thereof. Thus, R-proteins "guard" central cellular processes and monitor perturbations thereof. Hence, the same R-protein could be used to monitor perturbations of the same cellular process by effectors from different pathogens. To date, examples of both direct- (Deslandes et al. 2003, Dodds et al. 2006) and indirect recognition (Bhattacharjee, et al. 2011, Kim et al. 2005b) of effectors by R-proteins have been found.

Several studied R-proteins seem to work in pairs in a type-unspecific manner, sharing downstream signal transduction. This includes the TIR-NB-LRR type RPP2A and RPP2B that recognize oomycete effectors (Eitas and Dangl 2010). There are also those that have a broader role such as the RPS4 and RRS1. They form a TIR-NB-LRR pair that recognizes effectors of both bacterial- and fungal origin (Narusaka et al. 2013). Many effectors have multiple targets in the plant, evidenced by the ability of AvrRpt2 to promote virulence in *rin4* mutants and studies that have shown that AvrB targets the chaperone RAR1 in addition to RIN4 (Belkhadir et al. 2004, Lim and Kunkel 2004, Shang et al. 2006)

TIR-NB-LRR type R-proteins depend on the protein EDS1 (Falk et al. 1999) and mutants lacking EDS1 do not induce a defense reaction in response to the cognate effectors (Zhang et al. 2004). The TIR-NB-LRR R-protein RPS4 senses the action of the bacterial effector AvrRps4 on EDS1 in Arabidopsis (Bhattacharjee, et al. 2011). EDS1 and RPS4 forms a complex with AvrRps4, EDS1-RPS4-AvrRps4, that migrates into the nucleus to initiate transcriptional changes, possibly by interacting with another TIR-NB-LRR protein, RRS1, and members of the WRKY family transcription factors (Garcia et al. 2010, Heidrich et al. 2011, Schon et al. 2013). Thus, EDS1 functions both as a virulence target and as an active component of TIR-NB-LRR mediated ETI through the EDS1-RPS4-AvrRps4 complex.

In contrast, CC-NB-LRR type receptors like RPM1 are with a few exceptions not dependent of EDS1. Instead, most CC-NB-LRRs rely on NDR1 (NO DISEASE RESISTANCE 1) NDR1 resides in the plasma membrane and likely acts as an adaptor between RIN4, R-proteins and possibly other components (Day et al. 2006, Gassmann and Bhattacharjee 2012, Hatsugai et al. 2009). Thus, EDS1 and NDR1 represent two signal transduction pathways for effector triggered responses that R-proteins from TIR-NB-LRR and CC-NB-LRR classes depend on respectively.

The two signalling nodes EDS1 and NDR1 differ in mode of action for activating HR-PCD. EDS1 seem to be activating mainly the autophagic like machinery of the cell, through the *ATG* (Autophagy related genes) genes (Hofius et al. 2009). There is still some controversy to the mechanism of autophagy genes involvement as ATGs seems to promote both pro-death and pro-cell survival processes (Hofius et al. 2011).

Cell death activation downstream of NDR1 entails recruitment of the proteasome degradation machi-

nery, resulting in fusion of the tonoplast and plasma membrane. The fusion event is dependent on the PBA1 caspase-like activity, as part of the proteasome, possibly degrading negative regulators of the process (Hatsugai, et al. 2009). This releases vacuolar processing enzymes, VPEs that exhibit caspase1-like activity and help degrade cell content (Hatsugai et al. 2004). In addition, a pair of proteases, known as metacaspases (MC1 and MC2), are believed to be involved in regulation and activation of HR-PCD downstream CC-NB-LRRs (Coll et al. 2010). MC1 act positively on cell death activation and MC2 negatively by regulating MC1 activity. Metacaspases themselves do not have any actual caspases-1 like activity, since their active site prefers lysine or arginine instead of aspartic acid (Lam and Zhang 2012). However, only MC1 requires an active catalytic site for active functionality, whereas MC2 does not (Coll, et al. 2010).

Loss of the PBA1 and VPEs causes an actual loss of resistance against some bacterial assailants, whereas other caspases-like genes seems dispensable (Coll, et al. 2010, Hatsugai, et al. 2009, Rojo et al. 2004). How the metacaspases contributes to regulation and activation of cell death has been studied to some extent. Metacaspase 1 (MC1) physically interacts with the protein LSD1 (lesions simulating disease resistance response), a zinc-finger transcription factor (Coll, et al. 2010). LSD1 was originally found as a lesion mimetic mutant, hypersensitive to multiple stimuli. It initiates cell death upon contact to ROS-, light- and pathogens treatment (Dietrich et al. 1994, Jabs et al. 1996). Mutants in LSD1 accumulate high levels of superoxide and salicylic acid and are dependent on this for their hypersensitivity, and dependent on MC1 for the mechanistic activation of this cell death (Coll, et al. 2010). Hence, LSD1 is a cellular hub that negatively regulates cell death by its interaction with MC1, bZIP10, catalases and presumably several other mechanisms (Kaminaka et al. 2006, Li et al. 2013).

This difference in cell death activation is further strengthened by the results of **Paper VII**. The *pen3* mutant was shown to elicit less HR-PCD as compared to Col-0 when subjected to *Pst*:AvrRpm1 and *Pst*:AvrRps4. These effectors are recognized by RPM1 and RPS4 respectively. Another paper investigating the same mutant (Kobae et al. 2006), found it to have no apparent phenotype against *Pst* expressing the effector AvrRpt2. While RPS4 are solely dependent on TIR-NB-LRR downstream signalling RPM1 has been suggested to activate both TIR- and CC-NB-LRR signalling (Hofius, et al. 2009). In contrast, RPS2 which recognizes the action of AvrRpt2 is entirely dependent on CC-NB-LRR signalling. Thus, PEN3 appears to be involved in one but not the other type of cell death activation. This is further supported by slower cell death response against *Hpa* in **Paper VII**.

4.6 PART VI - The hypersensitive response, the heart of effector triggered immunity

There is no strong demarcation between the MTI and ETI in plant immune responses, as is illustrated by the similar biphasic production of PA and ROS, cytosolic Ca^{2+} increase and MAPK signalling (Andersson et al. 2006b, Lamb and Dixon 1997, Yamaguchi, et al. 2005). ETI represents a strong, prolonged, fast, and genetically robust response whereas MTI on the other hand initiates earlier but are dependent on a few key proteins. (Cui, et al. 2014).

Transcriptional regulation of ETI

There is likely a rapid transcriptional regulation already minutes after effector recognition as a consequence of R-protein mediated activation of defense responses. As these changes likely set the stage for the whole HR, **Paper III** pursued further investigation of this.

Paper III identified important transcriptional activity already 15 minutes after recognition of AvrRpm1. These changes have previously been overlooked by other studies that have focused on sampling after two hours and later. The well-defined temporal patterns of transcription of these genes vouch for a minute control of the events succeeding effector recognition. Three temporal profiles with an initial down regulation and two with an initial up-regulation were identified among the genes uniquely responding to AvrRpm1 recognition by RPM1. Among these, as well as those that responded with dissimilar expression depending on the presence or absence of RPM1, ten genes were selected for further investigation.

The selection criteria resulting in the ten genes included genes responding strongly positive to effector

recognition and genes that not been ascribed an established role in early ETI. In order to rapidly screen for involvement in defense reactions a method that required no stable knockout of genes i.e. transient oligonucleotide mediated silencing was used. This technique relies on oligonucleotides with a phosphorothioate (PTO) modification, which prevent breakdown by nucleases. The oligonucleotides are designed in a fashion such that they selectively bind to mRNA of selected genes. The ten genes were assayed for involve-ment in HR, as measured by release of electrolytes. This was performed by exposing PTO silenced plants to *Pst*:AvrRpm1. This assay resulted in two genes that when treated with antisense oligosnucleotide displayed reduced cell death compared to sense oligonucleotide treatment. These genes encode a ring finger protein (RHC1A) and a WKRY transcription factor (WRKY54). Further studies are needed to establish the role of these two transcription factors in the early signalling of ETI.

Signalling during the hypersensitive response

Accumulation of ROS (Lamb and Dixon 1997) and PA (Andersson, et al. 2006b) occurs also during ETI signalling. The first wave peaks within the first hour of effector recognition and peter out during the second. The second pulse initiates at four hours after recognition, peaks at a much higher level and remains high for several hours. The initial pulse of PA is presumably produced chiefly by PLCs, whereas the second is dependent on PLDs (Andersson, et al. 2006b). PA then partakes in NO and ROS crosstalk in the same manner as in MTI. MTI associated penetration resistance seems to be partially dependent on the PLDδ isoform against both *Bgh* (Pinosa, et al. 2013) and *Ep* (**Paper IV**). Further exploration in **Paper IV** examined if this was the case also for effector triggered signalling against *Pst*:AvrRpm1.

As previously mentioned, n-butanol is known to specifically inhibit PLD activity by routing PLD enzymatic activity towards phosphatidylbutanol instead of PA (**Figure 6**). The preference of *n*-butanol over water of PLD was confirmed by quantification of phosphatidylbutanol in butanol treated plants. A level of 0.8% *n*-Butanol was sufficient to completely abolish the cell death response in Arabidopsis towards *Pst*:AvrRpm1 (Andersson, et al. 2006b), whereas tert-butanol did not reduce it significantly. T-DNA insertion mutants in genes encoding the 12 isoforms of PLD were inoculated with *Pst*:AvrRpm1 and subsequent monitoring of released electrolytes as well as measurement of *in planta* growth of bacteria. No single or any of the tested multiple mutants did exhibit any large alteration in terms of released electrolytes as compared to Col-0. Several single mutants instead had a minor shift compared to wild type. This can be interpreted as a high degree of redundancy among the PLD isoforms in effector triggered signalling, evidenced by no reduced ability of the plant with regards to resistance.

The overlap of PLD activity in ETI triggered by recognition of *Pst*:AvrRpm1 (**Paper IV**) stands in contrast to the sole contribution of PLDδ to PLD activity in MTI responses against powdery mildews. This might be expected by the robustness of the ETI response in contrast to that of MTI. This is reflected also by the full resistance against virulent *Pst* of all PLD mutants.

Oxidant and anti-oxidant systems

In addition to ROS being produced by NADPH oxidases and peroxidases during ETI, photosystems in chloroplasts and the respiration chains in mitochondria contribute. Hence, light play an important role in ROS accumulation and the outcome of ETI (Karpinski et al. 2003). Accumulation of ROS such as superoxide and consecutive cell death in wild type Arabidopsis is dependent on the EDS1-PAD4 complex, a basic leucine zipper transcription factor (bZIP10) and activation of cell wall peroxidases and peroxisomal catalase activity for several effectors (Bindschedler, et al. 2006, Kaminaka, et al. 2006, Li, et al. 2013, Rusterucci et al. 2001). Catalase proteins are responsible for detoxification of cellular hydrogen peroxide into oxygen and water thus serves as a safeguard during oxidative bursts.

It is believed that the singlet oxygen ($^1O_2$) accumulating during ETI originates from the photosystems in the chloroplast and that it contributes to spontaneous lipid peroxidation in parallel to that mediated by lipoxygenases (LOX) (Zoeller et al. 2012). Hence, ETI activated in darkness results in reduced levels of ROS and thus reduced levels of PR gene transcripts compared to what is observed during defense responses acti-

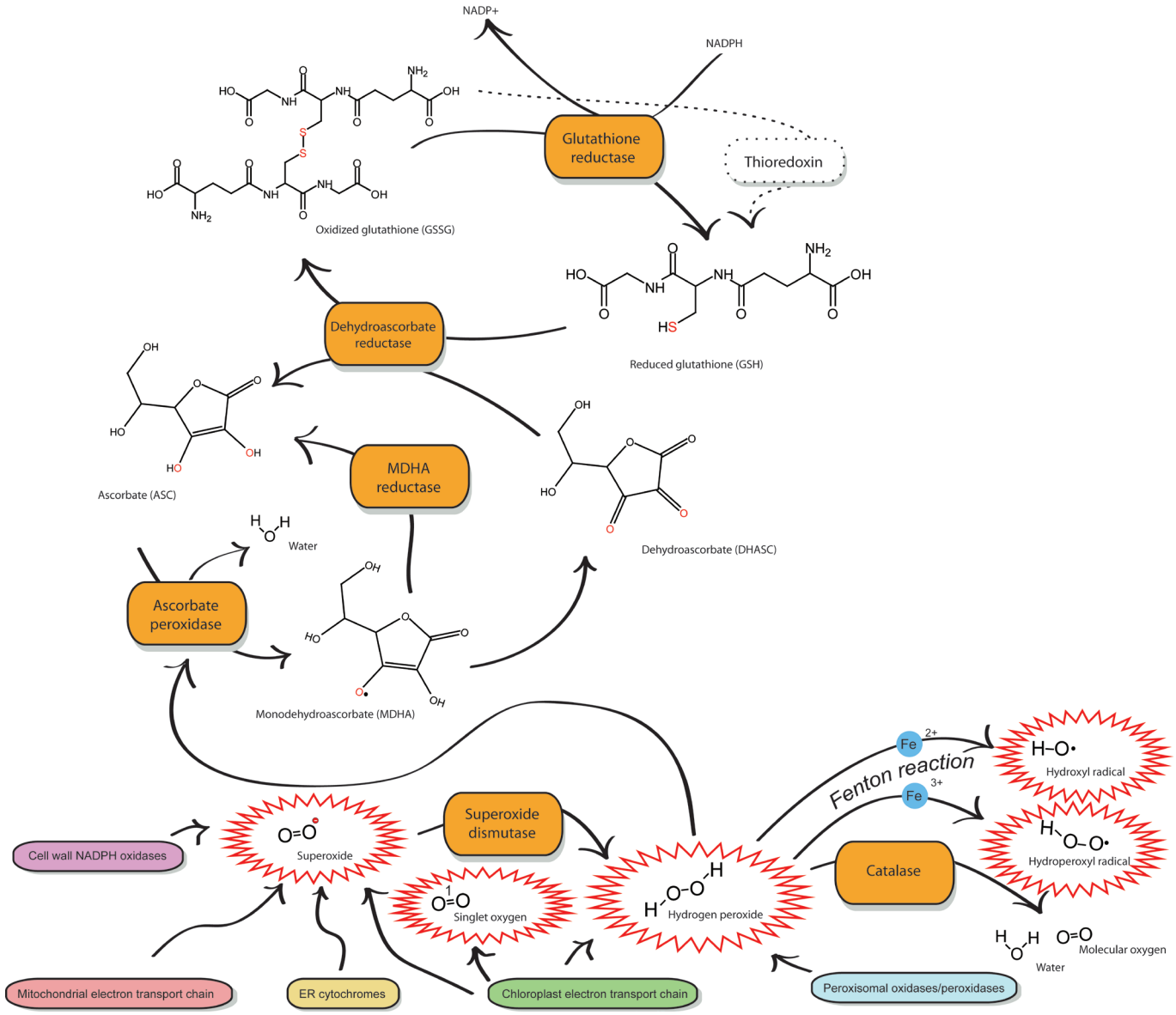

Figure 8. Several processes contribute to reactive oxygen species (Red pointy circles) in plants. Some of these can be converted into each other or taken care of by the glutathione-ascorbate antioxidant system. Orange boxes indicate enzymes and red letters on atoms indicate where on the molecule the reaction takes plase

vated during daylight (Genoud et al. 2002).

Production of ROS during ETI activates an antioxidant system consisting of a redox flux from NADPH through reduced (**GSH**) and oxidized glutathione disulfide (**GSSG**) to reduced ascrobate (ASC) and oxidized dihydro-ascorbate (DHASC) (Spoel and Loake 2011), **Figure 8**. To some extent, the glutathione system can be replaced by the NADPH dependent thioredoxin system (Marty et al. 2009). Presence of glutathione biosynthesis is however required for proper elicitation of programmed cell death (Hiruma et al. 2013). The respective redox pairs are not interchangeable with each other, in contrast, they are dependent on directional electron flow (Spoel and Loake 2011). These antioxidant systems serve as an important buffer for cellular redox status, preventing an accidental cell response to the inadvertently produced ROS and provide a redox threshold for signalling. Levels of ascorbate and glutathione are regulated by light and diurnal rhythms, and both serve multiple purposes, glutathione primarily as a sulfur- or electron donor in various biosynthetic reactions and ascorbate as an enzyme cofactor (Bartoli et al. 2006, Dutilleul et al. 2003, Foyer and Noctor 2011). To generate the massive ROS bursts, plants generate molecules that affect and diminish the respective antioxidant system pairs. **Paper VIII** aimed to isolate compounds that could mediate a signal for triggering

local cell death upon pathogen elicitation. This search resulted in such a compound.

By submerging leaf tissue containing the DEX:AvrRpm1 system in water and fractioning the water solution after elicitation, the molecule 4-methylsulfinylbutyl isothiocyanate (sulforaphane) was identified. Sulforaphane is conjugated to a sugar group in unstressed cells and belongs to a group of compounds known as glucosinolates. Glucosinolates are restricted to species, primarily within the Brassicaceae family (Agerbirk and Olsen 2013). The presence of these compounds in plants has been known since the 1960s (Larsen 1965) and they are characterized by a thioglucose group bound to a carbon atom that is attached to a variable group and an sulphonated ketoxime group (Natella et al. 2014). Breakdown of glucosinolates are initiated by hydrolysis of the sugar group. The sugar group makes the glucosinolate a more stable storage compound and enables quick activation upon wounding or pathogen defense.

The biosynthetic pathway for sulforaphane is not fully understood. Hence, lack of mutants in biosynthetic genes posed a difficult obstacle when investigating its role in ETI. To resolve this, two different sets of mutants was used to investigate the role of sulforaphane during ETI in Arabidopsis, *myb28* and *myb29* that acts as transcription factors for aliphatic glucosinolate biosynthesis (Beekwilder et al. 2008) and the *tgg1* and *tgg2* mutants that lacks the myrosinases involved in glucosinolate breakdown into aglycones (Barth and Jander 2006). Both sets of double mutants produced very low levels of sulforaphane in response to *Pst*:AvrRpm1 and as a result possess reduced capacity to trigger cell death against *Pst*:AvrRpm1 and *Hpa*, and reduced resistance against *Hpa*.

In **Paper VIII** it is suggested that sulforaphane could act as a signalling compound at the local level of pathogen infection. Sulforaphane signalling presumably does not work in a ligand-receptor fashion but instead presumably through sulforaphane binding to GSH. This in turn would deplete its antioxidant capacity as has been suggested in mammalian systems (Valgimigli and Iori 2009), thus speeding up ROS triggered immune responses by inducing a shift in redox state.

Transcriptional behavior of the *PEN* genes against biotic stress encouraged an investigation of their role in effector triggered signalling further. Loss of function mutants in the *PEN* genes display an attenuated cell death response against *Pst*:AvrRpm1, *Pst*:AvrRps4 and the *Hpa* isolate Cala2 (**Paper VII**). Mutations in *PEN1* and *PEN3* have known indirect effects with regard to the SA pathway being upregulated (Stein et al. 2006, Zhang et al. 2007). Enhancement of the SA pathway could prime the plant prior to the bacterial assay, resulting in resistance rather than cell death. No increase in SA levels was observed in the plants, priming or other indirect effects from losing the genes cannot completely be ruled out as explanation.

Thus, **Paper VII** focused on pursuing the pathway PEN2 is a part, that of indole glucosinolates and their breakdown products. One of the compounds downstream of indole glucosinolate hydrolysis is indole acetonitrile, IAN (Wittstock and Halkier 2002). Infiltration of IAN, was shown to induce cell death in plants (**Paper VII**). It is tempting to speculate that IAN have similar mode of action as that of sulforaphane. This could possibly suggest a role of indole glucosinolate breakdown products in ROS signalling, i.e. by contributing to reduction of the glutathione pool during the HR. This notion is not very far-fetched, as it is already known that IAN binds GSH in the biosynthesis of the secondary metabolite camalexin (Su et al. 2011). Moreover, two mutants in genes in the indole glucosinolate biosynthesis chain (*ugt74B1* and *cyp79B2/cyp79B3*) display significantly less cell death when inoculated with *Pst*:AvrRpm1 compared to wild type. .

The *PEN* genes have previously been shown to be involved in cell wall based MTI responses, **Paper VII** extends these roles to include also effector triggered signalling against *Pst* and *Hpa*. In addendum to **Paper VII**, exposure to indole acetonitrile does indeed induce *ATG6*, *PR1*, *GST1* and *VPEγ* transcripts (**Figure 9**) further strengthening its involvement in HR signalling. The same system do not appears to be active during cell death triggered by recognition of effectors produced by the oomycete *Hpa*, as *pen2* does not appear to trigger less cell death than wild type against the *Hpa* isolate Cala2. This system was instead shown to depend on PEN3 for full cell death and resistance.

Plant species lacking glucosinolates presumably have other electrophilic compounds with functional similarities. This is evidenced by the presence of benzoxazinoid compounds in many grass species such as wheat and maize (Pedersen et al. 2011). These compounds, including 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-

3-one (DIMBOA) from maize are often stored in the vacuole conjugated to sugars in a similar manner as many glucosinolates. Moreover, DIMBOA has been shown to deplete the GSH pool to prevent its antioxidant capacity (Dixon et al. 2012).

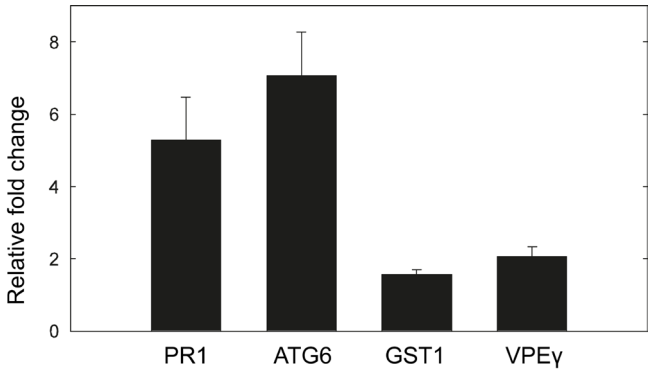

Figure 9. Relative fold change of genes involved in effector triggered signalling 24 h post exposure to IAN to that of mock treated plants. qPCR performed as described in **Paper VI**. Error bars indicate SD, n=4.

Lipid signalling

Membrane lipids are an integral part of cell structure and signalling during defense responses (Shah 2005). Chloroplast membranes contains a high proportion of lipid species containing one (monogalactosyl diacylglycerol, **MGDG**) or two consecutive galactose heads (digalactosyl diacylglycerol, **DGDG**) or in some cases a more exotic sulfoquinovosyl group (sulfoquinovosyl diacylglycerol, **SQDG**) esterified to a glycerol backbone. In addition to the head group, two fatty acids are esterified to the glycerol molecule. The head group and the two fatty acids are highly variable in terms of length, structure and level of saturation, giving rise to a variety of lipids. A portion of these structural lipids are converted upon abiotic and biotic stress. Hence, the hitherto presented PA is but one of several lipids induced by plants during HR.

Lipids with oxygenated fatty acids, oxylipins, are produced during the HR (Vu et al. 2012, Zoeller, et al. 2012, **Paper II** and **VI)**. One group of oxylipins with a prominent role in defense is the jasmonates. Jasmonates are important signalling compounds in wounding and several developmental processes, including flowering and germination in addition to their role in HR (Wasternack and Hause 2013). They are formed through several consecutive enzymatic steps (**Figure 10**). First, 13-lipoxygenases (13-LOX) produces a hydroperoxide group on a polyunsaturated fatty acid by addition of molecular oxygen. Second, hydroperoxides are transformed into epoxy groups by AOS (Allene Oxide Synthase) and cyclized by AOC (Allene Oxide Cyclase). These steps results in production of oxo-phytodienoic acid (**OPDA**) and are performed in the chloroplast (Wasternack and Hause 2013). OPDA can then be reduced and undergo chain shortening in the peroxisomes to yield the phytohormone JA. The produced JA can be further converted into volatile methyl jasmonate that translocases throughout the plant to induce stomatal closure (Gehring et al. 1997, Seo et al. 2001). JA is also transformed into an isoleucine amino acid conjugates (JA-lle), the active form of JA (Fonseca et al. 2009). JA-lle is then perceived by binding to the JA receptor COI1 (Coronatine insensitive 1) that works in association with the E3 ubiquitin ligase complex SKP1-Cullin (Pieterse et al. 2012). Upon binding JA-lle, the complex initiates ubiquitination and degradation of repressor genes and subsequent transcription of JA responsive genes.

OPDA (C18), its C16 analogue (dinor (dn)-OPDA) and JA each have signalling properties of their own (Dave and Graham 2012, Taki et al. 2005). Hence, OPDAs do not act as mere intermediates, supported by the notion that OPDA is not perceived by this COI1, explaining the different set of genes being activated upon OPDA exposure as compared to JA (Wasternack and Hause 2013). Instead OPDA and other oxylipins have been suggested to act as electrophiles (Farmer and Davoine 2007), a similar mode of action as that of sulforaphane and IAN (**Paper VII** and **VIII**). OPDA conjugated to GSH are produced and transported to the vacuole upon exposure to *Pst*:AvrRpm1, proposing regulatory mechanisms between ROS and OPDA/JA signalling during HR (Davoine et al. 2006, Ohkama-Ohtsu et al. 2011). If OPDA is formed on bound fatty acids (Nilsson, et al. 2012) it is likely cleaved off by a phospholipase before exhibiting any signalling properties. MGDG, SQDG and DGDG can all undergo head group acylation in response to cold and biotic stimuli (Vu et al. 2014, Vu, et al. 2012). Species throughout the plant kingdom attaches acyl chains to the head group of lipids (Acyl-MGDG, Acyl-DGDG, Acyl-SQDG etc.). **Paper V** report that while head group

acylation seem widespread, oxidized lipid species (OPDA and dnOPDA) on either head or glycerol backbone appear to be confined to a narrow range of plants. Many but not all members of the *Brassicaceae* family produce galactolipids with oxidized fatty acids during HR (Andersson et al. 2006a, Hisamatsu et al. 2005). The function of these compex lipids, known as arabidopsides, are not fully known. Arabidopsides have antimicrobial properties on their own, as well as acting as signalling lipids and possibly as reservoirs for OPDA (Kourtchenko et al. 2007).

Presence of acylated galactolipids in plants have been known for several decades but their synthesis has remained enigmatic, until recently, when this was pursued in detail in **Paper V**. Oat was shown to produces high levels of lipids with acylated head groups upon wounding. Fractionated oat protein extracts were assayed for acyl transferase activity. An active fraction was isolated, digested and subjected to mass spectroscopic analysis. Peptide fragments were matched against an oat EST sequence database. This resulted in a hit of a putative phospholipase with an orthologue in the Arabidopsis genome (At2g42690). Two independent T-DNA insertion mutants in this gene in Arabidopsis were unable to produce lipid species with acylated head groups. Furthermore, the coding sequence of the gene from Arabidopsis was transformed into *E.coli* fused to a hexahistidine tag. The enzyme was overexpressed and extracted from *E. coli* and subjected to affinity- and size exclusion chromatography. Head group acylation activity was confirmed *in vitro* by incubating MGDG from Spinach (*Spinacia oleracea*) with the purified protein extract. The T-DNA insertion mutants did not display any apparent morphological differences compared to wild type. Previous reports have indicated that the very same gene might be involved in UV-light response (Lo et al. 2004). Array data retrieved from GeneVestigator supports a role in light response, and also towards several pathogens. When subjected to *Pst*:AvrRpm1 the mutant leaks slightly more electrolytes compared to wild type. Though, T-DNA insertion mutants in the gene coding for the acyl transferase do not appear to affect resistance either to *Pst*:AvrRpm1 or the herbivore *Spodoptera littoralis*.

In conclusion, acylated lipids are formed in plants during HR, both by interaction of virulent and non-virulent pathogens (**Paper V**) and this investigation thus resolves the old question of how acylated lipids are formed in plants. Supporting this conclusion are that the cloned AGAP1 undoubtedly have acyl transferase activity on MGDG *in vitro*, as monitored by extraction of lipids and quantification by LC-MS/MS and that T-DNA insertion mutants in the gene produce negligible levels upon wounding.

The enzyme LOX2 is the main contributor of 13-LOX activity during HR, while the other three 13-LOXes are only able to produce minute amounts, and their activities are not induced upon effector recognition (Chauvin et al. 2013). It is well established that lipid signalling comprises an important part of HR (Testerink and Munnik 2011). **Paper II** showed that AOS produces a specific set of lipids upon recognition of AvrRpm1 *in planta* that is absent in the *dde2-2* mutant. Investigation of the HR lipidome proposes a specific composition of lipids, dependent on LOX2 activity, being produced during HR, different from that dependent on AOS (**Paper VI**).

Lipid profiling of loss of function mutants in *LOX2* (*lox2-1*), *AOS* (*dde2-2*) and *FAD378* (*fad3 fad7 fad8*) in response to *Pst*:AvrRpm1 was performed using the method developed in **Paper II**. The result from electrolyte leakage upon exposure to *Pst*:AvrRpm1 encouraged this investigation. The *dde2-2* and *fad3 fad7 fad8* mutants did not display any alteration to the pattern of released electrolytes. However, the *lox2-1* mutant was delayed in this release of electrolytes by several hours. Consecutive electron microscopy and PAR measurements confirmed the delay both structurally and biochemically. This vouched for a role for LOX2 activity separate of that in OPDA and JA production. The specific composition of the lipidome obtained in *lox-2-1* supports such a parallel role (**Paper VI**). The simplest explanation would however be that fatty acids containing hydroperoxides actively binds GSH and perturb the GSH/GSSG system as proposed for sulforaphane and IAN (**Paper VII, VIII**) (Davoine, et al. 2006), and that LOX2 is responsible for producing the majority of these during early HR. This mechanism has been suggested for 13-LOX products at later time points (Davoine, et al. 2006). Thus, production of singlet oxygen was monitored during HR by usage of a fluorescent probe. Indeed, the production singlet oxygen was delayed in *lox2-1*.

The notion that *lox2-1* shows delayed production of ROS in **Paper VI** suggests a redox mediated mechanism. Singlet oxygen induces transcription of *EDS1* and stimulates ET and JA biosynthesis (op den Camp et al. 2003). Both OPDA and JA previously have been ruled out as downstream compounds of singlet oxygen signalling (Przybyla et al. 2008), this leaves EDS1 or other components of redox signalling responsible for the delay. However, future studies will have to determine if there is a causal relationship between the various ROS, light and LOX2 activity.

4.7 Post penetration – ETI or MTI?

Little is known about the cellular events that transpire in the plant post penetration of a fungal spore. It is known that EDS1 play an important role in post penetration resistance against non-adapted powdery mildews, and that localized cell death of infected epidermal cells is key in stopping spores from proliferating (Lipka, et al. 2005, Stein, et al. 2006). It has also been suggested that the PEN proteins could have a role in post penetration in addition to their role in cell wall based penetration defense (**Paper VII,** (Collins, et al. 2003). **Paper IX** investigated the roles of EDS1 and other SA associated proteins in post penetration further.

To this end, *pen1* mutants in combination with mutations in *SID2, EDS1, FMO1* and *ALD1* in various combinations were used. The *pen1* mutation enables more than 90% penetration success rate and allow studies of defenses subsequent the penetration event. Only mutants containing *eds1* were affected in post penetration resistance against *Bgh*, whereas mutant lines including both *eds1* and *fmo1* independently, were compromised against *Ep*. Finally, an attempt was made to identify novel genes in post penetration resistance. Over 5000 seedlings from a EMS mutagenized M2 population of *pen1 eds1* plants were screened for increased post penetration resistance phenotypes against *Ep*., resulting in multiple plants with mutations causing increased susceptibility.

Paper IX thus complements previous knowledge of EDS1 involvement in post-penetration resistance, by adding the involvement of FMO1 in the Arabidopsis-*Ep* interaction. Two genes in addition to *EDS1* that contribute to post-penetration resistance both to *Ep* and *Bgh* were presented and has to be investigated further. As EDS1 has been shown to be targeted by *Pst* effectors (Bhattacharjee, et al. 2011), *in planta* growth of bacteria was monitored in the selected EMS mutants. Interestingly, four of these mutants were more susceptible to virulent *Pst* than *pen1 eds1*, suggesting a broad role in plant disease resistance. Subsequent studies will have to determine the genetic identity of these components and whether they trigger ETI or MTI based defenses.

4.8 Conclusions and outlook

Sequencing of plant and plant pathogen genomes as well as progress in biochemical and molecular methodology during the last decades has elucidated many key concepts of plant pathogen interactions. This thesis contributes with insight into several signalling routes during the hypersensitive response and the genetic components that mediate these. The first part of the thesis focused on the development and enhancing of techniques for analyzing HR (**Paper I**) and HR related lipid signalling (**Paper II**). The second part focused on early transcriptional regulation (**Paper III**), lipid signalling (**Paper IV, V**) and lipoxygenase activity (**Paper VI**). The third and final part examined components activated later during HR, which are involved in triggering cell death (**Paper VII, VIII** and **IX**).

However, as additional components are elucidated new questions can be posed. These will have to be investigated further by future studies. **Paper I** described the development of a vacuum method for infiltrating plant material with bacteria and the importance of bacterial culturing conditions and inoculation titer. How these parameters in detail affect the HR remain to be investigated. A metabolomic- or transcriptomic study of *Pst* prior and during infection could give insight as to what constitutes the difference between these

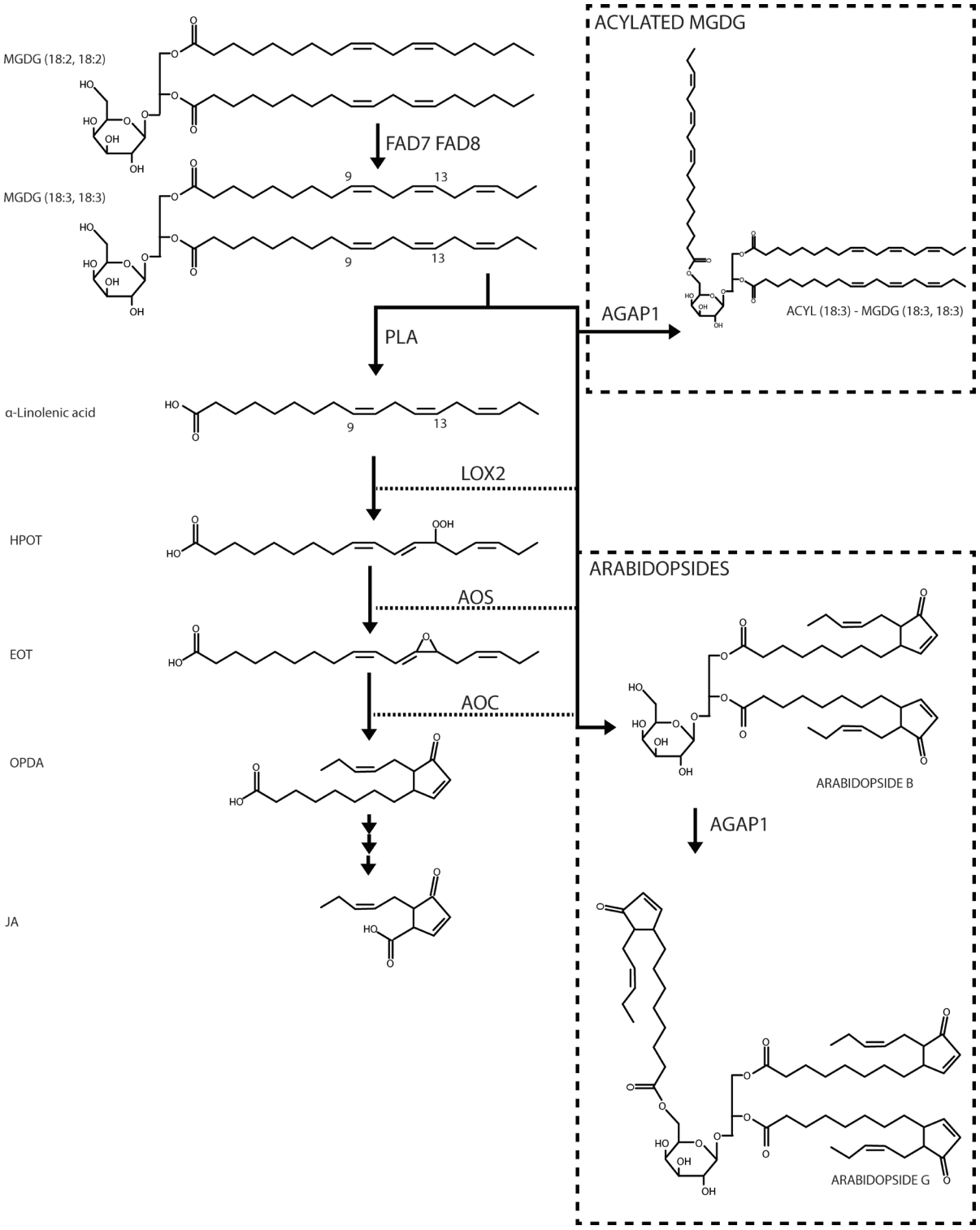

Figure 10. Biosynthetic chain of oxylipins in Arabidopsis. The fatty acid desaturases FAD7 and FAD8 introduces a third double bond on the fatty acids of MGDG (18:2, 18:2) to form MGDG (18:3, 18:3). These compounds can be cleaved of by phospholipase A (PLA) to yield free α-linolenic acid. The lipoxygenase LOX2 introduces molecular oxygen to form a lipid peroxides(hydroperoxyoctadecatrienoic acid, HPOT). AOS then creates an unstable epoxy group to form (oxidooctadecatrienoic acid, EOT). This compound is cyclized by AOC to yield oxophytodienoic acid, OPDA. OPDA can through several steps then be converted into JA. In parallel to free fatty acids, Arabidopsis has been shown to produce OPDA on bound fatty acids to yield a group of oxylipins known as Arabidopsides. Both MGDG and Arabidopsides can undergo head group acylation by the enzyme AGAP1 using either oxidized or non-oxidized fatty acids.

conditions. **Paper II** also describes the development a method. This proved effective in separating isobaric lipid species. This method was used to analyze lipidome of the HR in Arabidopsis. Several novel lipid species were identified, but what are the functions of these? This question was partially addressed in **Paper VI** but needs to be addressed by future studies using other mutants in the biosynthesis pathway and possibly by feeding experiments.

Paper III, IV, V and **VI** focused on early signalling. Interestingly there are several early patterns of transcription identified in **Paper III**. What role do the respective transcriptional patterns play? And what activates the transcription of these genes? Knockout of all of the genes in the respective cluster by constructing quadruple- or quintuple mutants would be an interesting project, however tedious. It would nevertheless help in answering what role they do play. The other question would easiest be answered using proteomics.

Lipid signalling is extremely important during early initiation of cell death as shown by the abolishment of this upon *n*-butanol addition. Several PLD isoforms are responsible for this as shown in **Paper IV**. What produced PA actually does in the cell would be an interesting follow up. It would however also be interesting to investigate how PLD isoforms contribute to insect chewing. **Paper V** identified the main contributor of galactolipid head group acylation in plants. The enzyme, AGAP1 was previously classified as a phospholipase, but this paper suggests it has dual roles. The study also delimited the presence of OPDA and head group acylated lipids throughout the plant kingdom. What is the role of the acylated lipid species? What other possible transferase activities does the enzyme possess? Knockout of *AGAP1* in Arabidopsis has not proven very fruitful, only minor phenotypes could be observed. It would therefore be interesting to create knockouts in other, distant relatives and investigate a role there. **Paper VI** shows that a certain lipoxygenase is crucial for timely induction of cell death in Arabidopsis. This is presumably due to less creation of lipid hydroperoxides that can antagonize the GSH/GSSG system. An interesting experiment would be to monitor redox changes in real time using cell cultures and optical tweezers in *lox2*-mutants and wild type, both transformed with the redox sensor RoGFP. This would offer a real time redox based system that can monitor the redox state throughout HR in the presence and absence of LOX2.

Thus, **Paper V** and **VI** together established knowledge to what parts of the JA biosynthetic chain (**Figure 10**) that are important for elicitation of HR-PCD during ETI. Loss of trienoic fatty acids (in *fad3 fad7 fad8*) as well as OPDA (*dde2-2*) was only associated with minor delay of HR-PCD, whereas loss of lipid 13-peroxides (*lox2-1*) was shown do delay HR-PCD with several hours. Loss of head group acylated lipids on the other hand (*agap1-1*) did instead confer increased HR-PCD and could possibly serve as a regulatory mechanism.

Paper VII extended the role of the *PEN* genes to include ETI. It also provided evidence that this partly could be attributed to loss of indole glucosinolate breakdown products. Several questions remain, do the pen1 and pen3 mutations have indirect effects that make them trigger less cell death. Do indole acetonitrile use the same mode of action as sulforaphane to affect cells? The mode of action of indole glucosinolates and their breakdown products are straightforward using feeding experiments combined with transcriptomics and redox sensors. The question regarding indirect effects of *pen* mutations is somewhat trickier to answer; one way to resolve this would be to use a quantitative proteomic approach. That could reveal absence or presence of proteins involved in priming defense. **Paper VIII** identified the isothiocyanate sulforaphane and described it a role in local defense responses through depletion of the GSH pool and thus shifting the redox status of the cells. What cellular signalling do sulforaphane invoke when perceived by surrounding cells? What role, if any, do the other antioxidants play in detoxification of sulforaphane? These questions need to be addressed in future studies. Studying sulforaphane *in vitro* in combination with other antioxidants or with mutants in biosynthetic genes of antioxidants could give clues as to their role, whereas a transcriptomic or proteomic level study could reveal what response feeding of sulforaphane triggers.

Post penetration defenses still have many questions that need addressing. To date it is not entirely clear whether it is resemble ETI or MTI. **Paper IX** identified novel components in the post penetration resistance and investigated the role of the SA associated gene network *EDS1, SID2, FMO1* and *ALD1*. Additionally **Paper IX** reports that cultivation conditions affect *Bgh* in a manner similar to that found for *Pst* in **Paper I.** What are the additional components of post penetration resistance against powdery mildews? How do

they relate to EDS1? Whole genome sequencing or backcrossing and mapping will reveal the identity of the components, and combinatory studies with EDS1 will disclose their relation, if any.

The field of plant pathogen interactions in general would benefit from further characterization of the mechanics behind execution of the programmed cell death in detail and elucidation of the relation of these and those of animal- and fungal cells.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

6

Växt-patogenförsvar: Signalering, resistens och celldöd

Växter producerar inte bara vackra blommor utan är den näringskälla alla landlevande djur är beroende av. Mikroskopiska organismer som bakterier och svampar är också beroende av denna näring. En del av dessa har anpassat sig till att direkt söka näring hos växter och orsakar därför död och sjukdom hos de växter de infekterar. Sjukdomsalstrande mikroorganismer är ett stort problem världen över och orsakar bortfall av i storleksordningen 10-20% av den totala skörden. Så varför ser man så få sjuka växter ute i naturen? Det beror bara delvis på att du är ute för lite, svaret ligger dock framförallt i att växter under miljoner år anpassat sig till detta och utvecklat ett effektivt försvar. Detta gör att de flesta växter är friska större delen av tiden. Men växter har väl inget immunförsvar kanske du tänker? Jodå, växter har ett immunförsvar som ger dom fullgott skydd mot de flesta sjukdomsframkallande mikroorganismer. Eftersom växter inte kan förflytta sig och dessutom saknar specifika immunceller behöver varje cell av växten kunna försvara sig själv. På cellnivå kan växtceller känna av när mikroorganismer finns närvarande och förstärka cellväggen, producera giftiga försvarsmolekyler och stänga sina klyvöppningar.

Mikroorgansimer som orsakar sjukdom har under evolutionen utvecklat sätt att ta sig runt växtcellernas försvar. Ofta genom att producera så kallade effektorproteiner som tar sig in i växtcellen där de försvårar och i vissa fall förhindrar försvaret från att fungera. Växten har i sin tur utvecklat proteiner som kan känna av dessa effektorer och signalera till cellen att aktivera ett starkare försvar. Denna förstärkta försvarsreaktion kallas den hypersensitiva responsen, HR och resulterar i effektormedierad immunitet. En central funktion under HR är för den infekterade växtcellen att begå självmord genom genetiskt programmerad celldöd. Det verkar kanske korkat att ta död på celler, men det är en mekanism som finns i alla organismer och som effektivt stoppar många inkräktare i växter. I min avhandling har jag studerat komponenter växtcellen behöver för att signallera att den borde starta denna process.

Den första delen av avhandlingen handlar om vidareutvecklingen av metoder för att infiltrera bakterier och mäta celldöd (**Papper I**) samt separera lipider med kromatografi (**Papper II**). Dessa metoder används sedan i resterande arbeten. Lipider låter ju märkligt kanske du tycker, men de är viktiga byggstenar i alla celler och bygger upp dess membran. Lipider består oftast av en ryggrad bestående av en glycerolmolekyl, en vattenälskande huvudgrupp och två fettälskande kolvätekedjor.

Den andra delen av avhandlingen handlar tidig signalering under HR, dels på genregleringsnivå (**Papper III**) dels på lipidnivå. Flera komponenter av lipidsignalering undersöktes. Dels de enzymer som är med och producerar signallipiden fosfatidsyra (PA) under HR (**Papper IV**), dels hur vanligt det är med oxiderade lipider i växtvärlden (**Papper V**), vilket enzym som är ansvarigt för att fästa fettsyror på den delen av lipiden som är vattenälskande, något som resulterar i acylerade lipider (**Papper V**). I **Papper VI** undersöktes dessutom hur ett enzym som fäster syre på lipider bidrar till att cellen initierar celldöden genom förändringar i växcellens redoxpotential. Ännu ett jobbigt ord, redoxpotential, förenklat kan man beskriva det som ett mått på hur oxidativ miljön är inne i cellen.

Den sista delen av avhanlingen beskriver hur två molekyler, indol acetonitril (**Papper VII**) och sulforafan (P**apper VIII**) och proteiner i deras produktionskedja föreslås påverka den cellulära miljön genom förändringar i redoxpotentialen och därigenom bidra till celldöd och försvar. Slutligen undersöktes hur olika delar av salicylsyra relaterade proteiner bidrar till den programmerade celldöden i försvaret mot svampar (**Papper IX**).

Sammanfattningsvis kan man säga att avhandlingen har gett insikt i hur olika aspekter av redox- lipid- och hormonsignallering bidrar till celldöden hos växter.

ACKNOWLEDGEMENTS

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Tack och hej

Åherrejävlar, vad skriver man i den enda del som ni kommer läsa? Det gäller i alla fall att formulera sig rätt och inte glömma allt för många personer. Så om ni inte ens orkade läsa den svenska sammanfattningen har jag i korthet infekterat fyrahundratjugoelvatusenmiljarder (bara ett räkneexempel) runda bladbitar med sjukdomsalstrande mikroorganismer och undersökt vad som händer i dom.

Först, **Mats**, tack för allt. Det känns om du aldrig riktigt tvivlat på att det skulle ordna sig, det var rather bra. Du har trots tidsbrist alltid funnit tid för mina frågor, tankar och projekt. Tack inte minst för att jag fått härja så fritt i labbet.

Andra medlemmar i växt-patogengruppen nuvarande och tidigare skall ha ett extra tack. Tack-tack. **Anders**, tack för otaliga experiment i lab och diskussioner under kafferaster, de har varit mycket uppskattade. Inte minst tack för läsning av avhandlingen. **Per**, tack för många intressanta diskussioner och inte minst att du skött mina växter! **Nathalie**, thanks for teaching me all the fungal work. Without you several papers in this work would have been impossible. **Francesco**, I'm thankful for all advice and discussion in the lab and your contribution to all the papers. **Elham**, your enthusiasm is inspiring, thanks for all help in the lab and for all discussions. **Mats E**, tack för att du introducerade mig till växt-patogenområdet under mitt masterarbete, det fick mig att vilja fortsätta på det spåret. Thanks to **Olga** for great collaboration with the TF manuscript!

Frida, tack för assistans med ditt och datt, allt rörande proteinjobb och trevligt sällskap och spelsnack!

Anders T, tack för intressanta inlägg om allt från himmel till jord, för läsning av avhandlingen. Jag uppmuntrar stort ditt nyfunna bryggarintresse.

I would like to thank all the people that were involved in the PCMB research area during my PhD studies (Especially **Aakash, Per S, Olle, Adrian, Nori, Panos, Henrik, Sazzad, Cong Fei, Cornelia**, **Björn, Jenny C, Andrei, Azeez, Lan, Hugues, Milton, Mohamed** and **Nadir**) for advice, borrowed stuff and discussions.

Emelie, Lisa delad frustration är halverad frustration? Tack för trevligt sällskap och diskussioner under åren.

Hela fikagruppen (**Johanna, Jenny, Henrik, Mikael, Norun, Åsa, Monica och Vivianne**) skall ha tack för trevligt sällskap.

Sven och **Niclas**, utan er stannar Botan, tack för snabb hjälp med mången ditt och datt under åren.

Tack till alla studenter som arbetat i vårt lab under min tid som doktorand (**Javi, Tarek, Dilara, Megiel, Eliza, Inta, Lina**) både er arbetsinsats och möjligeten att få dela med sig av kunskap. Speciellt tack till **Lovisa** som bidragit till många trevliga diskussioner på fika och lunch.

Stort tack till de som bidragit ekonomisk till mina projekt: Stiftelsen Lars Hiertas Minne, Kungl. Vetenskapsakademien, Willhelm och Martina Lundgrens Vetenskapsfond och P A Larssons fond samt till de företag som erbjuder gratisversioner av programvara (R, ImageJ, ApE, MzMine, ACDLabs – Chemsketch) och andra digitala verktyg (Oligocalc, Athena, Tair, T-DNA express mfl.) ornaments (TrueMitra) och fonter (Fiolex software, TrueFonts).

Extra stort tack till familj och vänner för att stöd och pepp under dessa år, inte minst dig min älskade **Sofia**!

PAPERS

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