Wounding- and Pathogen-Induced Defense Responses in Plants

ANDERS K NILSSON

GÖTEBORGS UNIVERSITET

FACULTY OF SCIENCE
DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

Till Kajsa

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Anders K Nilsson

Göteborg University, Department of Biological and Environmental Sciences
Box 461, SE-405 30 Göteborg, Sweden

ABSTRACT

Microbial pathogens and herbivores that cause disease or inflict damage to plants are ubiquitous in nature. To withstand and counteract invasions by these, plants have evolved several overlapping layers of defense. A preformed barrier consisting of physical impediments and toxic secondary metabolites limits the progress of most attackers. If these are overcome, a second line of inducible defense responses can be activated in plants through the recognition of non-self structures. Enormous progress has been made in the field of plant pathology over the last decades. Many of the mechanism by which plants perceive pathogens and pests, and the downstream signaling events that ultimately lead to immune responses have been characterized on a molecular level. Yet a comprehensive understanding for how plants can fend off invaders and achieve immunity with such finesse remains to be attained.

Two aspects of plant immunity are addressed in this thesis: I) the cell-to-cell communication that governs local defense and II) the genetic machinery and the biochemical processes that underlie wounding and pathogen-induced accumulation of complex lipids.

One of the most effective plant defense strategies against parasites is termed hypersensitive response (HR) and involves programmed cell death in infected and neighboring cells. In here, evidence is presented that the glucosinolate breakdown product sulforaphane is released from *Arabidopsis thaliana* cells undergoing HR induced by the bacterial effector AvrRpm1, and that sulforaphane can cause cell death when infiltrated into naïve tissue (Paper II). Hence, sulforaphane is identified as a novel regulator of plants' local defense. Plants unable to synthesize sulforaphane displayed impaired HR response and enhanced pathogen susceptibility. A proposed mode of action for sulforaphane is that it binds glutathione and thereby affects the cellular redox status.

Galactolipids containing the phytohormone 12-oxo-phytodienoic (OPDA), also called arabidopsides, are formed quickly and to high concentrations following mechanical wounding and pathogen elicitation in Arabidopsis. Data presented show that lipid-bound OPDA is formed while the fatty acid remains attached to the glycerol backbone (Paper III), and that all steps in this synthesis are enzyme catalyzed (Paper IV). Paper VI reports on the development of a LC-MS based method for the profiling of plant glycerolipids. This method was subsequently used to investigate natural variation in arabidopside accumulation (Paper IV), delimit the occurrence of OPDA-containing and acylated galactolipids in the plant kingdom (Paper V), and for the phospholipid profiling of the HR in Arabidopsis (Paper VI). Some of the findings from these studies include support that the gene *Hydroperoxide lyase* (*HPL*) is involved in arabidopside formation, that acylated MGDG species are omnipresent in the plant kingdom, and that a not previously described class of acylated OPDA-containing phosphatidylglycerols is induced during effector-triggered HR in Arabidopsis. Taken together, these results show that the plant membrane lipid composition is highly dynamic and that distinct lipids profiles are generated during different types of defense responses.

Crop losses due to diseases-causing pathogen and pests are estimated at around 30% globally. Understanding the mechanisms that determine resistance in plants and how plant diseases can be controlled is therefore of great value. The work presented in this thesis is my contribution to a deepened understanding of the plant innate immune system.

LIST OF PUBLICATIONS

This thesis is based on the following papers which are referred to by their Roman numerals in the text:

I. Johansson ON[1], **Nilsson AK**[1], Backhaus T, Andersson MX and Ellerström M. (2013). A quick and robust method with improved throughput for quantification of the hypersensitive response in plants. *Manuscript*

II. Andersson MX[1], **Nilsson AK**[1], Johansson ON, Adolfsson LE, Pinosa F, Garcia Petit C, Aronsson H, Mackey D, Tör M, Hamberg M and Ellerström M. (2013). The isothiocyanate sulforaphane: a signaling molecule in Arabidopsis local defense response against microbial pathogens. *Manuscript*

III. **Nilsson AK**, Fahlberg P, Ellerström M and Andersson MX. (2012). Oxo-phytodienoic acid (OPDA) is formed on fatty acids esterified to galactolipids after tissue disruption in *Arabidopsis thaliana*. FEBS Lett 586(16): 2483-2487[*]

IV. **Nilsson AK**, Fahlberg P, Hamberg M, Andersson MX and Ellerström M. Natural variation in arabidopsides in *Arabidopsis thaliana*. *Manuscript*

V. **Nilsson AK,** Kommuri M, Fahlberg P, Töpel M, Modarres M, Ellerström M and Andersson MX. Characterization of MGDG acyl transferase in higher plants. *Manuscript*

VI. **Nilsson AK,** Steinhart F, Ellerström M and Andersson MX. Glycerolipid profiling of the hypersensitive response induced by AvrRpm1 reveals novel acylated oxo-phytodienoic acid containing phospholipid species in *Arabidopsis thaliana*. *Manuscript*

[1] Both authors contributed equally to this work
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List of abbreviations

16:3	All-*cis* 7,10,13-hexadecatrienoic acid
18:3	All-*cis* 9,12,15-octadecatrienoic acid (linolenic acid)
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
DAG	Diacylglycerol
DAMP	Danger-associated molecular patterns
DGDG	Digalactosyl diacylglycerol
dnOPDA	Dinor-oxo-phytodienoic acid
EFR	Elongation factor Tu receptor
ETI	Effector-triggered immunity
FLS2	Flagellin sensing 2
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HPL	Hydroperoxide lyase
HR	Hypersensitive response
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
NHR	Non-host resistance
OPDA	12-oxo-phytodienoic acid
PA	Phosphatidic acid
PAMP	Pathogen-associated molecular pattern
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PIP	Phosphatidylinositol phosphate
PLC	Phospholipase C
PLD	Phospholipase D
PRRs	Pattern recognition receptors
PTI	PAMP -triggered immunity
R proteins	Resistance proteins
RLK	Receptor-like kinase
RLP	Receptor-like protein
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
T3SS	Type III secretion system

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1. INTRODUCTION

Plants are the main producers of biomass on land. They use the energy from sunlight to form energy-rich chemical compounds through the process of photosynthesis. Most other land-living organisms, microscopic and macroscopic, indirectly or directly, depend on organic carbon from plants for their survival. To withstand and counteract attacks from pathogenic microorganisms and pests, plants have evolved an advanced immune system. A preformed defense layer consisting of physical impediments and toxic secondary metabolites limits the progress of most invaders. If breached, a second line of inducible plant defenses can be activated through the recognition of non-self structures by pattern recognition receptors. Over the last decades, enormous progress has been made in our knowledge of the molecular mechanisms that underlie plant immunity.

With a world population expected to reach nine billion by the year 2050, food security is arguably the most critical global challenge of our time. To feed a growing population and avoid malnutrition, food production has to increase almost two-fold over the next forty years (1, 2). At the same time, the arable land available for crop cultivation is believed to be reduced by 8-20% as a consequence of urbanization (3). There is no simple solution to this challenge and several strategies must be used to prevent a potential food crisis. As an example, the UN proposes a substantial worldwide diet change - away from animal products towards plant-based food - as part of a solution (4). This implies that we must radically change how we grow plants, what type of plants we cultivate, and how we best make use of the harvest. Today, pre-harvest and post-harvest crop losses due to diseases and pests in the world are estimated at around 30% (5). In this light, understanding the mechanisms that determine resistance in plants and how plant diseases can be controlled are undoubtedly of paramount importance.

The work presented in this thesis is my contribution to a deepened understanding of **the plant innate immune system**. It can be anticipated that plant science in general, and molecular plant pathology in particular, will have a major role to play in the future development of plants with increased disease resistance, and thereby limiting crop losses and reduce the use of toxic pesticides.

The first section of this thesis introduces the basic architecture of the plant innate immune system. The systems by which plants recognize pathogens directly or indirectly via so-called PRR and NB-LRR receptors are described in detail. Plant responses triggered by wounding and herbivores are also discussed. The second part is devoted to describing the outcome of wounding and pathogen elicitation, i.e. plants responses that lead to a halt in the infection and immunity. In this section the results from the research articles that lay the foundation for this thesis are presented and discussed. **Paper I**, **II** and **VI** focus on the role of lipid-derived signals and secondary metabolites in plant pathogen responses. In **Paper III**, **IV** and **V** the biosynthetic pathways and the function of complex lipids in wounding responses are explored.

2. THE ARCHITECTURE OF THE PLANT INNATE IMMUNE SYSTEM

The vast diversity and complexity in lifestyle and feeding behaviors of microbial pathogens have shaped the plant immune system through co-evolution over millions of years. Mainly, plant pathogens can be divided into two classes accordingly to how they derive nutrients from their host. Necrotrophic microorganisms kill their hosts during the infection and feed from the dead tissue. Thus, necrotrophs are only dependent on avoiding recognition or suppressing the plant immune system during the early phase of an infection. Plant defense responses to necrotrophs are in many respects similar to those induced by wounding and animal feeding. Biotrophs, on the other hand, rely on live host cells either completely or partially in their life cycle. Therefore, biotrophs must constantly suppress plant immunity during the colonization and keep the host alive in order to survive and proliferate. Biotrophic pathogens that only depend on living cells for the initial stage of the infection and then switch to a necrotrophic lifestyle are called hemi-biotrophs. Plants deploy a battery of inducible defense responses to help them cope with both bio- and necrotrophic microorganisms.

At least four criteria have to be met by an effective immune system of any organism: I) recognition of antigens or non-self structures must be highly specific II) self-reactivity (auto-immunity) must be avoided III) responses must be under strict regulation to minimize fitness costs and IV) the resistance must be durable and not lost over time.

The most advanced and best studied immune systems are found in jawed vertebrates. The innate immune system of these animals consists of a preformed layer of cellular defense mechanisms that are rapidly activated upon infection. This innate immune system is triggered by structures that are shared by groups of related microbes and the response to repeated infections is essentially the same. Innate immunity consists of both non-mobile cell types and mobile cell types that roam the circulatory systems in search for invaders (6). The innate immune system is complemented by a second line of defense, known as specific or acquired immunity. This highly adaptive type of immunity consists of cells (B- and T-lymphocytes) carrying receptors that are produced by somatic recombination of gene segments and leads to an almost infinite antigen-specificity (7). Through clonal expansion of cells with antigen-binding receptors, the responses of the specific immune system are increasingly stronger and more effective for each encounter of a particular antigen. In this way, a molecular memory is created that primes the immune system for future attacks from the same organism.

Plants do not have such an elaborate immune system as they lack both a circulatory system and specialized immune cells. In plants, each individual living cell of the plant body must be able to respond autonomously to a wide range of potential pathogens. Nevertheless, plants are capable of perceiving a wide range of antigens with high specificity, avoiding reactivity to self, and to ward off potentially dangerous microbes. Plants are also capable of immunizing tissue distal to an infection, creating a "memory" that can be maintained over generations (8). It thus appears that the

immune systems of vertebrates and plants must at least partly use alternative mechanisms to deal with infectious disease. This leads us to the question: How do plants perceive intruders and accomplish immunity?

2.1 The Two Branches of Plant Immunity: MTI and ETI

For a pathogen to successfully colonize a host plant and acquire nutrients, several independent and complex networks of defense have to be overcome. The first line of inducible defense in plants is mediated through surface-localized **pattern recognition receptors (PRRs)**. PRRs perceive pathogens directly via "non-self" molecules or indirectly through the detection of danger associated "self" signals. The direct sensing of microbes is activated by the recognition of general elicitors, **Pathogen-Associated Molecular Patterns (PAMPs)** or **Microbe-Associated Molecular Patterns (MAMPs)**, which are characteristic for entire groups of microbes, such as bacterial peptidoglycan or fungal chitin. Since PAMPs are often common to both pathogentic and non-pathogenic microbes, the wider term MAMP will be used throughout this text. Attempted infections may lead to the indirect recognition of the pathogen through host derived danger signals, **Danger-Associated Molecular Patterns (DAMPs)**, that arise from wounding or injury. These include plant peptides released from the cell wall. MAMP-binding and DAMP-binding activate the PRRs and induce multiple defense responses in the plant cells resulting in **MAMP-triggered immunity (MTI)** (9). Moreover, PRR receptors are required for the discrimination of "non-self" during the establishment of beneficial interactions with symbiotic bacteria and mycorrhiza fungi (10, 11). Due to the relatively low selectivity and broad range response of MTI, the immunity it confers was previously referred to as basal resistance. The pattern-perception systems of plants are conceptually similar to that of the innate immune system of animals. However, at a molecular level, the antigen epitopes perceived are not shared and the receptor molecules involved differ, although they often respond to common microbial elicitors. Evidence therefore suggests that plant and animal signaling systems have emerged independently through convergent evolution (12).

To evade MTI, adapted pathogens secrete **effector molecules** into the plant cells that interfere with PRR signaling and suppress pattern-triggered responses (13). Effectors may also enforce metabolic shifts on the host plant which are beneficial for the attacker (14, 15). In turn, plants express intracellular **resistance (R) proteins** that directly interact with the effectors or sense their presence through perturbation of endogenous effector targets (16-18). The resulting **Effector-triggered immunity (ETI)** is a much faster and stronger immune reaction then those triggered by MAMPs (8). ETI and MTI responses are often overlapping although distinct differences exist. For example, the **hypersensitive response (HR)**, a type of localized programmed cell death, most often follows R-mediated resistance, while callose deposition and cell wall fortification are commonly associated with PRR-triggered resistance. As an evolutionary twist to the system, pathogens have developed effectors that render the R proteins

useless. These effectors may in turn be sensed by another set of R proteins, reflecting an evolutionary arms race between the plant and the microbe (the "zigzag model", 19).

2.2 Non-host and Race-specific Resistance

Most plant pathogens are specialists restricted to one or a few closely related host species. Consequently, all other plants are "non-host plants" to a certain pathogen. The phenomenon of **non-host resistance (NHR)** confers durable protection to plants against the vast majority of potential pathogens (20, 21). Since plants only rarely develop disease, this is also the most common type of resistance. NHR can be divided into two subclasses depending on whether or not HR-associated cell death is initiated in the plant. Type I NHR is analogous to basal resistance and takes place when non-adapted pathogens are unable to overcome the responses of MTI. The underlying mechanism may be that the pathogen is incapable of delivering its set of effector molecules, or that the effectors are ineffective on the new host, resulting in failure of pathogen growth. This type of resistance does not cause any visible symptoms and is the outcome of most attempted attacks. In type II NHR, effector molecules from non-adapted pathogens trigger ETI and hypersensitive cell death. The result can be observed as macroscopic lesions in leaves. One or both types of resistance mechanisms can be triggered by the same pathogen, working independently or in parallel (22).

On the other end of the scale of plant resistance we find **race-specific resistance** (also called cultivar level resistance). This type of resistance is the result of co-evolution between host and pathogen and is frequently determined through recognition of a single effector by a cognate plant R protein. Race-specific resistance is narrow and often varies considerable between plant cultivars and pathogen races. To exemplify this, the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* is adapted to tomato and a non-pathogen to the model plant *Arabidopsis thaliana* (hereafter Arabidopsis). By removing the gene encoding the effector protein AvrRpt2 from *P. syringae*, Arabidopsis plants are unable to mount ETI and lose their resistance to the pathogen (23). Likewise, plants mutated in the RPS2 gene, encoding the plant AvrRpt2 cognate R protein, are susceptible to *P. syringae* (24). Pathogens that are able to evade recognition and cause disease in plants are said to be **virulent** whereas pathogens that trigger ETI and fails to colonize the plant are referred to as **avirulent**.

2. 3 First Encounter - The Leaf Surface

Plants are equipped with pre-existing physical barriers that limit damage by herbivores and pest. Bark, trichomes, thorns and other specialized organs act as impediments to many types of organisms. However, these defense layers only have limited effect against the advancement of pathogens.

Biotrophic plant pathogens rarely enter host cells. Instead they proliferate in the intercellular space within the tissue, the apoplast. Once the apoplast is reached, their surface epitopes may betray them to the plant immune receptors. But even before a prospective pathogen reaches the sentinels of the MTI and ETI systems, several physical

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and chemical barriers have to be fought. The hydrophobic cuticle of plant leaves is rich in allelochemicals and waxes, constituting an inhospitable environment for microbes. Although many pathogens are able to live epiphytically for some time, the sooner they can enter the tissue interior to derive nutrients the better. In this context, it is not surprising that many plant surface molecules act as determinants for fungal spore germination (25-27). Another obstacle for the pathogen to overcome is the plant cell wall, a highly dynamic structure that provides mechanical support and connects the living protoplast with the plant body through the apoplastic space. If an infection is sensed and stopped at this level, no further defense actions are needed from the plant.

Phytopathogens use various strategies to cross the surface of the plant host and reach the intercellular space from where they can feed. Viruses and bacteria, for example, are dependent on insect vectors, wounds or natural openings (e.g. stomata and hydathodes) for their entry. Fungal and oomycete pathogens can either enter the plant tissue through openings or by directly penetrating both the cuticle and cell walls. They do so by forming an appressorium, a hyphal structure that exerts an extremely high pressure on the underlying cell wall. Once breached, the fungus develops the haustorium, a feeding organ consisting of host plasma membrane invaginations (28). To improve their penetration success, fungi and oomycets are known to secrete hydrolytic enzymes that degrade cell wall polysaccharides of the host (e.g. endo-polygalacturonases, cutinases and pectin lyases). These enzymes are believed to be essential for pathogenecity and are often encoded be several functionally redundant genes. Cell wall fragments released by such enzymes are the classic example of DAMP signals in plants; exogenous application of oligogalacturonides (OGs) and cutin monomers have well documented effect as defense elicitors (29-31). The systems by which plant perceive these danger signals are however still elusive.

Another strategy for pathogens to gain access to the plant interior is to manipulate plants' own gates to the apoplastic space, the stomata pores. Recent studies have shown that certain pathogenic bacteria and fungi play an active role in the regulation of plant stomata aperture (32). Several pathovars of the bacterium *P. syringae* secrete coronatine, a phytotoxic compound structurally similar to the plant hormone jasmonoyl-isoleucine (JA-Ile). When delivered into the plant tissue, coronatine promotes opening of stomata and provides an entry route for the bacteria (33). As such, the leaf surface constitutes a battleground for the chemical warfare between microbe and host.

2.4 MAMP Induced Signaling

MAMPs are highly conserved molecules that are shared among several classes of microbes. They include lipopolysaccharides and flagellin from gram-negative bacteria, peptidoglycans from gram-positive bacteria, chitin, ergosterol and β-glucans from oomycetes and fungi. As many of the MAMPs represent vital components for microbial life, they are not *per se* important for pathogenicity. MAMPs serve as molecular cues for surface localized pattern-recognition receptors (PRRs) that relay the signal of an

attack to the plant cell interior. Principally, the PRRs identified so far can be divided into receptor-like kinases (RLKs) or receptor-like proteins (RLP), both belonging to the RLK/Pelle superfamily of protein kinases. RLKs consist of a ligand-binding extracellular region, a single membrane spanning domain, and a cytoplasmic kinase domain. RLPs differ from RLKs in that they lack the kinase domain and only have a short cytoplasmic tail. Therefore, RLPs require the interaction with accessory proteins for signal transduction. In addition to their role in translating the presence of pathogens, RLK/Pelle proteins have key roles in development, growth and perception of hormones (34). The extracellular region of RLKs/RLPs shows great diversity and more than 20 structurally distinct domains exist (35). This large versatility in amino acid sequence has been ascribed the need for plants to quickly adapt to the ever-changing structures of microbial elicitors (36). Most of the PRRs with known function in plant defense contain a leucine-rich repeat (LRR) or a lysine motif (LysM) ectodomain. *In silico* analyses of the Arabidopsis genome have identified 56 RLPs and more than 600 RLK sequences, of which 216 contain LRR domains (37, 38).

2.4.1 FLS2 – Signaling through RLK receptors

To date, only eleven PRRs with known ligands have been characterized from plants. The majority of these receptors sense patters from pathogenic microbes, but some are also involved in the recognition of symbionts (39). The first to be identified, and probably the best studied PRR receptor is FLAGELLIN SENSING 2 (FLS2). FLS2 belongs to the group of LRR-containing RLKs and detects a conserved amino acid stretch of the bacterial MAMP flagellin (40). The ability to respond to the flagellin *flg22* epitope is evolutionary preserved among all major groups of higher plants (41). Interestingly, the vertebrate receptor TLR5 (Toll-like receptor 5) recognizes another conserved flagellin epitope, suggesting that plants and animals have evolved their own unique system for flagellin perception (42).

Within minutes after binding to *flg22*, FLS2 loses its lateral mobility in the plasma membrane and forms oligodimers with BAK1, another LRR-RLK protein (43, 44). BAK1 was originally indentified as component of the brassinosteroid signaling pathway, hence the name Brassinosteroid insensitive 1- Associate Kinase (45). Upon stimulation with *flg22*, BAK1 phosphorylates cytoplasmic kinases, including BIK1, which in turn transphosphorylates the BAK1-FLS2 protein complex for dissociation. The downstream signaling events of BAK1-FLS2 complex formation include a MAP kinase cascade and the activation of calcium-dependent protein kinases (46). The signal is attenuated by the ubiquitination, internalization and degradation of the complex (47, 48). It is possible that the internalization may not only facilitate attenuation of the signal, but that it is also important for distribution of the signal within the cell. To restore original FLS2 levels, *de novo* protein synthesis is required. Recently, several of the components involved in FLS2 signaling were found to be required for responses to other elicitors. The elongation factor Tu receptor (EFR) that recognizes the bacterial protein EF-Tu is dependent on BAK1 and BIK1 for its function (44). Also, BAK1 is important for signaling

involved in responses to the elicitors Ve1 and LeEix1/2. In contrast, chitin signaling by CERK1 is independent of BAK1, suggesting that at least two separate pathways for PRR mediated signaling exist (46).

Several lines of evidence further support the idea that RLKs use common conserved signaling pathways: chimeric protein with the EFR outer domain, and FLS2 transmembrane- and signaling domain retained responsiveness to the *elf18* epitope (49); heterologous expression of the Arabidopsis EFR receptor in *Nicotiana benthamiana* and tomato (plants lacking endogenous EF-Tu perception systems) confer enhanced disease resistance against several genera of virulent bacteria (50); silencing of the BAK1 homolog in *N. benthamiana* enhanced the susceptibility to the late blight oomycete pathogen *Phytophthora infestans* (51). These discoveries, together with the notion that MAMP responses often are quantitative and complementary, make RLK receptors interesting from an agricultural point of view. An attractive approach to create durable, broad-spectrum resistance would be to stack several known PRR receptors (called pyramiding) from different plant families into one crop. This provides challenges, but also exciting opportunities, for plant engineers of the future.

The high degree of similarity in MAMPs over species and family borders implies that they are indispensable for the microbes. Therefore, modification or loss of genes encoding them would be expected to severely affect the fitness of the pathogen. Indeed, introduction of mutations in flagellin inactivated FLS2 signaling, but also negatively influenced microbe adhesion, motility and virulence (52). In another study, several mutations in the flagellin-encoding gene *fliC* were indentified in isolates of *Pseudomonas syringae* pv. *tomato*, suggesting that this MAMP is under at least some selective pressure (53). Overall, PRR receptors equip plants with a robust detection system for a wide range of microbial pathogens.

2.5 Effector Stimulation and NB-LRR Signaling

To counteract and suppress plant responses evoked by PAMP triggered immunity, adapted pathogens secrete effector molecules that increase their virulence. Plants resistance (R) proteins perceive effectors by direct physical interaction or indirectly through effector modification of host targets (54). Although many types of genetically and structurally unrelated receptors can be activated by effectors, the largest class of R proteins is the **nucleotide binding-leucine rich repeat (NB-LRR)** receptors. Activation of NB-LRR receptors results in a fast and strong response that has been termed effector-triggered immunity (ETI). This type of defense is also called gene-for-gene resistance, because a single plant R protein can confer resistance through recognition of a matching **avirulence (Avr) protein** of the pathogen (55).

Much of our current knowledge on effectors and their host targets come from studies conducted on two major groups of Gram-negative bacteria, *Pseudomonas syringae* and *Xanthomonas* spp. Gram-negative bacteria inject a repertoire of effector molecules into the plant cell via their type III secretion system (T3SS) (56). A typical phytopathogenic strain of *P. syringae* expresses around 15-30 effectors that are

secreted during the infection (57). Several studies have identified components involved in PRR signaling as effector targets. AvrPphB, a cysteine protease from *P. syringae*, and AvrAC, an uridylyl transferase from *X. campestris* pv. *campestris*, both target the BIK1 kinase of the FLS2/EFR/CERK1 signalosome (58, 59). AvrPto is an E3 ligase that promotes degradation of the FLS2 receptor by catalyzing polyubiquitination of the kinase domain in Arabidopsis (60). Similarly, the MAP kinase pathways downstream of PRR activation are targeted by multiple effectors (61, 62). Also later events in the plant defense reaction have been identified as effector targets; the HopZ effector was found to enhance pathogenicity by degrading an enzyme involved in isoflavonoid biosynthesis in soybean (63). However, not all effector molecules associates with, and interfere with protein function. TAL effectors from *Xanthomonas* bacterial pathogens contain domains that are characteristic for eukaryotic transcription activators. TALEs bind host DNA with high sequence specificity and induce expression of target genes, also termed *disease susceptibility genes* (64). Target genes for transcriptional reprogramming by TAL effectors include transcription factors and SWEET sugar transporters (64, 65). SWEET proteins mediate glucose transport and up-regulation of the encoding genes may help the pathogen to fulfill its nutritional needs (14)

2.5.1 Structure and function of NB-LRR proteins

Plant NB-LRR receptors can be categorized accordingly to their N terminal domain: **TIR-NB-LRR** with a Toll/interleukin 1-like receptor domain, and **CC-NB-LRR** with a coiled-coil domain. The multi-domain structure of NB-LRRs permits them to simultaneously act as sensors and response factors of pathogen elicitation (66, 67). In the absence of pathogen produced effectors, the NB-LRR proteins are maintained in an inactive but primed state through a complex fold that is stabilized by domain-domain interactions (54). Small molecular perturbations may easily switch on the receptors from this stage and initiate signaling. To avoid auto-reactivity, the stability and the turnover of these receptors are kept under tight control by chaperones and ubiquitin E3 ligases. Failure to regulate NB-LRR receptor titer has been associated with autoimmune responses in plants (68, 69). The LRR domain seems to have a dual function, namely, as a sensor of pathogen stimuli and as an intramolecular signal transducer. In the inactive state, the NB domain interacts with the N-terminal part of the LRR and forms a closed nucleotide-binding pocket. During activation, the NB domain is released allowing exchange of ADP for ATP, alternatively ATP hydrolysis (70), and enables the protein to assume an open conformation (71). It is still unclear which domain(s) is required for downstream signaling that leads to the execution of the defense response. Evidence suggest that different subdomains are required to accomplish this role in different NB-LRR proteins (67 and references therein).

2.5.2 The Guard Model

Direct binding between a NB-LRR receptor and pathogen-derived effector has yet only been described in a few cases (72). Instead, it appears that NB-LRR receptors and other

R proteins act as guards by monitoring the targets of pathogen effectors. NB-LRRs are activated if the integrity of the effector target, the "guardee", is altered and downstream signaling is initiated. This **Guard Model** explains how several functionally unrelated effectors can be recognized by a single NB-LRR if they share a common target (73). The model also explains how a relatively low number of NB-LRRs proteins, 150 in Arabidopsis and 600 in rice (74, 75), can confer resistance to a virtually endless repertoire of pathogen-encoded effectors. Recently, this model has been challenged and it was suggested that some effector targets act as baits or plant decoys for effector detection by R proteins (76). The authors proposed that gene duplication of true effector targets, or evolution of effector target mimics, could result in decoys that are strictly involved in effector perception. It seems reasonable to believe that these concepts are not mutually exclusive and that plants have evolved several ways to perceive effector action from a general set of components.

A molecularly well characterized guarded effector target is the protein RIN4 of Arabidopsis. RIN4 is a negative regulator of MAMP signaling that is under the surveillance of at least two CC-NB-LRR receptors, RPM1 and RPS2 (77, 78). The R protein RPM1 specifically detects phosphorylation of RIN4 by the *P. syringae* effectors AvrRpm1 and AvrB, whereas RPS2 recognizes proteolytic cleavage of RIN4 by the AvrRps2 effector (77, 79). Effector stimulated activation of either RPM1 or RPS2 result in effector-triggered immunity. Interestingly, peptide fragments of RIN4 that were produced by AvrRps2-mediated degradation could suppress MTI in plants, supporting the hypothesis that RIN4 is indeed a virulence target and not a host decoy (80). Emerging evidence suggests that other effector targets, as the LeEix2 receptor for the fungal elicitor EIX, can act as pathogen bait and prevent virulence (81).

2.5.3 NB-LRR signal integrators

Two proteins, NON-RACE SPECIFIC DISEASE RESISTANCE-1 (NDR1) and ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), have been identified to integrate signaling from several CC-NB-LRRs and TIR-NB-LLRs, respectively (82). NDR1 resides in the plasma membrane where it interacts with the RIN4/RPM1/RPS2 complex via its cytoplasmic tail. Loss-of-function mutations in NDR1 render plants susceptible to *P. syringae* expressing any of the effectors AvrRpm1, AvrRpt2 or AvrB (83). It therefore appears that the role of NDR1 is to act as an adaptor protein that assures proper localization and assembly of immune receptors (82). *EDS1* encodes a lipase-like protein that is required for signaling through the TIR-NB-LRR resistance proteins RPS4 in Arabidopsis and L6 in Flax (*Linum usitatissimum*) among others (84-86). As for NDR1, mutations in EDS1 result in reduced resistance to virulent pathogens. EDS1 is found in two distinct subcellular pools, one in the cytoplasm associated with PAD4 and one in the nucleus in complex with PAD4 and SAG101 (87, 88). Translocation of EDS1 between those cellular compartments seems to be important for relaying TIR-NB-LRR signaling and for the activation of ETI (89). Thus, protein nodes as NDR1 and EDS1 can coordinate diverse signals into a limited number of downstream targets.

2.5.4 R Genes in an Evolutionary Perspective

Pathogens are under strong evolutionary pressure to evade recognition by plant receptors, and consequently, effector genes are highly polymorphic in sequence. To this background, it is noteworthy that R genes are often found in gene clusters within the plant genome. It is believed that such clusters are the result of an arms race that has led to gene duplication and diversification, allowing greater specificity and effectiveness among the R proteins (8). R gene-rich regions show higher rates of mutations and recombination events than the genome average (90). Some reports even suggest that pathogen pressure can promote the formation of new R genes through epigenetic destabilization of the genome (91). This genomic flexibility arms plant with new protein variants that can serve as sentinels for previously unencountered epitopes of microbial pathogens.

2.6 What Happens After Pathogen Recognition?

The intracellular signaling events that follow MTI and PTI have been intensively studied. Many of the components of this sophisticated regulatory network have been identified on a genetic and biochemical level. In general, plant perception of specific effectors and MAMP molecules lead to the activation of similar intracellular signaling cascades (92). Also responses to MAMP and effector elicitation overlap considerably and ETI has been described as "an accelerated and amplified MPI response" (19). However, specific elicitor-receptor interactions may activate certain subsets of the signaling machinery and employ distinct sets of signal transducers. Moreover, even though common signaling routes are triggered by several types of elicitors, amplitude and timing of the responses are known to differ substantially (93).

2.6.1 Minutes after elicitation – Second messengers and protein modification

One of the first detectable physiological responses of pathogen stimulation is the activation of membrane localized ion channels. Within minutes after elicitation, an influx of H^+ and Ca^{2+}, and an efflux of K^+ and Cl^- results in depolarization of the plasma membrane (94). Ca^{2+} originating from the apoplastic space serves as a second messenger in the cytoplasm that further activates ion transporters and other calcium-dependent proteins, including calmodulins and transcriptional activators (95). Another important element of the early responses is the oxidative burst which constitutes the generation of reactive oxygen species (ROS) at the infection site. ROS are produced by extracellular NADPH oxidases and cell wall peroxidases and depend on transient increase in cytosolic calcium levels (96, 97). ROS may act directly as microbial toxins or they may work as activators of other defense signals through protein modifications (98). In addition, nitric oxide (NO), a reactive gaseous radical, accumulates in the plant tissue in response to pathogens and acts in cooperation with ROS to execute defense programs (99).

Lipids and lipid derived molecules are also associated with early defense signaling. Ca^{2+} and hydrogen peroxide formed from the oxidative burst can activate

phospholipase C (PLC) and phospholipase D (PLD) that produce the lipid second messenger phosphatidic acid (PA) (100-102). PLC cleaves off the headgroup of the membrane lipids PIP and PIP_2, which can then be phosphorylated by diacylglycerol kinase (DAGK) to PA. PLD catalyses the hydrolysis of the headgroup from structural lipids like PC and PE and directly forms PA. Pharmacologic inhibition or genetic silencing of PA production in plants result in reduced pathogen responsiveness (100, 102, 103). How PA relays the signal to downstream targets still remains an open question. Elicitor activation of PLCs generates, in addition to PA, inositol phosphates ($InsP_2$ and $InsP_3$ that can be phosphorylated to bioactive $InsP_5$ and $InsP_6$) (104). Inositol phosphates further stimulate influx of calcium into the cytosol, thereby creating a feed-forward loop (102).

Multiple post-translational modifications of proteins are identified as yet another early event of plant pathogen responses. As in animals, phosphorylation mediated through mitogen-activated protein kinases (MAPKs) is known to play a central role in biotic stress signaling in plants. The Arabidopsis genome contains 20 MAPKs that are under the regulatory control of 10 MAPK kinases (MAPKKs), which in turn are the substrate of approximately 60 MAPKK kinases (92). Stimulation with the flagellin *fls22* epitope in Arabidopsis was shown to initiate a MAPK cascade that culminated in the activation of MPK3 and MPK6, leading to the subsequent activation of WRKY proteins (a family of transcription factors that serve as key players of plant immunity) (105). In other studies, dozens of proteins have been found to be phosphorylated in a *flg22* dependent manner. Interestingly, RbohD, one of the enzymes responsible for pathogen induced ROS formation, was identified as one of these proteins (41 and references therein).

A consequence of the oxidative burst and ROS accumulation is the induction of cellular redox changes. These alterations in redox can be sensed by reactive cysteine residues of regulatory proteins. For instance, NPR1 is found as oligomeres in the cytoplasm under steady-state conditions. In response to pathogen evoked redox changes and accumulation of salicylic acid, internal disulfide bonds are reduced and NPR1 oligomers can dissociate into monomers. NPR1 monomers are then free to translocate to the nucleus and activate a subclass of TGA transcription factors (95). Other protein targets subjected to modifications during defense responses include histones which may be acetylated or deacetylated (106). Taken together, these and other studies present a direct link between transcriptional control and pathogen perception.

2.6.2 Minutes and hours after elicitation – Transcriptional reprogramming

The series of alarm signals that a pathogen triggers ultimately reaches the nucleus where substantial transcriptional reprogramming occurs. Large-scale expression profiling has provided us with detailed information of transcriptome regulation in plants with high spatial and temporal resolution.

Pathogen infection is known to alter expression in up to 25% of the host's genome (107). In general, responses to different pathogens, avirulent as well as virulent, target

overlapping sets of genes (108). This observation is in line with the notion that signals generated from several types of immune receptors converge at some level (see above). Analogous to elicitor-evoked signaling, transcriptional output is quantitative rather than qualitative and depends on the input stimulus. Reprogramming of defense-associated genes is known to take place earlier and with greater amplitude following effector recognition compared to MAMP perception (107).

The global transcriptional switch that follows pathogen recognition establishes a transition from normal to a defense-orientated metabolism (95). These changes comprise the up-regulation of genes encoding components of the pattern recognition and signaling machinery, and enzymes involved in production of secondary metabolites. Down-regulated genes include those who govern cell division and other housekeeping functions (95, 109).

2.6.3 Hormones in plant immunity

Plant hormones, phytohormones, play essential roles in all stages of plant life and reproduction. Immune responses are no exception. Precise regulation of hormones in time and space allows plants to accurately respond and react to a wide range of external stimuli. Three hormones in particular, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are crucial for plant immunity. This phytohormone triad also has a well-documented role in responses to abiotic stresses like wounding (110-113). In addition, recent studies have identified abscisic acid (ABA), auxin, brassinosteriods (BR), cytokinin (CK), gibberellic acid (GA) and peptide hormones as important regulators of immune responses (114).

Overly simplified, SA is primarily induced by and confers resistance against biotrophic and hemi-biotrophic pathogens. By contrast, the JA and ethylene pathways are generally induced by and effective against necrotrophic pathogens and chewing insects. Mutants impaired in the accumulation of any of these hormones exhibit enhanced susceptibility to bacterial and fungal pathogens (115). Crosstalk between the SA pathway and the JA/ET pathway has been observed in many plants (116). Evidence for both synergistic and antagonistic interactions between SA and JA are reported (117-119). The details of JA synthesis and its role in plant defense are discussed in one of the following sections.

2.6.4 Systemic acquired resistance

Infection and defense activation in one part of a plant is frequently associated with the induction of resistance responses in distal organs. This phenomenon is known as **systemic acquired resistance (SAR)** and helps plants to withstand secondary infections. The local cell death of the hypersensitive response is usually the inducer of SAR but is not obligatory required for the production of mobile SAR signals (120-122). Systemic responses to pathogens were first reported more than half a century ago (123). Over the years, a range of candidate molecules has been proposed to act as the long-distance signal from the infection site to the healthy tissue. Methyl salicylate, jasmonic

acid, a glycerol-3-phosphate dependent factor, azaleic acid, abietic acid, dehydroabietinal, and most recently pipecolic acid are some of the substances reported to mediate SAR. The reader is directed elsewhere for excellent reviews on the subject (124-127).

2.7 Wounding Signaling and Responses

Feeding animals inflict physical damage to plants. Also freezing and other abiotic stresses may cause injury and tissue disruption. The signaling components needed for wounding responses are mainly the same as for pathogen recognition. These include membrane depolarization and proton fluxes, ROS formation, activation of Ca^{2+} channels and calcium-dependent proteins, PA accumulation, changes in protein phosphorylation patterns, and regulation of phytohormones (128, 129). The plant hormone JA and other related signaling compounds, collectively known as **jasmonates**, are of special importance for wounding responses (130, 131).

Just as pathogens, herbivorous insects and arthropods secrete elicitors or promote the release of plant endogenous danger signals during feeding (132, 133). Relatively little is however known about the molecular recognition events that trigger responses to herbivores.

The transcriptional activation that follows wounding leads to production of proteins directed to I) healing the damaged tissue (thereby limiting secondary infection by pathogens) II) produce compounds that restricts growth of the attacking herbivore (e.g. by reinforcing physical barriers or by producing toxins) III) generate signals that amplify the local defense or systemic responses (129). All plants produce volatile organic compounds in response to insect herbivore damage. The cocktail of substances released is often specific for each herbivore species. In many cases, the volatiles act as cues for natural enemies (predators or parasites) and direct them to the attacking herbivore (130). Volatiles also serve as intraplant and interplant communication agents that further activate defense mechanisms. Thereby, damaged plants can warn their neighbors of imminent attacks (134).

In summary, pathogen perception and mechanical injury often activate common signaling cascades. Wounding frequently results in the production of host compounds that strengthen immunity and prime uninfested tissue for rapid responses.

3. SCIENTIFIC AIMS

The two overall aims of this thesis are to I) gain insight into the cell-to-cell communication in plants that governs local defense responses, and II) elucidate the genetic machinery and the biochemical processes that underlie wounding and pathogen-induced accumulation of complex lipids.

Specifically, the following questions are addressed:

- How do we best quantify the extent of effector triggered immunity in a controlled, quick and reproducible fashion? **(Paper I)**
- How can cells adjacent to infected cells perceive the intrusion and activate defense responses? **(Paper II)**
- What changes occur in the composition of plant glycerolipids during hypersensitive response? **(Paper VI)**
- Are arabidopsides, a group of oxidized membrane lipids, formed from esterification of oxidized fatty acids or are they the result of the direct enzymatic modification of bound fatty acids? **(Paper III)**
- Is there any difference in the ability to accumulate arabidopsides in different subspecies of *Arabidopsis thaliana*? And if so, which genes control this variation? **(Paper IV)**
- How widespread is the ability to produce acylated and OPDA-containing glycerogalactolipids in the plant kingdom? **(Paper V)**
- Which protein(s) is responsible for the acyl transferase activity associated with synthesis of acylated glycerogalactolipids in plants? **(Paper V)**

4. EXPERIMENTAL SETUP

4.1 Pathogen Triggered Responses

During an adequate plant immune response the infection is stopped at an early stage before the prospective pathogen can colonize the host tissue. This implies that only a limited number of plant cells are directly involved in the defense reaction. A standard approach to scale up the plant immune reaction, so it involves the whole leaf tissue, is to inject a pathogen suspension directly into the leaf mesophyll using a needleless syringe. One of the earliest recordable effects of pathogen elicitation is the loss of electrolytes from the plant tissue caused by changes in cell membrane permeability (135). If the inoculated plant material is placed in water, the magnitude of the defense reaction will be proportional to the electrolyte leakage and can be measured as a change in conductivity over time (Figure 1B). ETI is frequently associated with a fast increase in conductivity whereas a more moderate raise in electrolytes follows MTI. The electrolyte leakage assay has been extensively used and many papers on the topic of plant pathology include one or several such experiments (e.g. 136). Hand inoculation of leaves using a syringe does however come with some limitations. For example, only one sample at a time can be inoculated and if not performed with care the leaf may be injured.

Two strategies have been used to circumvent these problems in the work presented in this thesis. A simple and robust vacuum-based inoculation procedure suitable for the quantification of ETI by electrolyte leakage is presented in **Paper I** (Figure 1). In the report a systematic comparison between vacuum infiltration and syringe inoculation in terms of wounding and reproducibility in Arabidopsis is performed. It was found that the method did not cause more wounding than syringe inoculation whereas throughput and reproducibility were markedly improved. Using vacuum inoculation, the influence of bacterial titer on programmed cell death (electrolyte leakage) was investigated after treatment with the Gram-negative bacterium *Pseudomonas syringae* DC3000 expressing the type III effector AvrRpm1. Moreover, the method was used as a means to quantify induction of the lipid second messenger phosphatidic acid after AvrRpm1 recognition. The vacuum method was subsequently used in **Paper II** for the infiltration of bacteria and the secondary metabolite sulforaphane, for determining hypersensitive cell death and cellular redox, respectively.

The other strategy employed for scaling up ETI is the use of a transgenic system with *in planta* expression of an effector protein. In this system, Arabidopsis plants have been transformed with a dexamethasone inducible copy of the *P. syringae* effector AvrRpm1 (78, 102, 137, 138). When treated with the chemical inducer dexamethasone, the *avrRpm1* gene is transcribed and translated by the plant. AvrRpm1 recognition by the cognate plant R protein RPM1 results in a signaling cascade that ultimately triggers resistance responses. Alternatively, the AvrRpm1 effector is expressed in the *rpm1.3*

mutant background which lacks the ability to recognize the effector and mount defense (139). The ectopic expression of the bacterial effector takes away the need to use an actual pathogen. Thereby, responses evoked by the effector can be separated from those triggered by other pattern recognition events. In **Paper II**, this transgenic system was used to isolate signaling compounds released by plants during the hypersensitive response. This system was further employed for phospholipid profiling of the hypersensitive response in **Paper VI**.
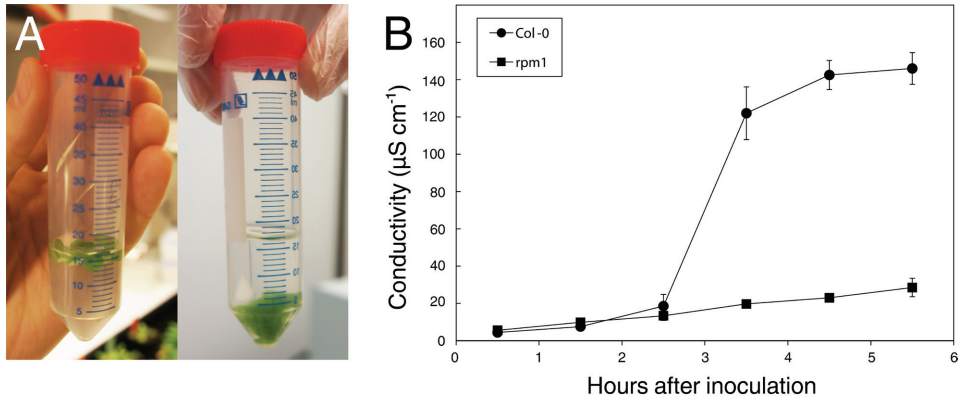
Figure 1. Vacuum infiltration of Arabidopsis leaf discs. (A) Discs before (left) and after (right) vacuum infiltration of a bacterial suspension. Fully infiltrated discs are easily distinguishable as they lose their buoyancy. **(B)** Electrolyte leakage after a high titer suspension of the bacterial pathogen *P.syrigae avrRpm1* has been vacuum infiltrated into leaf discs from wild-type Col-0 and *rpm1.3* plants. The *rpm1.3* mutant lacks recognition of the bacterial effector and fails to mount hypersensitive response.

4.2 Wounding Triggered Responses

In nature, plants are subjected to mechanical injuries by chewing insects or larger herbivores. To try to reconstitute such interactions in the laboratory is challenging and no method is without its limitations. A standard approach to induce wounding responses in plants is to crush the leaf tissue using a hemostat (for example 140). This technique is applicable for many but not all types of experiments. Some of the drawbacks of this method are that the tissue is not uniformly wounded and a mix of intact and crushed cells is generated; determination of the tissue weight is difficult once the tissue is crushed; it is not possible to simultaneously wound large amounts of plant tissue.

We heard from colleagues (Mike Pollard, personal communication) that a freeze-thaw cycle could trigger something reminiscent of a wounding response in plants. This was tested by freezing Arabidopsis tissue in liquid nitrogen and then left it to thaw at room temperature. Freeze-thawing was found to be comparable hemostat wounding in terms of production of free and esterified OPDA (see below), lipids generally associated

with wounding responses in Arabidopsis (137, 140-142). This freeze-thaw method has been used throughout this work as an inducer of wounding responses (**Paper I, III, IV and V**). The method allows uniform and synchronized tissue disruption in multiple samples simultaneously. Freeze-thawing could obviously not be expected to perfectly emulate responses inflicted by pests or herbivores. Freeze-thawing only generates completely disrupted cells and not the complex pattern of damaged and live cells caused by feeding herbivores. It is firmly established that artificial wounding does not reproduce the volatile cocktail and transcription profile generated by herbivore feeding (133, 143, 144). Moreover, freeze-thawing does not trigger the accumulation of JA and JA-Ile (145; **Paper I**), compounds produced in response to hemostat wounding, suggesting that intact cells are required for their synthesis. Nevertheless, the freeze-thaw method provides a reproducible response that shares many characteristics with herbivore feeding and mechanical wounding.

# 5. PLANT RESPONSES TO WOUNDING AND EFFECTOR ELICITATION

It can be difficult, and under certain circumstances perhaps also irrelevant, to distinguish immune responses from signaling events that lead to immunity. Components of the defense machinery can act to relay or amplify a signal, or to act as allelochemicals depending of the given situation. To illustrate, reactive oxygen species associated with the oxidative burst are pivotal for the establishment of hypersensitive response in plants (146). Furthermore, ROS species produced by plants can be directly harmful to microbes. In order to survive inside the host during the colonization, the bacterial pathogen *P. syringe* must produce virulence factors that detoxify hydrogen peroxide (147, 148). Finally, ROS can activate transcription of plant pathogenesis related genes and promote the production of phytoalexins that further limit growth of the attacker (149).

In the upcoming sections the results from the articles included in this thesis are summarized. As will become apparent, several of the substances discussed may have this dual role - acting as both signal transducers and pathogen toxins.

5.1 Death as a Last Resort: the Hypersensitive Response

The recognition of microbial effectors by plant NB-LRRs typically leads to a halt in the infection, and frequently, **hypersensitive response (HR)**, a type of programmed cell death at the site of attempted pathogen entry (150). The HR phenomenon was first described almost a century ago by Elvin C Stakman (151). He noted that cereal crops resistant to isolates of the black stem rust fungus *Puccinia graminis* developed a type of abnormally fast cell death at infection sites. The formation of local HR-lesions has been postulated to represent a scorched earth tactics of the plant that confines the intruder to a restricted area and stops further infection (152). Although HR is commonly associated with ETI, typical MAMPs can also contribute to the hypersensitive reaction (153). Moreover, HR is not a criterion for resistance as plants defective in programmed cell death may still be able to combat infections (see below).

As previously discussed, the intracellular signaling components required to stimulate ETI, and therefore also HR, are qualitatively similar to those of MPI but induced faster and to greater extent. Consequently, accumulation of ROS, NO, Ca^{2+} and SA, as well as the activation of MAPK cascades and defense gene expression coincide with HR (150). Despite all efforts that have been put in to elucidating the molecular mechanisms that lead to HR, a comprehensive model for how HR is executed and how resistance can be achieved in cells adjacent to the lesion is still missing.
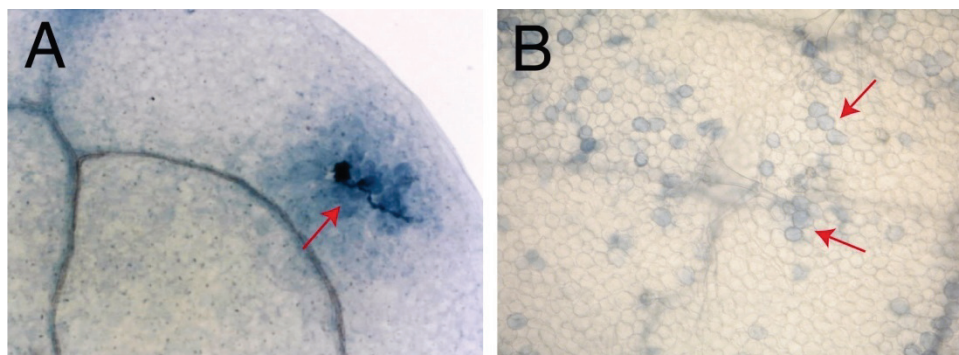
Figure 2. Spread of cell death during hypersensitive response. Arabidopsis plants were stained for dead cells with Trypan blue after inoculation with the oomycet *H. arabidopsidis* race Cala2 **(A)** or the bacterium *P. syringae* expressing AvrRpm1 **(B)**. Not only host cells that are in direct contact with the pathogen undergoes programmed cell death but also neighboring cells (marked with arrows). For *P. syringae* this is apparent as patches of dead cells in the host. Thus, a cell death inducing signal must be released by the primary infected cell that triggers cell death and other responses in the surrounding tissue (picture credit Gülin Boztaş and Oskar Johansson).

Natural HR-lesions may be either microscopic, involving only single or few interconnected cells, or macroscopic, visible as large patches of dead cells in leaves. The size of the HR lesion and the timing of the HR depend on the type of pathogen, the specific effector repertoire of the pathogen, and the infection strategy of the pathogen. Although dissimilarities exist, common to all HR lesions is that they must be initiated at the primary infected cell(s) and then spread to surrounding cell layers. This is particularly apparent during infections by obligate biotrophs such as the oomycete *Hyaloperonospora arabidopsides* on Arabidopsis (Figure 2). The propagation of the HR implies that a signal must be released from the initial cells undergoing HR that activates the cell death program in neighboring cells. Also, the spread of the cell death must be attenuated and other defense responses initiated in cells further away from the point of attempted pathogen ingress. Signals that act on a local tissue scale during HR have so far largely been neglected. We set out to identify small diffusible molecules involved in the cell-to-cell signaling that can propagate programmed cell death in Arabidopsis (**Paper II**). The system described above, with transgenic expression of the AvrRpm1 effector, was used to scale up the HR reaction. A "diffusate" collected from plants undergoing HR was tested for cell death activity in naïve tissue. From this diffusate, the isothiocyante **sulforaphane** was identified as a potent inducer of cell death in previously unchallenged tissue (Figure 3, see also the Glucosinolates section). Sulforaphane is found in several cruciferous plants and has been intensively studied for its nutraceutical properties in humans (154). Specifically, sulforaphane consumption has been associated with induction of cytoprotective proteins (previously called "phase 2 enzymes"). In mammalian cells, sulforaphane is metabolized via the mercaptic acid pathway. In the initial step of this pathway, sulforaphane is conjugated to the thiol-

containing tripeptide glutathione, a reaction catalyzed by glutathione transferase (GST) (155). Glutathione is the most abundant antioxidant agent in both plant and animal cells, contributing to the neutralization of ROS and free radicals. It exists in reduced (GSH) and oxidized (GSSG) states, and the ratio between them influences cellular redox potential (156). Evidence for the involvement of glutathione during HR is increasingly growing. For example, exogenous application of GSH together with avirulent bacteria effectively suppresses HR (157, 158). Furthermore, mutants that are impaired in glutathione synthesis display enhanced susceptibility to pathogens (159). Changes in the reduction potential of glutathione have even been suggested to be a universal marker for plant stress and inducer of programmed cell death (160). A possible mode of action of sulforaphane in plants is that it binds glutathione and thereby affects cellular redox and downstream responses as shown in mammalian systems. To investigate this, Arabidopsis leaf tissue was vacuum-infiltrated with sulforaphane and analyzed for GSH and GSSG (**Paper II**). The sulforaphane treatment led to a severe depletion of both reduced and oxidized forms of glutathione. Accordingly, this led to a substantial increase in redox potential of the glutathione pool. One plausible mode of action of sulforaphane is therefore that it acts as molecular switch through the glutathione system by changing the state of protein intermolecular disulfide bridges. These modifications may result in rearrangement of tertiary and quaternary protein structures and thereby change their activity (161). If sulforaphane is directly conjugated to glutathione, and if such conjugation is non-enzymatic or whether it depends on proteinaceous co-factors remains to be investigated.

Several studies have drawn parallels between different types of programmed cell death in animals and that of the HR in plants. Although some hallmarks are shared between animal programmed cell death and plant HR, overall, HR represents a distinct form of plant specific cell death (162). HR and animal apoptosis are similarly associated with cytoplasmic shrinkage, chromatin condensation and the release of mitochondrial Cytochrome *c*. On the other hand, plant HR can be executed independently of ATP and is usually not associated with DNA laddering as reported for animal apoptosis (150 and references therein). Activation of a family of cysteine-proteases, known as caspases, are intimately linked with apoptotic cell death in animals (163). Recently, plant Type I Metacaspases with homology to caspases were identified as regulators of programmed cell death in plants (164). It was reported that Arabidopsis metacaspase AtMC1 and AtMC2 antagonistically control HR-associated cell death. Loss of Type I Metacaspase activity did not influence proliferation of avirulent bacteria in the host, uncoupling HR cell death from resistance. Similarly, the Arabidopsis mutants *myb28myb29* and *tgg1tgg2* (165, 166)*,* defective in sulforaphane synthesis, did not lose resistance to *P. syringae* expressing AvrRpm1 despite that HR was impaired (**Paper II**). Several other defense-related mutants are known to display similar phenotypes: defective in HR but functional or even enhanced resistance (167; Oskar Johansson, personal communication). Thus, it still remains unclear if HR associated cell death is the result of an unstoppable defense reaction, or whether it represents an active defense strategy of the plant.

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In summary, the locally acquired resistance generated by HR, together with the protection that SAR provides, enables plants to stop infections before the pathogen is able to proliferate. Sulforaphane or analogous glutathione-scavenging compounds are proposed to be one of the key components of the local defense, and thereby, potentially also important for the development of resistance in distal tissue.

5.2 Glucosinolates – the Mustard Bomb

By definition, secondary metabolites are molecules produced by plants that are not necessary for growth and reproduction. The versatility in these low-molecular-weight compounds is astonishing and well over 100,000 different structures are known (168). Some secondary metabolites are specific to certain families of plants whereas others are more general or found within particular phylogenetic groups. Their production and occurrence is often restricted to certain cell types, tissues or organs, and varies during time of day and developmental stage. Herbivory and attempted infections commonly results in either *de novo* production or activation of stored secondary metabolite precursors. While many phytochemicals serve as repellers or direct toxins to microbes and herbivores, emerging evidence indicate that they also function in defense-related signal transduction within the plant (169).

One major class of secondary metabolites found in Arabidopsis and other plants of the order Brassicalesis is the **glucosinolates**. The hydrolysis products generated from glucosinolates give brassicaceous vegetables such as cabbage, broccoli and radish their pungent flavor. In plants, biologically inactive glucosinolates are physically separated from β-thioglucosidase enzymes (also known as **myrosinases**). The myrosinases are either localized in stomatal guard cells (166) or in specialized myrosin cells in the phloem parenchyma (170). Upon tissue damage, the glucosinolates come in contact with myrosinases which rapidly hydrolyses them to unstable aglucone intermediates. The aglucones can then rearrange into various reactive compounds, typically isothiocyanates, thiocyanates and nitriles (Figure 3)(171). Due to the extremely quick production of glucosinolate metabolites upon loss of cellular integrity, the system has been called the Mustard bomb (172). The structure of the hydrolysis product mainly depends on the glucosinolate side chain, but pH and protein factors also influence the end product (173). To date, more than 120 distinct glucosinolate structures have been described (154), enabling the production of several hundred different hydrolysis products.

The role of glucosinolate breakdown products as defense compounds against insect herbivory is well established (132). Several reports have provided evidence that glucosinolates and their metabolites also contribute to innate immunity in Arabidopsis (**Paper II**, 174, 175, 176). Unlike glucosinolate metabolites produced during insect interactions, production induced by microbial pathogens does not seem to depend on the compartmentalization system of substrate and myrosinases described above.
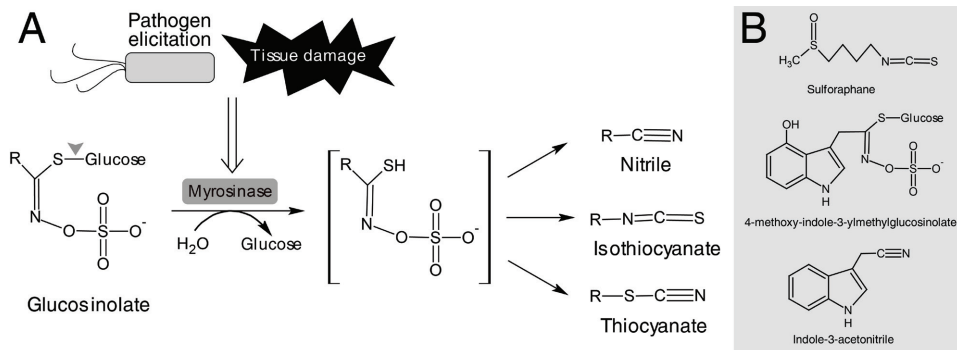
Figure 3. The glucosinolate-myrosinase system. (A) Biologically inactive glucosinolates are hydrolyzed by myrosinases upon tissue damage or pathogen elicitation. The instable aglucones then rearranges into nitriles, isothiocyanates or thiocyantes. The chemical structure of the isothiocyante sulforaphane, 4-methoxy-3ylmethyl glucosinolate (4MI3G) and indole-3-acetonitrile (I3AN) are shown in **B**.

Clay and colleagues reported that the double mutant *cyp79B2cyp79B3*, which completely lacks indole glucosinolates, was impaired in callose responses provoked by the MAMP *flg22*. The hydrolysis products of 4-methoxyindol-3-ylmethyl glucosinolate (4MI3G) generated by the PEN2 myrosinase were ascribed to this (Figure 3B). Unfortunately, the structure of the active hydrolysis product could not be identified. Indole glucosinolates have also been linked to non-host resistance in Arabidopsis. The fungi *Blumeria graminis hordei* and *Erysiphe pisi* cause powdery mildew on grasses and pea species, respectively, but are non-adapted to Arabidopsis and do not cause disease. Inoculation of *B. g. hordei* and *E. pisi* in the *cyp79B2cyp79B3* background revealed penetration rates similar to that in *pen2*. (174). Again, providing support that 4MI3G hydrolysis products generated by PEN2 are important for Arabidopsis pathogen defense. More recently, Fan *et al.* put forward evidence that the aliphatic glucosinolate product **sulforaphane** is induced by, and contributes to resistance against non-adapted strains of bacterial pathogens in Arabidopsis (Figure 3B) (175). To overcome the bacteriostatic effect of this isothiocyante, adapted strains of *P. syringae* were found to harbor so-called *SAX* genes that function as virulence factors by detoxifying sulforaphane produced by the plant. The molecular mechanism by which the *SAX* gene products neutralize sulforaphane still remains unknown. In **Paper II** the Arabidopsis ecotype Col-0 was shown to release high concentrations of sulforaphane after elicitation with the effector AvrRpm1 from *P. syringae*. The role of sulforaphane in race-specific resistance in Arabidopsis was investigated using two independent T-DNA mutant lines. The *myb28myb29* mutant lacks transcription factors necessary for the synthesis of aliphatic glucosinolates and has severely reduced levels of sulforaphane (165, **Paper II**). The other line, *tgg1tgg2*, is mutated in genes encoding myrosinases that catalyze the cleavage of the inactive glucosinolates into sulforaphane and other breakdown products (166).

When plants are inoculated with high titers of avirulent bacteria, all plants cells come in direct physical contact with the pathogen and quickly induce programmed cell death (**Paper I**). Since sulforaphane was identified as a mobile signal that propagates cell death from primary infected cells to adjacent cell layers, plants impaired in sulforaphane production were hypothesized to show a more pronounced HR phenotype at lower pathogen inocula. The sulforaphane deficient mutants *myb28myb29* and *tgg1tgg2* were therefore inoculated with two different titers of *P. syringae* expressing AvrRpm1. One high titer (OD_{600}=0.1) where all plant cells physically interacts with the pathogen, and one lower titer (OD_{600}=0.01) where only some of the plant cells come in contact with the pathogen and induce programmed cell death. In accordance with the hypothesis, the extent of HR cell death in *myb28myb29* and *tgg1tgg2* plants did not differ from that of wild-type after inoculation with the high titer of bacteria (measured by electrolyte leakage). Conversely, when plants were treated with the lower concentration of bacteria, electrolyte leakage was reduced by approximately 25% in the two mutant lines (**Paper II**).

The role of sulforaphane as a contributor to local defense was further examined using HR-inducing isolates of the obligate biotrophic oomycete *Hyaloperonospora arabidopsides*. The *H. arabidopsides* isolate Cala2 triggers a rapid cell death response in wild-type Col-0 plants and only occasionally manages to outgrow the HR defense of the host and cause trailing necrosis. The sulforaphane deficient mutants and wild-type plants were inoculated with Cala2 and the extent of cell death was determined after trypan blue staining. The frequency of trailing necrosis interactions was found to be 7-10 times higher in the double mutants compared with wild-type plants (**Paper II**). In a few instances the oomycete was able to completely outgrow the trailing necrosis in the *myb28myb29* plants, something never observed in wild-type. Mutant and wild-type plants were also inoculated with the *H. arabidopsides* isolate Emwa1. Both *myb28myb29* and *tgg1tgg2* lines displayed increased susceptibility to Emwa1 evident as a sixfold increase in the number of oomycete sporophores formed compared to wild-type plants.

As discussed above, a possible mode of action of sulforaphane is that it promotes HR-type cell death by creating an oxidative cellular environment. *P. syringae* DC3000 has at least four genes that code for effectors which can detoxify plant produced H_2O_2 (177). It is tempting to speculate that *SAX* gene products, in addition to provide protection from the direct toxic effect of sulforaphane, can function in concert with other bacterial virulence factors to suppress the plant's oxidative burst and in so doing delay or abolish plant immune responses.

The composition of glucosinolates and their breakdown products varies considerably between Arabidopsis ecotypes (178). The commonly used ecotype Col-0 is extraordinarily rich in the sulforaphane precursor glucoraphanine (178, 179). A reasonable assumption is that other ecotypes that lack glucoraphanine and sulforaphane can produce glucosinolate breakdown compounds with similar properties as sulforaphane during ETI. To test this, several glucoraphanine-deficient ecotypes were infiltrated with *P. syringae* expressing AvrRpm1. 3-indole-acetonitrile (3IAN) and other

nitriles that did not accumulate in Col-0 were found to increase in abundance in other ecotypes following AvrRpm1 recognition, although not to the same extent as sulforaphane in Col-0 (Figure 3B and 4A). Furthermore, several isthiocyanates and 3IAN caused cell death when infiltrated into Col-0 leaf tissue (**Paper II** and Figure 4B). Interestingly, stimulation with the MAPK kinase MKK9 or infection by the fungus *Botrytis cinerea* lead to the production of I3AN-glutathione conjugates in Arabidopsis (180). Thus, it seems plausible that ecotypes unable to synthesize sulforaphane produce other glucosinolate breakdown products that can carry out a similar function. One approach to investigate this further could be to do a similar search for cell death promoting molecules as performed in Paper II, but in a different genetic background. Both L*er* and Ws-0 are poor producers of isothiocyntas and potential candidates for such a forward screen.
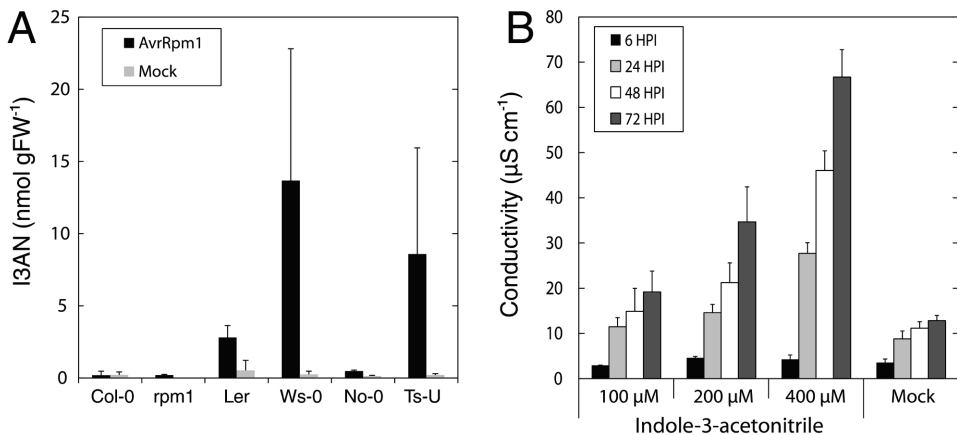
Figure 4. Indole-3-acetonitrile (I3AN) and cell death. (A) Plants were treated with *P. syringae* harboring AvrRpm1 at OD_{600}=0.1 or mock inoculated with 10 mM $MgCl_2$. The amount of I3AN released from the plant tissue was quantified by GC-MS 6 hours after infiltration. **(B)** Arabidopsis leaves were inoculated with I3AN at different concentrations or mock treated with water. Leaf discs were made and left to float in the same solution as they were inoculated with. Conductivity of the bathing solution was measured at indicated time points. Means and standard deviation of triplicate samples are shown for both experiments.

Far from all plants contain glucosinolates and myrosinases. Nevertheless, all plants are fully capable of mounting HR. It is possible that plant species that lack glucosinolates and their breakdown products produce other reactive compounds that can promote HR by affecting cellular redox status through the GSH-GSSH system. HR-like lesions were observed when sulforaphane was infiltrated into the leaves of plants that do not produce glucosinolates (Figure 5). This finding lends some support to the idea that reactive electrophilic compounds in general can trigger programmed cell death in plants.

In conclusion, glucosinolates and their hydrolysis products represent a new class of signaling molecules involved in plant innate immunity. The isothiocyante sulforaphane was identified as a potent inducer of cell death in plants and shown to be important for race-specific resistance in Arabidopsis.
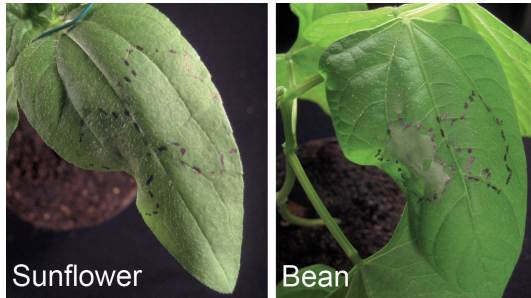
Figure 5. Effect of sulforaphane in plants lacking glucosinolates. Sunflower and bean leves were syringe infiltrated with 0.5 mM sulforaphane (left marked area) or water (right marked area). Pictures were taken 24 h after infiltration.

5.3 Lipids and Lipid Signaling

Lipids are essential to life as they fuel metabolism and give membranes their structure. More than that, lipids are involved in developmental processes, cell differentiation, growth and responses to various stresses (181). The role of lipid molecules and lipid-derived signals in plant immune responses is being increasingly recognized. In particular, the signaling activity of **phosphatidic acid (PA)** and **oxylipins** are of major interest to molecular plant pathologist (110, 128). Spatial and temporal distribution of lipid mediators are tightly controlled under stress conditions by lipid-modifying enzymes such as lipases, lipoxygenases, dehydrogenases, phosphatases and kinases (182). Using genetic and biochemical approaches, we have investigated how various phospho- and galactolipids change in abundance over time in response to wounding and pathogen treatment. Our findings are presented and discussed in the following sections.

Emerging techniques have made it possible to reconstruct the lipid species composition in crude extracts of small biological samples (183). To be able to separate and quantify plant membrane glycerolipids with high accuracy, a new reverse phase liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) method was developed (**Paper VI**). This method allows us to quantitatively determine the presence of a broad range of lipids with diverse chemical properties, ranging from lyso-galactolipids to oxygenated and acylated phospholipid species. Using this method, phospholipids such as PA, PC, PG and PE, and galactolipids of the classes MGDG and DGDG can be analyzed in a single run. The advantage of coupling liquid chromatography to mass spectrometry, as opposed to direct infusion mass spectrometry, is that truly isobaric or near isobaric lipid species with similar masses can

be separated. To illustrate, the respective ammonium adduct of the oxylipin arabidopside A and the chloroplast membrane lipid 18:3,18:3-MGDG have the molecular masses of 792.6 Da and 792.7 Da, and are difficult to separate in direct infusion experiments using a low resolving mass spectrometer such as triple quadrupole (QqQ). However, in combination with liquid chromatography the two species can be separated and quantified. Thus, a relatively low-resolution instrument as quadrupole mass spectrometer can be used for *de novo* lipid discovery and complex lipid profiling studies. An example of truly isobaric lipid species that can be separated by retention time using the method described in Paper VI is 18:3,16:0-PG and 18:2,16:1-PG. Both molecules have a mass of 744.5 Da (monoisotopic weight) and are indistinguishable using solely mass spectrometry. Variants of this LC-MS method have been used throughout the work presented here and details are found within the manuscripts.

5.3.1 Oxylipins and jasmonates

The thylakoid membranes and the inner envelope of chloroplasts are predominantly composed of the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG) (Figure 6). MGDG and DGDG are rich in polyunsaturated fatty acids that can serve as substrates for the synthesis of a diverse group of lipid oxidation products, collectively called **oxylipins** (184, 185). The oxygenation process can be either enzymatically driven or occur through non-enzymatic reactions (186, 187). **Jasmonates** are a group of plant specific oxylipins that are derived from enzymatic oxidation of plastidial α-linolenic acid (18:3, number of carbon atoms: number of *cis* double bonds) or hexadecatrienoic acid (16:3) (188). The best characterized jasmonates include **jasmonic acid (JA)**, methyl jasmonate (MeJA), jasmonyl-isoleucine (JA-Ile), and their precursor molecules **12-oxo-phytodienoic acid** (**OPDA**, 18C) and **dinor-OPDA** (**dnOPDA**, 16C) (131).

Synthesis of the bioactive form of JA is complex and involves three cellular compartments. (dn)OPDA is produced in the plastids and transported to the peroxisomes where it undergoes chain shortening by β-oxidation (189). The formed JA is then translocated to the cytosol where it can be methylated or conjugated to amino acids. JA-Ile is reported to be the form with the highest biological activity (190). However, many other jasmonates, including OPDA and dnOPDA, have signaling properties by themselves (185, 191-194). It should be mentioned that the T-DNA mutant *opr3*, that was thought to produce (dn)OPDA but not JA and used in many experiments, was recently shown to be able to splice out the T-DNA containing intron and produce full-length OPR3 transcripts provided strong enough transcriptional activation (195). Thus, some of the signaling activity ascribed to OPDA may in fact be due to other jasmonates. Nevertheless, there is still convincing evidence for a distinct role of (dn)OPDA independent of JA.

The importance of jasmonates in plant defense against insect herbivores and necrotrophic pathogens is widely recognized (130, 196). Jasmonates are also functional

in defense responses to many biotrophic microorganisms (115, 197). Furthermore, it was reported that Eastern Hermann's tortoises preferred jasmonate-free over wild-type Arabidopsis plants, ascribing jasmonates a role in defense against vertebrate herbivores (198).

In 2001, Stelmach and co-workers showed that OPDA does not only exist in its free fatty acid form, but is also found as esters on the glycerol backbone of MGDG. In the years to follow, several more OPDA and/or dnOPDA containing glycerogalactolipids were identified and given the trivial names **arabidopside A-G** (Figure 6) (137, 138, 140, 199, 200). With the rapid evolution of mass spectrometry lipidomics tools, many more lipids with covalently bound (dn)OPDA have been discovered (**Paper III**, **Paper IV**, **Paper VI**, 141, 142). Several types of abiotic and biotic cues trigger arabidopsides to accumulate in plants, including wounding (137, 140-142), senescence (201), cold treatment (141) and bacterial effector elicitation (137, 138, 141).

The biosynthesis of jasmonates starts in the plastid with the enzymatic oxygenation of free (or bound, see below) 18:3 or 16:3 fatty acids by lipoxygenases (LOXs). LOXs are a group of non-heme iron containing dioxygenases which can be subdivided accordingly to their positional specificity of oxygenation. Type 1 LOXs introduce molecular oxygen at carbon 9 (9-LOX) of the fatty acid chain and have so far only been found in extra-plastidial compartments. Type 2 LOXs target carbon atom 13 (13-LOXs) of the fatty acid and are exclusively localized to plastids (202). Thus, 13-LOXs control arabidopside and JA synthesis. Arabidopsis carry four isoforms of 13-LOXs: LOX2, LOX3, LOX4 and LOX6. All four isoforms contribute to wounding-induced production of jasmonates (203). However, only the activity of LOX2 is necessary for the synthesis of arabidopsides (145). The *lox2-1* loss-off-function mutant display severely reduced formation of arabidopsides after wounding (145; **Paper IV**). The immediate products generated by 13-LOXs are 13(*S*)-hydroperoxides which function as substrate for several competing enzymes including allene oxide synthase (AOS) and hydroperoxide lyase (HPL) (Figure 6) (204). Both enzymes belong to an atypical subfamily of cytochrome P450 monooxygenases that neither requires molecular oxygen nor NADPH-reductase for their activity (205). Jasmonates and arabidopsides are synthesized via the AOS branch. AOS produces an instable 12,13-epoxide that can either spontaneously rearrange into, or be enzymatically converted to (dn)OPDA by allene oxide cyclase (AOC) (206). Enzymatically driven cyclization of the unstable allene oxide gives rise to optically pure 9S,13S-OPDA whereas spontaneous cyclization generates a mixture of 9R,13R-OPDA and 9S,13S-OPDA enantiomers (207). All lipid-bound OPDA that accumulate in response to wounding and AvrRpm1 effector elicitation in Arabidopsis have the 9S,13S configuration and are thus produced from enzymatic conversion of the unstable allene oxide by AOC (**Paper IV**, not shown). In the HPL branch, the 13(*S*)-hydroperoxide is cleaved into a C_6 aldehyde and a ω-oxo fatty acid (205).

Two alternative biosynthetic pathways have been postulated for the production of arabidopsides (208). In the first scenario, free (dn)OPDA produced from the "normal"

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JA synthesis pathway is esterified to the glycerol backbone by an acyl transferase. The other possibility is that trienoic fatty acids are converted into oxylipins while still attached to the galactolipid. The discovery of an arabidopside-specific LOX isoform strengthens the latter idea (145). Moreover, ACT1 (also known as ATS1), the 3-P-glycerol acyl transferase that catalyzes the synthesis of plastidial diacylglycerol lipids, is dispensable for the production of arabidopsides (137). However, direct experimental evidence for the conversion of glycerolipid-bound fatty acids to (dn)OPDA has been lacking. This was addressed in **Paper III**. Three lines of evidence are presented that support the hypothesis that arabidopsides are formed without any free fatty intermediates: I) The synthesis of esterified OPDA is extremely fast – two thirds of all available 18:3 fatty acids are converted to OPDA within two minutes after freeze-thaw treatment; II) No increase in free fatty acids was observed during the accumulation of esterified OPDA in response to tissue disruption by freeze-thawing; and III) ^{18}O from ^{18}O-labelled water was not incorporated into esterified OPDA after freeze-thawing, as would have been the case if a cycle of hydrolysis and re-acylation had occurred. It is concluded that arabidopside accumulation in response to wounding does not require acylation of free OPDA or other free fatty acid intermediates. Interestingly, bioactive eicosanoids attached to phospholipids in mammalian cells can be formed through both of the above described pathways (209). Therefore, it should not be ruled out that other oxidized membrane lipids can be synthesized from free substrates, or that free fatty acids can contribute to arabidopside accumulation in response to other stresses in plants.

Arabidopsis is rather unique in its ability to produce (dn)OPDA esters with glycerolipids. In fact, arabidopsides are only found in a closely related group of plants of the Brassicaceae family (**Paper V**, 185). So why do these plants produce arabidopsides? And to such an extent!? A large part of the esterfied 18:3 is converted to OPDA within minutes after wounding in Arabidopsis (**Paper III, Paper IV**). So far, arabidopsides have been shown to possess antimicrobial and antifungal properties *in vitro* (137, 138), to promote senescence (210), and to be linked to defense responses triggered by insect herbivory (211). Kourtchenko *et al.* proposed that arabidopsides can function as storage compounds for "true" signaling compounds such as OPDA and dnOPDA (137). Consistent with this idea, two Arabidopsis acyl hydrolases, PLAI and pPLAIIα, were shown to use arabidopsides as substrates and catalyze the release of free OPDA (212, 213). Furthermore, *plaI* knockout mutants displayed enhanced susceptibility to the necrothrophic fungus *Botrytis cinerea* (212). Another function of oxylipins and arabidopsides during immune responses might be to alter the permeability of membranes such as the chloroplast envelope. Oxidation of acyl chains increase the average area per lipid and thereby reduces the bilayer thickness and membrane permeability (214). To further our knowledge on the function of these complex lipids, the distribution of arabidopsides and related lipids within the *Arabidopsis thaliana* species (**Paper IV**), and in other plants (**Paper V**) were explored.
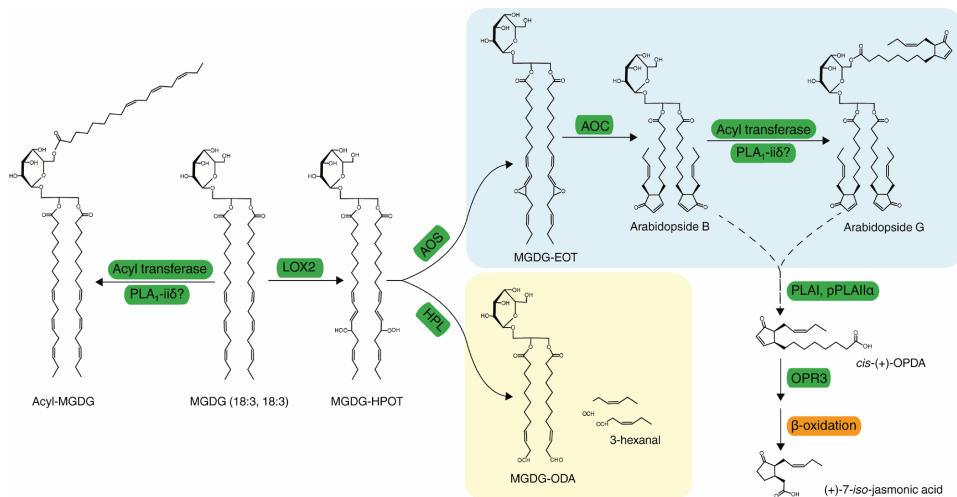
Figure 6. Proposed model for arabidopside and acyl-MGDG biosynthesis. Lipoxygenase 2 (LOX2) introduces molecular oxygen to trienoic fatty acids esterified to monogalactosyldiacylglycerol (MGDG) forming MGDG-13(*S*)-hydroperoxy-octadecatrienoic acid (MGDG-HPOT). In the Arabidopsis Col-0 accession, all MGDG-HPOT is transformed into arabidopsides via MGDG-12,13-epoxy octadecatrienoic acid (MGDG-EOT) catalyzed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) (highlighted in blue). In C24 and other Arabidopsis accessions, the MGDG-HPOT is partially cleaved by hydroperoxide lyase (HPL) to form MGDG-12-oxo-dodecenoic acid (MGDG-ODA) and C_6 aldehydes (shown in yellow). Arabiopsides can potentially be digested by the acyl hydrolases PLAI and pPLAIIα to yield free 12-oxo-phytodienoic acid (OPDA) (212, 213). OPDA can be reduced by OPDA reductase 3 (OPR3) and further modified though β-oxidation to form jasmonic acid. In jasmonate mutants, and in non-cruciferous plants, a substantial part of the MGDG pool is transformed into acyl-MGDG by an unknown acyl transferase in response to wounding. PLA$_1$-iiδ was identified as a candidate enzyme for the digestion of fatty acids and acyl transfer. Potentially, the same acyl transferase catalyzes the synthesis of acyl-arabidopside (e.g. arabidopside E and arabidopside G).

Arabidopsis occurs naturally throughout Europe and Asia. The plant has adapted to many different habitats giving rise to a plethora of subspecies, also known as ecotypes or accessions (215). The genetic variation among such accessions was used to identify genes linked to arabidopside accumulation in response to wounding (**Paper IV**). The accession C24 was identified as a poor producer and Col-0 as a high producer of arabidopsides. Both accessions did however metabolize MGDG in a *LOX2*-dependent manner after freeze-thaw wounding, suggesting that a difference in enzyme(s) acting on oxylipins existed between Col-0 and C24. To test whether any of the known enzymes involved in OPDA synthesis were differentially expressed in the two accessions, and if such a difference could explain the low production of arabidopsides in C24, quantitative real-time quantitative PCR (qPCR) was performed for *LOX2*, *AOS* and the four isoforms of *AOC*. Expression levels of all six genes were surprisingly similar between the two accessions. This finding led us to search for other genetic factors that might influence arabidopside formation. Col-0 was crossed with C24 and the F_2 progeny subsequently

used for PCR-based mapping. A small region on chromosome 4 consisting of only 21 genes was found to be strongly linked to the capacity to accumulate arabidopsides. One of the genes in this region, *HPL*, has a well characterized role in oxylipin synthesis (introduced above) and was therefore further investigated. Col-0 contains a deletion in the first exon of *HPL* that causes it to code for a truncated protein variant (216). C24 do not carry this mutation. However, L*er*, another Arabidopsis accession, has a functional *HPL* allele but can nevertheless accumulate substantial amounts of arabidopsides, although less than Col-0 (**Paper IV**) (216). To address this discrepancy, *HPL* transcript levels in all three accessions were monitored by qPCR. Intriguingly, *HPL* expression was approximately 70-fold higher in C24 compared to L*er*. A L*er* line carrying the non-functional Col-0 *HPL* allele also accumulated more arabidopsides compared to wild-type plants after tissue damages. Based on these observations, a model is proposed (Figure 6). In this model, LOX2 oxidizes galactolipid-bound α-linolenic acid to form 13(*S*)-hydroperoxylinoleic acid. From this point, two alternative fates for the hydroperoxide exist. In Col-0, the consecutive action of AOS and AOC results in the formation of arabidopsides. In C24 and other accessions, some of the hydroperoxides are digested by HPL to produce C_6 geen leaf volatiles and 12-oxo-dodecenoic acid (ODA)-containing galactolipids (205). Given that both AOS and AOC are enzymatically active on lipid-bound acyl chains (**Paper IV**), and due to the high degree of similarity between AOS and HPL (217), it seems reasonable to assume that also HPL can accept lipid-bound substrates. Surprisingly, no ODA galactolipids could be detected in C24, or substantial amounts of any other MGDG-derivatives after tissue disruption. A possible explanation for this is that HPL products activate other lipid modifying enzymes, such as lipases, that metabolize galactolipids.

At this point it cannot be exclude that additional loci in the mapped region contribute to the formation of arabidopsides. Col-0 T-DNA knockout lines of the most interesting genes are currently being analyzing for esterified OPDA in response to freeze-thaw treatment. Of the genes investigated so far, none seem to influence arabidopside accumulation. Furthermore, the role of HPL in arabidopside accumulation was established using a L*er* line carrying a non-functional *HPL* allele. Experimental evidence for that the C24 *HPL* allele affects arabidopside accumulation is still missing. In an attempt to address this, the C24 *HPL* allele with its native promoter was cloned and transformed into the Col-0 genetic background. Unfortunately, the gene was not expressed in the Col-0 line although its presence could be confirmed by BASTA selection and PCR amplification (not shown). It remains unclear why the gene was transcriptionally inactive in the Col-0 background.

In conclusion, the capacity to synthesize OPDA-containing galactolipids varies but is maintained in all Arabidopsis accessions. Hydroperoxid lyase was identified as a potential rate-limiting enzyme in the biosynthesis of arabidopsides.

5.3.2 Acylated MGDG

The presence of MGDG species with an extra fatty acid attached to the sugar headgroup was first reported in plants more than 45 years ago (Figure 6). Heinz identified that acyl-MGDG was formed in spinach leaf homogenates when prepared in low pH or unbuffered solutions (218, 219). The enzyme that catalyzes the acyl transfer from the glycerol backbone of one galactolipid to the sugar moiety of another lipid molecule was characterized on a biochemical level (218, 220). Since then, work devoted to clarifying the role of acylated galactolipids in plants has been scarce. With the discovery of arabidopsides containing oxidized fatty acids on the galactose headgroup (e.g. arabidopside E and G), interest in acylated galactolipids has been revived (142).

We set out to investigate the occurrence of acyl-MGDG and arabidopsides in the plant kingdom (**Paper V**). Representative members from all major groups of land plants and one species of cyanobacteria (*Synechococcus elongatus*) were selected for the study. Lipids were extracted before and after freeze-thaw wounding and analyzed by LC-MS/MS. In accordance with previous studies (221), arabidopsides were exclusively found in brassicaceous plants. Acyl-MGDG, on the other hand, could be detected in all investigated plants and also in cyanobacteria. Acyl-MGDG was found during basal conditions in all species and accumulated in response to freeze-thaw wounding in some. Interestingly, the Arabidopsis *lox2-1* mutant accumulated large amounts of acyl-MGDG in favor of arabidopsides after freeze-thaw treatment (**Paper IV**). The rate by which the mutant produced acyl-MGDG was higher than wild-type plants produced acylated arabidopside species. Whether this is due to substrate specificity for non-oxygenated fatty acids over OPDA of the acyl transferase, or more likely, if it reflects higher amounts of available substrates in *lox2-1* plants remains an open question.

Oat was found to be particularly prone to produce acyl-MGDG, and was therefore selected as a source of material for characterization of the transacylation reaction and for isolation of the active enzyme (**Paper V**). As most other plants, oat lacks the prokaryotic lipid synthesis pathway and is consequently unable to form significant amounts of 18:3,16:3-MGDG (222). As a result, acyl-MGDG synthesized in oat from exogenous applied 18:3,16:3-MGDG can easily be distinguished from acyl-MGDG made from endogenous galactolipids. The acyl transfer activity *in vitro* was tested by mixing oat leaf tissue extracts with 18:3,16:3-MGDG purified from spinach leaves. Samples were analyzed by LC-MS/MS and the presence of acyl-MGDG with one or two 16:3 fatty acids were used as indicators of enzyme activity. An enzymatically active fraction could readily be obtained from oat leaves. The enzyme catalyzing the acyl transfer was found in the pellet after ultracentrifugation at $100.000g_{max}$, indicating a membrane localization. This is in agreement with a previous report that the enzyme resides in the chloroplast envelope (220). For further purification, oat membrane proteins were detergent solubilized and subjected to gel filtration. Three out of 40 fractions retained acyl transfer activity when tested *in vitro*. The fraction with the highest activity was subjected to trypsination and tandem mass spectrometry in order to determine the polypeptide composition. Peptide sequences were searched against an oat EST library

(Sikora and Olsson, to be published) which revealed the presence of 345 proteins in the isolated fraction. The best candidate for catalyzing the acyl transfer among these was a polypeptide annotated as phospholipase a1-ii 5-like. This enzyme is relatively conserved throughout the plant kingdom and the gene with the highest sequence similarity in Arabidopsis (*At2G42690*) encodes the chloroplast localized PLA$_1$-iiδ enzyme (223). The identification of the enzyme catalyzing the acyl transfer will hopefully shed light on the role of acylated MGDG species in plant immunity and wounding responses. The use of T-DNA mutants in Arabidopsis and reverse genetics will likely be instrumental to this work.

The data presented in Paper V point to that the ability to form acylated galactolipids is conserved throughout the plant kingdom whereas arabidopsides are limited to a few genera of the Brassicaceae family. It still remains elusive why only Arabidopsis and closely related plants are able to produce arabidopsides. Many plant species contain *LOX2* orthologs and other genes required for arabidopside synthesis (224, 225). One possible explanation is that the Arabidopsis LOX2 enzyme is the only with substrate specificity towards glycerolipid-bound fatty acids. In agreement with this hypothesis, LOX2 displays the lowest oxygenase activity of the Arabidopsis lipoxygenases on free linolenic acid *in vitro* (225). On the other hand, lipoxygenase-2 in soybean was found to exclusively oxygenate linoleic and α-linolenic esters present in isolated biomembranes (226). Although not tested, it seems unlikely that soybean produces arabidopsides. Another scenario is that the components of the jasmonates machinery (LOX2, AOS and AOC) in Arabidopsis forms a complex that is recruited to the chloroplast membranes and facilitates the use of acyl lipid bound substrates. All three enzymes are associated with the thylakoid membranes in potato and at least AOS in Arabidopsis is localized to the thylakoids (221, 227).

In an interesting report from the lab of Kenji Matsui, a soy bean 13-lipoxygenase was shown to add oxygen to fatty acids of MGDG and produce hydroperoxides when co-applied with a detergent (228). A possible mechanism for the formation of OPDA-containing lipids in Arabidopsis could thus be that a substance with surfactant properties is released/produced upon loss of cellular integrity that facilitates LOX-dependent oxygenation of bound substrates.

To clarify LOX2´s involvement in arabidopside formation, the *LOX2* gene from *Brassica rapa spp chinensis* (Chinese cabbage) was cloned. *B. rapa* is a member of the Brassicaceae family and closely related to Arabidopsis, albeit importantly, incapable of accumulating arabidopsides (**Paper V**). The *B.rapa LOX2* gene has been transformed into the *lox2-1* background of Arabidopsis and will soon be tested if it complements the mutated plants. If not, domain-swapping with the native Arabidopsis *LOX2* can be performed to reveal what elements of the gene (if any) promote arabidopside production. An alternative approach would be to transfer the Arabidopsis *LOX2* into non-arabidopside plants.

In summary, the ability to produce acylated MGDGs is preserved in all plant species, suggesting an old evolutionary origin. The high conservation infers that acyl-MGDG and/or the enzyme responsible for their synthesis has an important physiological role to play, probably under both basal condition and stress responses. The acyl transferase is likely localized to the chloroplast envelope and is rapidly activated in response to tissue disruption in many plant species.

5.3.3 Phosphatidic acid in plant immunity

The lipid second messenger phosphatidic acid (PA) has emerged as an important signaling molecule in plants. Biotic and abiotic stresses can cause a rapid and transient accumulation of PA (128). Downstream targets of PA formation include MAP kinase activation and ROS production (229). There are two distinct biosynthetic pathways for stress induced PA formation: via the sequential action of phosphatidylinositol-specific phospholipase (PI-PLC) and diacylglycerol kinase (DAGK), or by the enzymatic activity of phospholipase D (PLD) (230). PLD cleaves off the headgroup of structural lipids like PC or PE to produce PA. PI-PLC hydrolyses phosphatidylinositol phosphate (PIP) to generate inositolphosphate (IP) and diacylglycerol (DAG), which can then be phosphorylated by DAG kinase to produce PA. Direct feeding of PA to Arabidopsis leaf discs causes HR-like symptoms (102). Furthermore, pharmacological inhibition of PLD activity efficiently blocks the HR (102 and not shown). Effector elicitation has been reported to induce PA formation in cell cultures and in a transgenic plant system (102, 231). Enzymes involved in PA production have also been shown to be the target of the effector protein AvrBsT from the bacterial pathogen *Xanthomonas euvesicatoria* (232). However, it has proven difficult to obtain good results of lipase activation in plants after syringe inoculation: wounding responses triggered by the inoculation procedure obscure any effect caused by the pathogen. The induction of PA was measured by two independent methods after vacuum infiltration with *P. syringae* expressing AvrRpm1 in Arabidopsis (**Paper I**). A fivefold significant increase in PA was observed 4 hours after pathogen treatment as quantified by TLC (^{33}P labeling) and LC-MS/MS.

The vacuum inoculation method opens up the possibility to further explore the contribution of different PLD isoforms to PA accumulation in race-specific resistance. Arabidopsis carries 12 isoforms of PLD divided into six gene families (233). In order to determine the involvement of specific PLDs in plant defense, T-DNA mutants represented in all classes of PLDs have been acquired. In addition, double and triple PLD knockout mutants have been generated. Recently, Pinosa *et al*. (submitted) were able to identity one isoform (PLDδ) as important for non-host defense against the fungal pathogen *Blumeria graminis* f. sp. *hordei*. It remains to be tested if the same gene or additional genes contribute to PA accumulation during race-specific responses.

5.3.4 Phospholipid profiling of the hypersensitive response

In **Paper VI** the results from a phospholipid profiling study of the hypersensitive response in Arabidopsis are reported. Plants carrying an inducible copy of the *P. syringae* effector AvrRpm1 in three genetic backgrounds were used: wild-type Col-0, *rpm1.3* and *dde2-2.* Mutant *rpm1.3* plants lack the recognition of the RPM1 effector and are unable to mount HR (139). *dde2-2* (delayed-dehiscence2-2) plants contain a transposon-induced mutation in the *AOS* gene (234) rendering them incapable of synthesizing jasmonates.

Leaf discs were inoculated with the inducing agent dexamethasone for 6 hours before lipid extraction and analysis by LC-MS/MS. Phospholipid species that accumulated both dependently and independently of *AOS* during HR were identified. Two not previously described OPDA-containing acylated phosphatidylglycerol (PG) species were found to increase in abundance after AvrRpm1 recognition (Figure 7). PGs containing OPDA are known to form in Arabidopsis during ETI and wounding responses (140, 141). Non-oxidized acyl-PG has been found in microorganisms (235-238), in mammals (239, 240), and in plants (241). This is however to the best of our knowledge the first time OPDA-containing acylated phospholipids are reported. Synthesis of acylated OPDA-PGs likely follows the two step reaction of acyl arabidopside formation. First, bound fatty acids are oxidized, probably in a LOX2 dependent manner, to OPDA. Secondly, the OPDA molecule from the glycerol backbone of one lipid (PG or MGDG) is transferred to the glycerol headgroup of a PG molecule. It will be interesting to see if the same enzyme can catalyze the formation acyl-PG species as well as acylated MGDGs and arabidopsides.
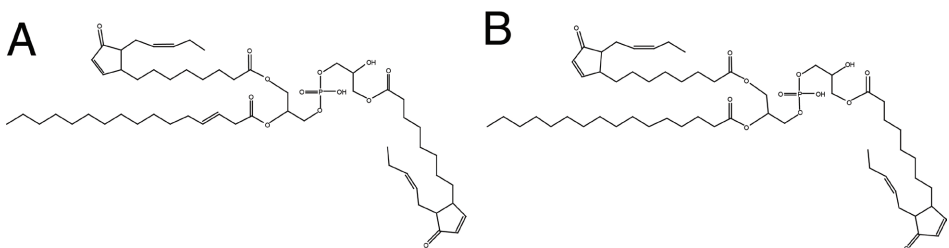
Figure 7. Putative structure of two OPDA-containing phosphatidylglycerol species induced during HR in Arabidopsis. (A) OPDA, 16:1-PG-OPDA and **(B)** OPDA, 16:0-PG-OPDA.

6. CONCLUSIONS AND PERSPECTIVES

Plants possess a suite of defenses that protects them from pathogen infections and animal herbivory. Tremendous progress has been made in the field of molecular plant pathology in recent years. However, a comprehensive understanding for how plants can fend off invaders and achieve immunity remains to be attained.

The first reports on hypersensitive reactions in plants date back to the early 20th century (150). It is now established that the HR is a complex multicellular defense mechanism that is activated by the recognition of pathogen avirulence factors by plant R protein receptors. Many of the components required for HR have been characterized on a genetic and biochemical level, but still, very little is known about the cell-to-cell communication that underlies local defense responses. In this thesis the list of inducible plant defense compounds produced during HR is extended by the introduction of the isothiocyanate sulforaphane. A proposed mechanism for sulforaphane is that it promotes cell death by affecting cellular redox through the glutathione system. Sulforaphane is but one of many small reactive molecules produced and released by plant cells during the HR (e.g. ROS, SA and OPDA). It can be envisioned that a cocktail of reactive and electrophilic compounds are released during the HR that contributes to an altered redox state of infected and neighboring cells. Such a cocktail may be highly specific and compose a distinct signature for different plant species. The mix of compounds would provide robustness to the plant defense as it would be difficult to detoxify by a single or a few pathogen virulence systems. If this is a general mechanism for the execution of HR cell death in plants remains to be experimentally verified. Moreover, the spread of cell death must be restricted and other defense mechanisms activated in cells adjacent to the site of attempted pathogen ingress. Cells surrounding the HR lesion are known to exhibit increased resistance to further pathogen attacks (242). Sulforaphane diffuses quickly and a concentration gradient from the infection site may contribute to prime cells surrounding the HR lesion and to establish local resistance. Sulforaphane-glutathione conjugates are potent transcriptional activators in mammalian cells (154). It would thus be interesting to do a transcriptional profiling study (e.g. DNA microarray or RNA sequencing) of plant cells treated with sulforaphane below the concentration limit that causes cell death. Such a screen may provide new insights to how pathogen challenged plants can acquire immunity.

The ability to form acylated galactolipids was found to be a ubiquitous feature of plants. Also phospholipids esterified with an extra fatty acid on the headgroup have been detected in several plant species. No physiological function for acylated galactolipids and/or the enzyme(s) catalyzing their production has yet been identified. Some but not all plant species tested accumulated acyl-MGDG in response to freeze-thaw wounding. A change in the composition of galactolipids from diacyl to lyso- and tri-acyl lipids likely has a profound impact on the curvature and other physical properties of membranes. To resolve the role of acylated lipids in wounding- and pathogen-induced responses in plants will be an exciting area for future studies. In

order to rigorously assign functions to these lipids, mutants impaired in their production will be needed.

OPDA-containing galactolipids are a rare flavor of Arabidopsis and a few other closely related plant species. These so-called arabidopsides accumulate to high concentrations in leaf tissue in response to wounding and pathogen elicitation. Arabidopsides possess antimicrobial properties *in vitro* (137, 138) and have been suggested to function in insect (211) and pathogens defense (208, 212). Preliminary data indicate that the first enzyme catalyzing arabidopside synthesis, LOX2, is required for a proper HR response to the bacterial effector protein AvrRpm1 in Arabidopsis (not shown). Whether this reflects the need for arabidopsides and/or other jasmonates in the HR response, or if the impaired HR is the result of decreased levels of reactive electrophile species from lipid peroxidation (186) is unclear and needs to be addressed. Finally, arabidopside synthesis has been shown to be strictly enzymatic. The question of how all enzymes in the arabidopside biosynthesis pathway can use lipid-bound fatty acid as substrates is still waiting to be answered.

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8. POPULÄRVETENSKAPLIG SAMMANFATTNING

Växter kan genom fotosyntesen använda solljus och koldioxid från luften för att bilda energirika kemiska föreningar som kolhydrater, proteiner och fetter. Precis som oss människor är de flesta djur och mikroorganismer beroende av den näring som växter producerar för sin överlevnad. För att kunna försvara sig mot angrepp från mikroorganismer, insekter och betande djur har växter utvecklat ett avancerat immunförsvar. Ett lager av vax, tjocka cellväggar och giftiga kemikalier hjälper växter att stå emot attacker från de flesta angripare. Vidare kan receptorer, förankrade i växternas cellmembran, känna igen kemiska strukturer hos inkräktare och aktivera flera typer av försvarsmekanismer. Somliga sjukdomsalstrande mikroorganismer, även kallade **patogener**, har i sin tur utvecklat strategier för att inaktivera det försvar de själva aktiverar i växten. Det pågår således en ständig kamp mellan växter och patogener om vem som har övertaget över den andre och vem som överlever.

Ungefär en tredjedel av alla de grödor som odlas världen över går idag förlorade på grund av skador orsakade av patogener och insekter. Detta gör forskningen kring växters immunförsvar till ett viktigt ämne. På lång sikt kan kunskapen användas till att ta fram grödor med ökad resistans mot skadedjur och därigenom minska förlusten på våra skördar och användningen av giftiga bekämpningsmedel.

Hypersensitiv respons (HR) är en välkänd försvarsmekanism i växter som för första gången beskrevs för över 100 år sedan. HR kan aktiveras i alla typer av växter och kan beskrivas som en brända jordens taktik. När en växtcell känner igen ett angrepp från en patogen aktiverar den en programmerad celldöd. Vid celldöden frigörs giftiga substanser som dödar den angripande mikroorganismen och förhindrar att infektionen sprids vidare till andra delar av växten.

I den forskning jag har bedrivit tillsammans med mina kollegor har vi undersökt vad som sker i växtceller vid HR orsakad av patogener och vid skada. Skaderesponser kan liknas vid vad som sker när växter blir angripna av betande djur och insekter som tuggar på bladen. I arbetet har främst växten backtrav (*Arabidopsis thailana*) använts. Backtrav är en liten växt som är släkt med senap och kål och som ofta används i laboratorier där den fungerar som en modellorganism för jordbruksväxter. I den första uppsatsen som finns inkluderad i den här avhandlingen presenterar vi en metod för att bestämma styrkan på den hypersensitiva responsen i växter. Denna metod kan till exempel användas för att identifiera vilka gener i växter som styr försvarsreaktioner mot patogena bakterier. I den andra uppsatsen studerades vilka molekyler som frigörs från växtceller vid HR, och som gör att inte bara celler i direkt kontakt med patogenen dör, utan även kringliggande celler. Vi upptäckte att ämnet **sulforafan** produceras och frigörs från växtceller i höga koncentrationer under HR. Sulforafan tillhör en klass

molekyler som ger kålväxter, så som broccoli och rädisa, dess speciella smak och har på senare tid fått stor uppmärksamhet för sina anticancerogena egenskaper hos människor. När blad infiltrerades med kemiskt rent sulforafan aktiverades celldöd som liknade den vid HR. Växter som saknade förmågan att bilda sulforafan visade sig ha en minskad motståndskraft mot infektioner av bakterier och en patogen algsvamp. Vi visar att sulforafan bidrar till HR genom att förbruka en viss typ av antioxidanter (glutation) som finns växtcellerna.

I uppsatserna 3-6 undersöktes hur sammansättningen av fettmolekyler, **lipider**, förändras i växter vid skada och vid patogenangrepp. Vi har särskilt tittat närmare på hur oxiderade lipider, det vill säga lipider som innehåller syre, bildas och vilken roll de har i växtförsvar. I den sista uppsatsen presenteras en enkel och snabb metod för att kunna identifiera och kvantifiera växtlipider, en metod som användes i samtliga arbeten.

För ett tiotal år sedan rapporterades det att den oxiderade fettsyran OPDA återfinns bunden i membranlipider i växter och ackumuleras i höga koncentrationer vid skada och patogen-aktiverad HR. Molekylerna gavs namnet **arabidopsider** (se figur). OPDA är kemiskt och strukturellt besläktad med växthormonet jasmonsyra (som för övrigt upptäcktes i jasmin och som ger växten dess karakteristiska doft, därav namnet). I uppsats 3 slår vi fast att bildningen av arabidopsider sker genom oxidering av bundna fettsyror, och

Kemisk struktur av tre membranlipider som finns i växten backtrav.

initieras bara sekunder efter det att en växt utsatts för skada. I den fjärde uppsatsen undersöktes om, och hur, förmågan att producera arabidopsider varierar mellan olika underarter av backtrav. Vi fann att förmågan att bilda arabidopsider varierade kraftigt mellan underarterna och att genen *HPL* bidrog till denna skillnad.

Normalt sett innehåller membranlipider två fettsyror men har i vissa växter rapporterats innehålla tre fettsyror, så kallade **acylerade membranlipider** (se figur). Vi samlade in växtmaterial från Göteborgs botaniska trädgård för att bestämma vilka arter som kunde bilda arabidopsider och vilka som kunde bilda acylerade membranlipider. Samtliga växter som undersöktes kunde bilda acylerade lipider medan endast ett fåtal

innehöll arabidopsider (uppsats 5). Det är fortfarande oklart vilken betydelse dessa lipider spelar för växtförsvaret. Upptäckten att acylerade membranlipider kan bildas i alla växter pekar mot att de har en viktig roll att spela vid skaderesponser. Arbetet med att identifiera det protein som reglerar produktionen av acylerade lipider har påbörjats och nyligen hittades en lovande kandidat. Identifiering och karakterisering av detta protein kommer förhoppningsvis att ge oss svaret på varför alla växter kan bilda acylerade membranlipider.

Sammanfattningsvis ger forskningsresultaten som presenteras i den här avhandlingen en ökad förståelse för de komplexa processer som styr växters immunförsvar och som bidrar till resistens. Jag hoppas att den här kunskapen kan ligga till grund för fortsatta studier inom ämnet och långsiktigt bidra till att ta fram växter med ökad motståndskraft - antingen genom klassisk växtförädling eller genom modern genteknik.

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