Plant Oxylipins and Lipid Transfer Proteins in Defense

- It's all about the fat

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Gothenburg 2017

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ISBN 978-91-88509-09-3
Online version: http://hdl.handle.net/2077/53430
Printed in Gothenburg, Sweden 2017
BrandFactory AB, Göteborg

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"I may not have gone where I intended to go, but I think I have ended up where I needed to be."

-- Douglas Adams

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ABSTRACT

Oxylipins, the oxygenated metabolites of polyunsaturated fatty acids (PUFAs), are found in many eukaryotic organisms. In plants, several enzymes can produce different types of oxylipins, and the chloroplast structural galactolipids mono- and digalactosyl diacylglycerol (MGDG and DGDG, respectively) are examples of sources of substrate PUFAs. In the model plant *Arabidopsis thaliana*, complex oxylipins known as arabidopsides are formed in response to different types of damage and pathogen elicitation. Similar substances known as linolipins are found in flax (*Linum usitatissimum*). If such substances are formed from free intermediates or directly from fatty acids esterified to complex lipids has been a matter of debate. The synthesis pathways of these substances were therefore investigated and the results show that the fatty acids remain esterified to the glycerol backbone during synthesis of arabidopsides (**Paper I**). It is also shown that all of the synthesis steps in Arabidopsis are enzyme catalyzed (**Paper II**). Formation of complex oxylipins could differ between plant species, but similar experiments on flax indicate that linolipins may also be formed from fatty acids bound to complex lipids. MGDG can have a fatty acid esterified to the galactose molecule, and in some plants, like Arabidopsis, this can be the oxidized fatty acid 12-oxo-phytodienoic acid (OPDA). It was investigated how common these lipids are in different plants, and what enzymes are involved in their synthesis. Samples from representative species of land plants were collected and screened, and non-oxidized acyl-MGDG were found to be omnipresent, while galactolipids with OPDA only exists in a few genera (**Paper III**). A protein responsible for this type of acyl transfer was identified in oat (*Avena sativa*), and an orthologue gene in Arabidopsis, that was named *AGAP1*. Knockout of the gene in Arabidopsis reduced the production of oxidized and non-oxidized acyl-MGDG to almost zero. *In vitro* experiments with protein expressed and purified from *E. coli* showed that the protein was able to catalyze MGDG acylation (**Paper III**). Investigations into the hypersensitive response (HR) in Arabidopsis revealed that the lipoxygenase LOX2, the enzyme responsible for the oxygenation of fatty acids in the arabidopside pathway, is involved in the initiation of the HR programmed cell death induced by effector triggered immunity (ETI). Mutant *lox2* plants had a delayed cell death response to *Pseudomonas syringae* pv. *tomato* (*Pst*) (**Paper IV**). Lipid transfer proteins (LTPs) are small proteins that can bind various lipids and non-polar molecules. Some of the lipid transfer proteins with a glycosylphosphatidylinositol (GPI)-anchor (LTPGs) were found to be involved in pre-penetration resistance against *Blumeria graminis* f. sp. *hordei* (*Bgh*), but not against the non-host mildew *Erysiphe pisi* (*Ep*) (**Paper V**). The reasons could be that fewer protecting or supporting substances that should end up in the protecting papilla are missing or less concentrated in the mutant plants.

ISBN: 978-91-88509-09-3

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

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

II. Nilsson, AK, **Fahlberg, P**, Johansson, ON, Hamberg, M, Andersson, MX and Ellerström, M. The activity of HYDROPEROXIDE LYASE 1 regulates accumulation of galactolipids containing 12-oxo-phytodienoic acid in Arabidopsis. Journal of experimental botany, 2016; 67: 5133-5144*

III. Nilsson, AK, Johansson, ON, **Fahlberg, P**, Kommuri, M, Töpel, M, Bodin, LJ, Sikora, P, Modarres, M, Ekengren, S, Nguyen, CT, Farmer, EE, Olsson, O, Ellerström, M, Andersson, MX. Acylated monogalactosyl diacylglycerol: prevalence in the plant kingdom and identification of an enzyme catalyzing galactolipid head group acylation in Arabidopsis thaliana. The Plant Journal, 2015; 84: 1152-1166*

IV. **Fahlberg, P**, Johansson, ON, Nilsson, AK, Bodin, L, Lundin, B, Andersson, MX. Chloroplast localized 13-lipoxygenase contributes to hypersensitive cell death in effector triggered immunity in Arabidopsis thaliana (Manuscript)

V. **Fahlberg, P**, Buhot, N, Johansson, ON, Andersson, MX. Involvement of GPI-anchored lipid transfer proteins in penetration resistance against a non-host powdery mildew in Arabidopsis thaliana. (Submitted to FEBS Lett.)

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Papers not included in thesis

- Pinosa, F, Buhot, N, Kwaaitaal, Mark, **Fahlberg, P**, Thordal-Christensen, H, Ellerström, M, Andersson, MX. Arabidopsis Phospholipase Dδ Is Involved in Basal Defense and Nonhost Resistance to Powdery Mildew Fungi. Plant Physiol. 2013 Oct; 163(2): 896–906.
- Nilsson AK, Johansson ON, **Fahlberg, P**, Steinhart F, Gustavsson MB, Ellerström M, Andersson MX. Formation of oxidized phosphatidylinositol and 12-oxo-phytodienoic acid containing acylated phosphatidylglycerol during the hypersensitive response in Arabidopsis. Phytochemistry. 2014; (May) 101: 65-75.
- Johansson ON, Fantozzi E, **Fahlberg P**, Nilsson AK, Buhot N, Tör M, Andersson MX. Role of the penetration resistance genes PEN1, PEN2 and PEN3 in the hypersensitive response and race specific resistance in Arabidopsis thaliana. Plant J. 2014 Aug;79(3): 466-76.
- Johansson, ON, **Fahlberg, P**, Karimi, E, Nilsson, AK, Ellerström, M, Andersson, MX. Redundancy among phospholipase D isoforms in resistance triggered by recognition of the Pseudomonas syringae effector AvrRpm1 in Arabidopsis thaliana. Front Plant Sci. 2014; 5: 639.

ABBREVIATIONS

16:3	Hexadecanoic acid
18:3	Octadecanoic acid
AOC	Allene oxide cyclase
α-DOX	α-dioxygenase
AOS	Allene oxide synthase
DGDG	Digalactosyl diacylglycerol
DES	Divinyl ether synthase
dnOPDA	Dinor-oxo-phytodienoic acid
EA	Etherolenic acid
ETI	Effector-triggered immunity
FAD	Fatty acid desaturase
FA	Fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPL	Hydroperoxide lyase
HPLC	High performance liquid chromatography
HR	Hypersensitive response
JA	Jasmonic acid
KOD	Ketodienes
KOT	Ketotrienes
LC-MS	Liquid chromatography-mass spectrometry

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
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerin
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLD	Phospholipase D
PUFA	Polyunsaturated fatty acid(s)
R protein	Resistance protein
ROS	Reactive oxygen species

CONTENT

1 INTRODUCTION

Without plants, the world would be quite different from what we know. Evolution of other organisms, like insects and animals, would not have happened the way it did, if colonization of land would have happened at all. This becomes especially obvious when considering that many organisms have co-evolved with plants, using them for food and shelter, growing dependent on their existence. At least since the Neolithic revolution (10000 BC), when human lifestyle started to change from nomadic hunter-gathering to farming settlers, plants have played a crucial role for the development of human civilization and culture (Richards, 2002). Of course, there are evidence of plants being used long before this, as medicinal plants were used in the Paleolithic era (some 60000 years ago) (Richards, 2002). The use of plants is just as important today, for human food and animal feed, textiles and other fibers, oils, etc. In addition, many of the modern medications are derived from plants, even if many can now be synthetically produced.

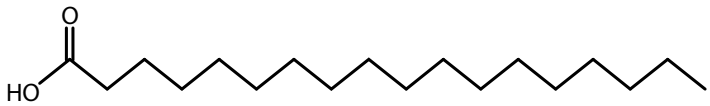
Plant pathogens, i.e. disease causing agents such as fungi, viruses, bacteria and other microorganisms have co-evolved with plants. As plants evolved defenses against the pathogens, the pathogens evolved ways to circumvent such defenses. Historically, pests have caused great economic loss as well as famine and death of millions of people. The infamous oomycete pathogen *Phytophtora infestans*, which cause potato late blight, is a prominent example. In the 1840s *P. infestans* caused catastrophic events around Europe, and was a major factor in the Irish potato famine that killed about a quarter of a million people, and led to mass emigration (Horsfall, 1956). It is still a problematic pest, leading to loss of about 16% of global potato yield, and it quickly adapts to overcome plants defenses (Vleeshouwers et al., 2011).

1.1 Plant membranes and lipids

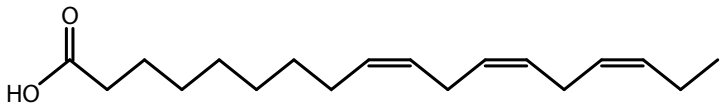
All living cells are separated and protected from the environment by a lipid bilayer membrane. Membranes are also found on the inside of eukaryotic cells, where they keep chemical processes, organelles and compartments separated from each other. The word “lipid” is derived from the Greek words lipos (fat) and eidos (form). The general definition of lipid is an organic compound that is soluble in non-polar organic solvents rather than water.

Fatty acids

Fatty acids (FA) are fundamental building blocks of most of the complex structural lipids of the membranes. They constitute the hydrophobic parts of the lipids in the lipid membranes of all living cells, and can be saturated or unsaturated (Figure 1). Unsaturated fatty acids (in *cis* formation) have "kinks" in the molecular structure, which prevents them from packing as closely as their saturated counterparts. This gives them lower melting points, making them more prone to be liquid at lower temperatures compared to the saturated fatty acids. This also makes them susceptible to oxidation. The nomenclature used to identify FAs is XX:Y, where XX is the carbon chain length and Y is the number of double bonds. There may also be a specification for the location of the double bonds counted from the carboxyl end of the carbon chain. This is written as delta (Δ) followed by the number of the carbon atom with the double bond. The α-linolenic acid in Figure 1 could accordingly be written as $C18:3^{\Delta 9,12,15}$.



Saturated fatty acid (stearic acid)



Unsaturated fatty acid (α-Linolenic acid)

Figure 1. Saturated vs unsaturated fatty acids. Skeletal formulas of the saturated stearic acid (above), and the unsaturated α-Linolenic acid (below).

Generally, *de novo* synthesis of fatty acids in plants is carried out in the plastid stroma. Acetyl-CoA carboxylase and fatty acid synthase are the two enzymes mainly responsible for *de novo* synthesis of fatty acids. Acetyl-CoA carboxylase adds a carboxyl group to the biotin prosthetic group of a biotin carboxyl carrier protein. The carboxyl group can then be moved by a carboxyltransferase to an acetyl-CoA molecule, which results in formation of malonyl-CoA. An acyl carrier protein (ACP) binds to the formed malonyl-CoA, and moves the molecule through a series of condensation reactions. This results in the formation of palmitate (16:0) and stearate (18:0), which can then be modified by desaturation or further elongation (Gurr et al., 2008). Desaturation of FAs is performed by fatty acid desaturases (FAD), enzymes that introduce double bonds into the carbon chain of the FAs. Most of the formed 18:0 from fatty acid

synthesis is desaturated by a Δ9 FAD to produce 18:1 (Ohlrogge and Browse, 1995; Schnurr et al., 2004). The fatty acids can then either remain for use in the chloroplast or be exported to the endoplasmic reticulum (the prokaryotic or eukaryotic pathway, respectively) (Ohlrogge and Browse, 1995). For export, the fatty acids are esterified to CoA in the outer plastid envelope (Schnurr et al., 2004). Other FAD in higher plants uses complex lipids and not free fatty acids as substrate for desaturation (Harwood, 1996). The two polyunsaturated fatty acids (PUFA) α-linolenic and linoleic acid ($18:2^{\Delta 9,12}$) are essential fatty acids for vertebrae animals. They lack the required FAD for endogenous synthesis and therefore must obtain these fatty acids from the diet (Wallis et al., 2002). They are also amongst the most common fatty acids in plants, where they are a substantial part of the chloroplast membranes (Ohlrogge and Browse, 1995).

Structural lipids

Fatty acids can form esters with glycerol, which makes acylglycerols. Since the glycerol molecule has three hydroxyl groups that can be esterified with fatty acids, triacylglycerol and other neutral fats can be formed (Ohlrogge and Browse, 1995). Membrane lipids usually have a molecular skeleton of glycerol or sphingosine, so called glycero- or sphingolipids, respectively. Membrane glycerolipids can have acyl groups esterified to the *sn-1* and *sn-2* positions of the glycerol molecule. Either a phosphate group or a galactose molecule can be esterified to the *sn-3* position, making glycerophospholipids or glycerogalactolipids, respectively. Glycerophospholipids usually have an additional group esterified to the phosphate, constituting a polar head group on the lipid molecule. Commonly found head groups in lipids of the plant plasma membrane are choline, ethanolamine, serine, glycerol and inositol. The acyl groups esterified to *sn-1* and *sn-2* are commonly PUFAs of C16/C18 type. Lipids that lack one acyl group are called lysolipids. Sphingolipids have a ceramide backbone, which is a long chain amino alcohol esterified to a fatty acid. Sphingolipids, while they are interesting parts of the membrane, will not be further discussed in this thesis. For the interested reader I leave the following references (Sperling and Heinz, 2003; Michaelson et al., 2016).

As previously mentioned, fatty acids can be saturated or unsaturated. For esterified fatty acids this will affect the way that the lipid molecules pack together in the membrane bilayer. Lipids with more unsaturated fatty acids will differ from those with more saturated fatty acids (with a more straight molecular structure) both in positioning and in increased

membrane fluidity (Los and Murata, 1998; Upchurch, 2008). The ratios of saturated and unsaturated fatty acids in the membrane bilayer will hence affect physiological properties, like freezing point, stability and permeability of the membrane (Upchurch, 2008).

The plasma membrane

The plasma membrane encompasses the entire individual cell and mainly consists of phospholipids, sphingolipids, sterols and proteins. It serves as a semipermeable protective layer, allowing controlled transport of compounds in and out, while keeping the inside separated from the outside of the cell. Phosphate from the phospholipids can also be used as a reserve if the plant cell needs more than the outer environment can provide. As the phosphate from phospholipids is released the remaining diacylglycerol is used to make galactolipids to replace the phospholipids in the membrane (Hartel et al., 2000; Andersson et al., 2003; Lin et al., 2009). Besides the structural and protective functions, the membrane bilayers also consist of different types of membrane spanning proteins, such as receptors and channels. These allow interaction with, and transport of substances to and from the outside environment. Synthesis of cellulose is also done by such proteins in the plasma membrane (Somerville, 2006).

Plastid membranes

Plastids are the result of some type of cyanobacterium-like prokaryote that was engulfed by a eukaryotic cell to form an endosymbiotic relationship, which would evolve into modern age plant cells (Yoon et al., 2004). The plastids were originally taken up into a eukaryotic cell by some type of invagination of the plasma membrane. Due to this, the lipid composition of the outer envelope membrane is more similar to the endoplasmic reticulum of the plant cell, while the inner envelope membrane is more similar to prokaryotic membranes, with more galactolipids than phospholipids (Block et al., 2007).

All plastids differentiate from proplastids, a type of precursor plastid, into one of several forms used for synthesis and storage. The most well-known type of plastid is the chloroplast, which has evolved into the light-harvesting organelles needed to make the now existing plant life possible. The inside of the chloroplasts is filled with fluid called the stroma, surrounding the stacks of thylakoids known as grana (Figure 2) (Waters and Langdale, 2009). Thylakoids are membrane enclosures that are interconnected, sharing the same inner space, known as the thylakoid space, or the lumen. The thylakoid membranes are the site of oxygenic

(i.e. oxygen generating) photosynthesis (Tomizioli et al., 2014). The thylakoid membrane is composed of around 70-80% of the galactolipids monogalactosyldiacylglycerol (MGDG, which accounts for more than half of the total amount of membrane lipids) and digalactosyldiacylglycerol (DGDG).

Other lipid components are sulfolipid sulfoquinovosyldiacylglycerol (SQDG) and the phospholipid phosphatidylglycerol (PG) (Block et al., 2007; Rast et al., 2015). There are reports on smaller amounts of phosphatidylcholine (PC) being found in all the membranes of the plastids. However, this is debated, and sometimes ascribed to be the result of contamination of prepared fractions (Dorne et al., 1990).

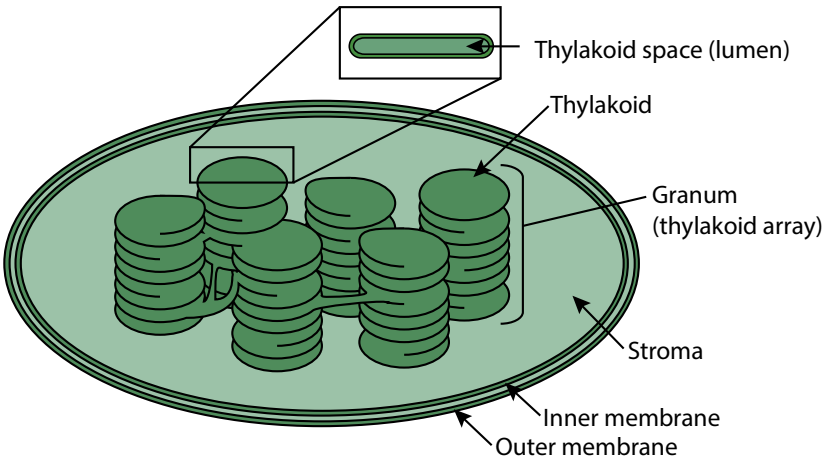


Figure 2. Illustration of chloroplast compartments and membranes

Lipid transfer proteins

Non-specific lipid transfer proteins (LTPs) were first isolated from potato tubers some forty years ago (Kader, 1975). They are relatively small proteins that have been found in all land plants, but not in green algae (*charophyta* and *chlorophyta*) (Edstam et al., 2011). LTPs have a hydrophobic cavity that *in vitro* has been shown to carry lipid molecules between lipid bilayers (Kader et al., 1984). They all have four or five α-helices and an eight cysteine motif (8CM, C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C) that forms four disulfide bridges stabilizing the molecular structure. These are characteristics that put the LTPs in the prolamin superfamily (Edstam et al., 2011). Since these enzymes are very resistant to both heat and proteolytic digestion, they can reach the intestinal tract and bind to IgE receptors (Zuidmeer and van Ree, 2007). This implicates

many LTPs in human true allergies. Most LTPs have an N-terminal signal peptide that targets them to the secretory pathway, directed to the apoplastic space. Some LTPs also have a motif that adds a glycosylphosphatidylinositol (GPI)-anchor (LTPGs) (Debono et al., 2009). The *in vivo* functions of LTPs in plants are still under investigation, but some suggestions for functions has been proposed (Salminen et al., 2016). The outer layer on the plant epidermal cells is the epicuticular wax that protects the tissues from dehydration, ultraviolet radiation and potentially against bacterial and fungal pathogens (Kunst and Samuels, 2003). The LTPGs have been implicated in formation of epicuticular wax (Debono et al., 2009; Lee et al., 2009; Kim et al., 2012), as well as sporopollenin and suberin (Edstam et al., 2013). LTPs have also been increasingly associated with plant disease resistance and have been classified as pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999; Liu et al., 2015). Several LTPs are known to have direct antimicrobial activity *in vitro* (Segura et al., 1993; Carvalho Ade and Gomes, 2007; Kirubakaran et al., 2008; Zottich et al., 2011; Finkina et al., 2016). A study by Bakan et al. (2006) showed that barley LTP1 can bind to the allene oxide oxylipin 9(S),10-epoxy-10,12(Z)-octadecadienoic acid. Perhaps this is a protective function, removing unstable allene oxide molecules to avoid damage.

1.2 Plant pathogen defense

The plant immune system

Plants are autotrophs, generating biomass from inorganic compounds through photosynthesis, which makes them the primary food for many other living organisms. Plants can also provide shelter and protection from weather and predators. Being permanently anchored to the substrate in which they grow, they cannot move to better locations to escape herbivores and pathogens. Instead, plants are forced to stand and fight against constant attacks, to which they are by no means defenseless. Plants have multiple layers of defenses that provide protection against a plethora of pathogens (Jones and Dangl, 2006). Cell walls provide rigidity to the tissues and also some protection against pathogens, chemical defenses with compounds that are toxic or deterrent, but also inducible defenses. These defenses can be put into two categories, depending on the type of molecules that senses infection and the downstream defense mechanisms (Jones and Dangl, 2006). When pathogens infect (or attempts to infect) a plant cell, certain molecules may be present, so called microbe-associated molecular patterns (MAMPs). MAMPs can activate

plant pattern recognition receptors (PRRs), which leads to a MAMP-triggered immunity (MTI, see Figure 3A) (Jones and Dangl, 2006; Nicaise et al., 2009; Macho and Zipfel, 2014).

Pathogens use disease promoting avirulence (Avr) genes, whose products, known as effectors, can elicit infection by suppressing the host cell defenses (Figure 3B) (Jones and Dangl, 2006; Gohre and Robatzek, 2008; Dodds and Rathjen, 2010; Trotta et al., 2014). To counteract the use of effectors, plants have evolved resistance (R) proteins that can detect such effectors and trigger a defense response, so called effector triggered immunity (ETI) (Figure 3C). This type of evolutionary warfare, where the pathogen Avr genes can be matched with plant R genes, was introduced as the so called "gene-for-gene theory" (Flor, 1971). Basically, a plant that has an R-gene matching the Avr gene product of a pathogen will be able to mount defenses against the pathogen, and the defenses may or may not stop the infection.

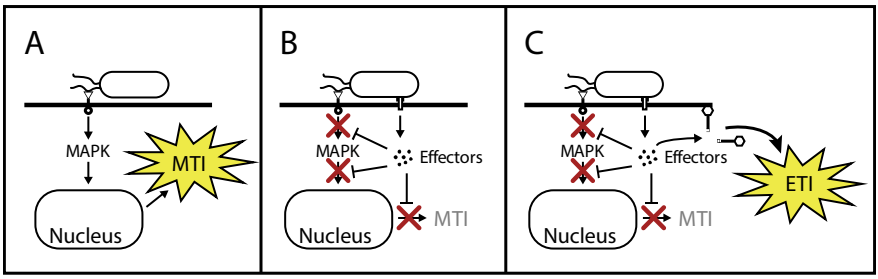


Figure 3. Plant cells can sense the molecules associated with infection and mount MAMP-triggered immunity (MTI) (A), leading to resistance against the pathogen. The pathogen can use effectors, proteins that facilitate infection, which can counteract the MTI response (B) and lead to disease. If the infected cell has R proteins that match the effectors, these can interact with the effector molecules or their actions, leading to Effector triggered immunity (ETI) (C), resisting infection. Redrawn and modified from (Chisholm et al., 2006).

Detection can be direct via interaction with the effector, or more commonly, indirect by changes affecting the R protein as the consequence of the effector molecules in the cell (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Dangl et al., 2013; Stuart et al., 2013). One generally efficient way to stop or inhibit further spread of the pathogen is to sacrifice affected cells. This is an outcome of the ETI, where a local form of programmed cell death known as the hypersensitive response (HR) is initiated (Jones and Dangl, 2006; Zurbriggen et al., 2010). The chloroplasts play crucial roles in the defense against pathogens. They provide the burst of reactive oxygen species needed for the initiation of

HR (Zurbriggen et al., 2010) and synthesize compounds involved in defense and signaling, like some of the oxylipins (Andersson et al., 2006), further described below.

The gene-for-gene type of protection against pathogens implies that the pathogen could overcome defenses of the plant cell by addition of one single Avr gene. The plant cell would also become susceptible if the specific R gene that confer defense was lost. Since the pathogens are technically able to infect but are stopped when triggering defenses, the defenses are sometimes called "host resistance". Since plants and pathogens have co-evolved, most pathogens have adapted to a narrow range of plant species that they are able to infect. This means that most plant species are immune to most pathogens, where plants and pathogens are called "non-host" to each other. This type of protection is due to non-host resistance (NHR) (Jones and Dangl, 2006). There can be several reasons for such immunity, such as chemical and structural differences between a host and non-host plant (Thordal-Christensen, 2003; Gill et al., 2015). NHR is generally thought of as a more robust immunity than host resistance. This is because NHR depends on more than one factor or single R/Avr gene interaction for immunity (Thordal-Christensen, 2003; Gill et al., 2015).

Tissue damage

Damage to the plant tissues can be caused by both biotic and abiotic factors. Pathogenic microorganisms and herbivorous insects have coevolved with plants, and the damage induced by insects feeding on plant tissues trigger the immune system in much the same way as the microorganisms do (Leon et al., 2001). Responses against herbivores include production of toxic compounds that may kill or have a deterring function or chemicals that attract natural enemies of insect herbivores. There can be physiological changes, such as thickening of leaf tissues or production of thorns, hairs or similar structures that can hinder or deter herbivores (War et al., 2012). Whether the wounding is inflicted by biotic or abiotic factors, it is important for the plant to repair the damage. At the same time they need to deter herbivores and keep microorganisms from infecting the damaged tissue (Leon et al., 2001).

Fungal pathogens and penetration resistance

When a fungal pathogen tries to infect a plant cell, it needs to breach the outer protective layers (i.e. the cuticle and cell wall) to obtain access to the plasma membrane and further infect the cell. If the plant cell detects such attacks, it may trigger MTI or ETI, like previously discussed.

However, the fungal pathogen may be stopped already when attempting penetration of the cell wall by the formation of a papilla by the affected cell (Figure 4). The papilla is a reinforcement to the cell wall consisting of substances such as callose, phenolic and antimicrobial compounds (Hardham et al., 2007). If the papilla fails to stop the penetrating hyphae, it will breach the cell wall and push down and invaginate into the plasma membrane to feed of the cell (Figure 4).

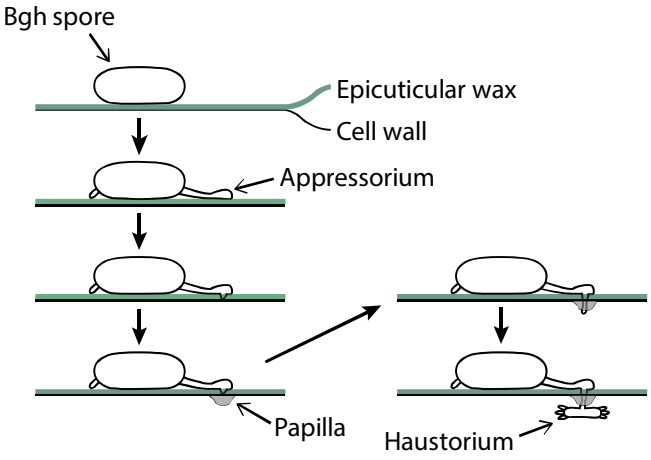


Figure 4. Infection on leaf surface by powdery mildew Blumeria graminis. The spore germinates and produces an appressorium, a structure that is specialized to penetrate the cell wall. The penetration attempt may be stopped by the formation of a papilla, or break through the papilla and proceed to infect the cell.

Amongst the more well studied genes involved in penetration resistance in the model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) are the penetration (PEN) mutants, *pen1* (Collins et al., 2003), *pen2* (Lipka et al., 2005) and *pen3* (Stein et al., 2006). Arabidopsis *pen* mutants display higher susceptibility to several pathogenic fungi, including penetration by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Thordal-Christensen, 2003; Underwood and Somerville, 2008). The PEN1 protein is a syntaxin involved in exosome secretion and formation of the papilla (Assaad et al., 2004; Nielsen and Thordal-Christensen, 2013). The myrosinase PEN2 produces active substances by hydrolysis of indole glucosinolates (Lipka et al., 2005; Bednarek et al., 2009) that are delivered into the apoplast by the ABC transporter protein PEN3 (Stein et al., 2006). Since the discovery of the PEN genes, several other gene products involved in penetration resistance has been found, like the phospholipase D delta isoform (PLDδ) (Pinosa et al., 2013).

The oxylipin pathways and pathogen defense

Oxylipins are a diverse group of oxygenated metabolites of PUFAs found in many eukaryotic organisms (Noverr et al., 2003). In mammals, perhaps the most well-known oxylipins are the cyclooxygenase generated prostanoids, involved in inflammation and inflammatory pain (Lee et al., 2013). The first step of oxylipin synthesis is the addition of oxygen(s) to PUFAs, in at least one step, transforming the fatty acids into fatty acid hydroperoxides. This oxidation can be done either enzymatically or non-enzymatically (Liavonchanka and Feussner, 2006). The enzymatic peroxidation in plants is catalyzed by lipoxygenases (LOX) or α-dioxygenases (α-DOX). The α-DOXs are enzymes catalyzing hydroperoxidation of the α-carbon (C2) of fatty acids (Hamberg et al., 1999) and have been shown to produce the antimicrobial substance 2-hydroxy-C18:3 (2-HOT) in response to bacterial infection in tobacco (*Nicotiana tabacum*) (Hamberg et al., 2003).

LOX enzymes are non-heme iron containing dioxygenases that catalyze addition of oxygen to PUFAs, producing fatty acid hydroperoxides (Porta and Rocha-Sosa, 2002; Andreou and Feussner, 2009; Mosblech et al., 2009). Plant LOXs can be categorized into two groups depending on subcellular localization, where 1-LOXs are extraplastidial and 2-LOXs are found in the plastids (Andreou and Feussner, 2009). LOXs are also categorized by which carbon in the fatty acid chain that the enzyme targets, and in plants there are 9- or 13-LOXs. 9-LOXs (type 1-LOXs) are enzymes that targets the 9th carbon from the carboxyl end of the fatty acid, while 13-LOXs (type 2-LOXs) targets the 13th carbon (Andreou and Feussner, 2009). In Arabidopsis there are six LOX isoforms, LOX1 to LOX6. LOX1 and LOX5 are 9-LOXs, while LOX2, LOX3, LOX4 and LOX6 are 13-LOXs.

The major substrates for oxylipin synthesis in plants are linoleic (18:2), α-linolenic acid (18:3) and hexadecatrienoic acid (16:3) (Mosblech et al., 2009). A major part of these fatty acids is derived from the galactolipids MGDG and DGDG, which contain high proportions of these fatty acids. The 9- and 13-LOXs produce 9- and 13-hydroperoxides from linoleic (9-/13-HPOD) and α-linolenic acid (9-/13-HPOT), respectively. The hydroperoxides produced by LOXs can be further processed by enzymes of the CYP74 family. Among these enzymes are divinyl ether synthase (DES), hydroperoxide lyase (HPL) and allene oxide synthase (AOS) (Figure 5). All plants species do not harbor all of these enzymes, and their expression and activity depends on factors like tissue, age and external stimuli (Yan et al., 2013). Enzymes in different plants may also use

different substrates, like only using the products from 9-LOX or 13-LOX, or being able to use both.

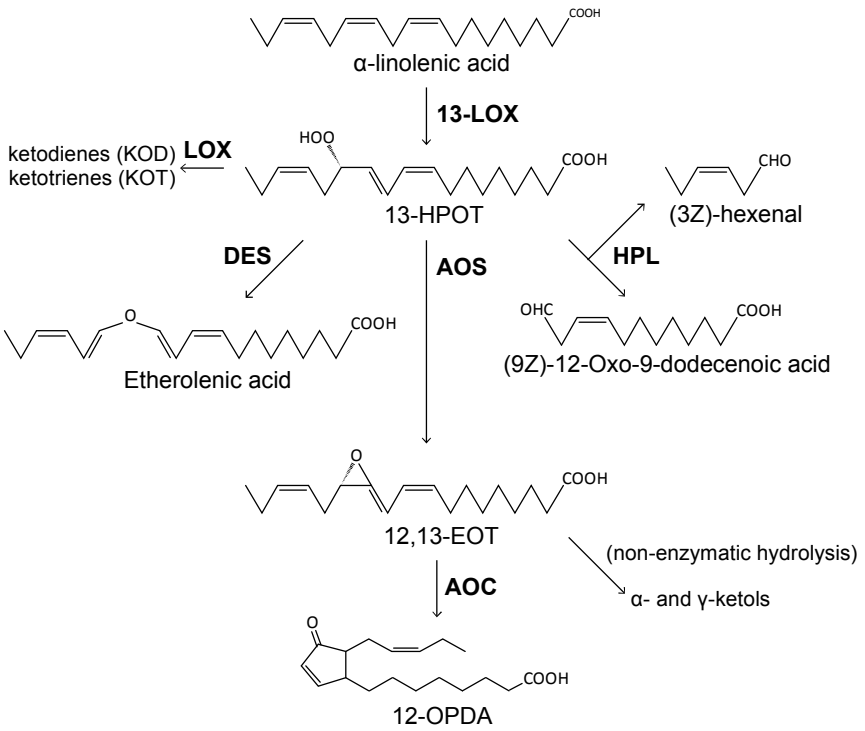


Figure 5. Pathways for oxylipin synthesis through divinyl ether synthase (DES), hydroperoxide lyase (HPL) and allene oxide synthase (AOS) from the 13-LOX product 13-hydroperoxy-linolenic acid (13-HPOT).

The LOXs can also use the produced hydroperoxides to yield keto fatty acids, ketodienes (KOD) and ketotrienes (KOT) (Feussner and Wasternack, 2002; Vellosillo et al., 2007). These have been shown to accumulate during HR induced cell death (Andersson et al., 2006) and to have *in vitro* antimicrobial properties (Prost et al., 2005).

Divinyl ethers

Divinyl ether oxylipins have been found in monocotyledons, such as garlic (*Allium sativum*) (Grechkin et al., 1995) and Lily of the valley (*Convallaria majalis*) (Ogorodnikova et al., 2008), and in dicotyledons, such as tomato (*Solanum lycopersicum*) (Itoh and Howe, 2001), potato (*Solanum tuberosum)* (Galliard and Phillips, 1972), and several species of *Ranunculaceae* (Hamberg, 1998; 2002; 2004). In some plants species,

such as tobacco, the expression of a 9-DES have been reported to be strictly pathogen induced. 9-DES convert 9-LOX generated hydroperoxides from linoleic and α-linolenic acid to colneleic and colnelenic acid, respectively (Fammartino et al., 2007). Colneleic and colnelenic acid have been found to accumulate in potato leaves infected with, and inhibit growth of, *Phytophthora infestans* (causing potato late blight), and they have also been found in tobacco leaves after infection with tobacco mosaic virus (Weber et al., 1999). In flax (*Linum usitatissimum*), the fatty acid hydroperoxides formed by a 13-LOX can be converted into etheroleic and etherolenic acid (EA) from linoleic and α-linolenic acid, respectively, by a 13-DES (Figure 5).

Jasmonates

Dehydration of fatty acid hydroperoxides by AOS results in formation of an unstable allene oxide that is the precursor of the jasmonates. These are a group of oxylipins including jasmonic acid (JA) and structurally related compounds (Browse, 2009; Wasternack and Song, 2017). Allene oxide cyclase (AOC) converts the allene oxide molecule into the JA precursors 12-oxo-phytodienoic acid (OPDA) or dinor 12-oxo-phytodienoic acid (dnOPDA) from C18 or C16 fatty acids, respectively (instances where both forms may be found will henceforward be abbreviated as (dn)OPDA) (Figure 5). The formed OPDA is then reduced by the OPDA reductase OPR3 in the peroxisome to form 3-oxo-2(2[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), that may be converted to (+)-7-iso-JA by beta-oxidation (Browse, 2009; Wasternack and Song, 2017). There are other OPRs, but OPR3 is the enzyme involved in biosynthesis of JA in Arabidopsis (Stintzi and Browse, 2000). JA can be further modified into several active compounds, including volatile methyl- or *cis*-jasmonate, or conjugated to amino acids like isoleucine (Ile) (Browse, 2009). Jasmonic acid isoleucine (JA-Ile) is responsible for development of, amongst other things, roots and pollen, and also regulates defenses against necrotrophic pathogens and insects (Wasternack and Song, 2017). Arabidopsis LOX2, a 13-LOX, is essential for the bulk formation of local wounding-induced JA, but not for JA levels found in unwounded tissue (Bell et al., 1995; Glauser et al., 2009). As a result, Arabidopsis plants lacking functional AOS or OPR3 gene cannot produce JA, and are therefore male sterile (Stintzi and Browse, 2000; von Malek et al., 2002), while silencing LOX2 does not render the plant infertile (Chauvin et al., 2013).

HPL Pathway

In the HPL pathway, the fatty acid hydroperoxide is converted to unstable hemiacetals. These spontaneously decompose into six carbon (C6)

aldehydes hexanal (from linoleic acid) or (Z3)-hexenal (from α-linolenic acid). The remaining aldehyde enols, such as 12-oxo-(Z)-9-dodecenoic acid, can isomerize to traumatin (Noordermeer et al., 2001; Grechkin and Hamberg, 2004). These substances are used in defense and signaling and form quickly after wounding (Zimmerman and Coudron, 1979; Savchenko et al., 2017). (Z3)-hexenal can be made into (2E)-hexenal by isomerization. Hexanal, (2E)-hexenal and (Z3)-hexenal can be converted into the corresponding alcohols by alcohol dehydrogenase (Bate and Rothstein, 1998). The six carbon products are collectively known as green leaf volatiles (GLVs). Substances produced in the HPL pathway have been found to be involved in resistance against fungal and bacterial pathogens (Croft et al., 1993; Shiojiri et al., 2012). Treatment of leaves with C6-aldehydes has been shown to induce lignification of cell walls and vascular bundles in Arabidopsis. This increased protection against the necrotrophic fungal pathogen *Botrytis cinerea* in Arabidopsis (Kishimoto et al., 2006). As previously discussed, formation of a papilla is an important protection to fight fungal pathogens trying to penetrate the cell wall. Since lignin is an essential part of the papilla, the aldehydes could be important components in the penetration resistance (Kishimoto et al., 2006).

Complex oxylipins in Arabidopsis and flax

The bulk (dn)OPDA in Arabidopsis is not found in free form, but esterified to galactolipids. The first molecule to be reported as an OPDA containing galactolipid was *sn1*-O-(12-oxophytodienoyl)-*sn2*-O-(hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O) (Stelmach et al., 2001). This discovery was followed by identification of two more species of MGDG with esterified (dn)OPDA. The first molecule was found to have OPDA esterified to *sn-1* and dnOPDA to *sn-2* of the glycerol backbone. The second identified molecule contained OPDA on both *sn-1* and *-2* positions, and these compounds were named arabidopside A and B, respectively (Hisamatsu et al., 2003) (Figure 6). (dn)OPDA was later found in the same positions as on arabidopside A and B but on DGDG, and these compounds were named arabidopsides C and D (Hisamatsu et al., 2005). Arabidopside F was described in 2006, as MGDG with 18:3 at *sn-1* and dnOPDA at *sn-2* (Nakajyo et al., 2006). OPDA has also been found in phosphatidylglycerol (PG) (Buseman et al., 2006; Nilsson et al., 2014), sulfoquinovosyl diacylglycerol (SQDG), and phosphatidylinositol (PI) species (Nilsson et al., 2014).

Some complex lipids can have an additional fatty acids esterified to the head group, and are in this thesis termed head group acylated. For

MGDG, the 6'-position of the galactose molecule can be esterified to FAs, which makes it acyl-MGDG. This was discovered fifty years ago to occur in spinach (Heinz, 1967). The same acylation is possible for DGDG, making acyl-DGDG (Heinz et al., 1974). Complex oxylipins with acylated head groups has also been found. Arabidopside E was identified in 2006 as an acyl-MGDG having OPDA residues esterified to *sn-1* of the glycerol backbone and to the galactosyl 6' position, and dnOPDA esterified to *sn-2* (Andersson et al., 2006). Arabidopside G is similar to E, but with an OPDA at *sn-2* (Kourtchenko et al., 2007). Arabidopsides have been found to accumulate as response to several types of stressors, like low temperature (Vu et al., 2012), damage and HR (Andersson et al., 2006; Kourtchenko et al., 2007). Arabidopsides have been found in a limited number of plant species, while acyl-MGDG containing non-oxidized fatty acids seems to be more common and has been reported to occur in several species. These include Arabidopsis (Ibrahim et al., 2011; Nilsson et al., 2014), tobacco (Matsuzaki et al., 1982), spinach (*Spinacia oleracea*) (Heinz, 1967) and tomato (*Solanum lycopersicum*) (Vu et al., 2014). There have previously been findings of OPDA-esters of the glycerol backbone of galactolipids in *Melissa officinalis* (Zabranska et al., 2012), *Cirsium arvense* (Hartley et al., 2015) and *Ipomoea tricolor* (Ohashi et al., 2005). Besides these studies, the ability to produce galactolipids containing (dn)OPDA has only been observed in a limited amount of plant species in the *Brassicaceae* family (Böttcher and Weiler, 2007; Kourtchenko et al., 2007).

Flax (*Linum usitatissimum*) is a plant species with strong DES activity, and complex oxylipins with esterified divinyl ether residues, called linolipins, have been found in flax leaves (Chechetkin et al., 2009). The first two isolated linolipins were named A and B, and are both MGDG species. While linolipin A has one α-linolenoyl residue esterified to *sn-1*, and one (ω5Z)-etherolenic acid esterified to *sn-2*, linolipin B has (ω5Z)-etherolenic acid esterified to both *sn-1* and *sn-2* (Figure 6). Linolipins C and D were recently described, and are DGDG analogs to linolipin A and B (Chechetkin et al., 2013). Like the arabidopsides in Arabidopsis, these complex oxylipins are induced by pathogen interactions and damage (Chechetkin et al., 2013). Linolipin A is the only linolipin detected in unstressed flax leaves (Chechetkin et al., 2009; Chechetkin et al., 2013).

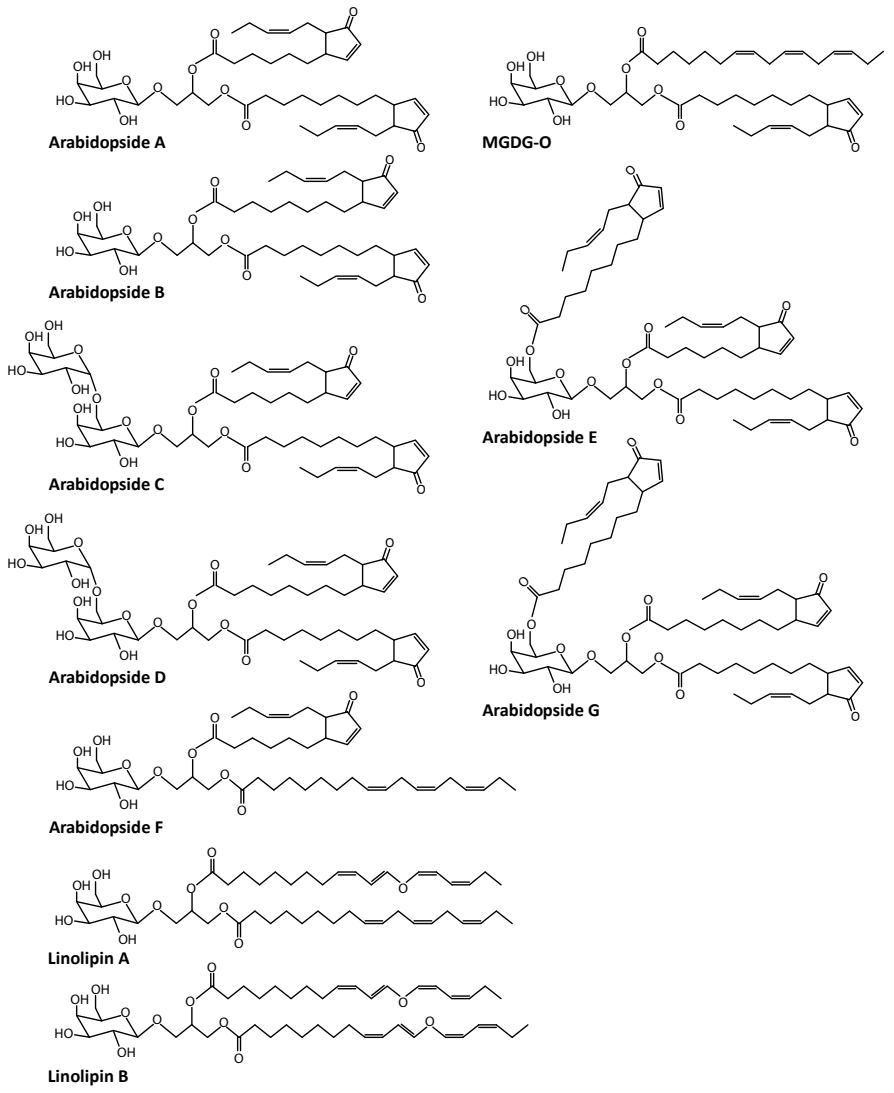


Figure 6. Arabidopsides and linolipins, complex oxylipins found in Arabidopsis and flax, respectively.

2 SCIENTIFIC AIMS

The overall aims for this thesis were to (1) investigate the biosynthesis of complex oxylipins and fill some of the knowledge gaps in the literature and (2) investigate defense mechanisms connected to oxylipins and other lipid species.

Specific aims:

- Are the oxylipins bound to glycerogalactolipids found in Arabidopsis (Paper I) and flax formed through free intermediates or while attached to the glycerol backbone?
- Investigate potential variation in ability to produce and accumulate arabidopsides in different Arabidopsis accessions, and the underlying causes for such variation. (Paper II)
- How common is production of acylated and OPDA-containing glycerogalactolipids amongst plants?
- What enzyme(s) is performing the acyl transfer? (Paper III)
- What are the roles of the different enzymes in the arabidopside synthesis pathway in hypersensitive response? (Paper III and Paper IV)
- Is there a connection between non-host resistance and the glycophosphatidylinositol anchored lipid transfer proteins? (Paper V)

3 EXPERIMENTAL SETUP

Arabidopsis, a model plant

Thale cress (*Arabidopsis thaliana* or Arabidopsis) is a member of the mustard family (*Brassicaceae*) and considered a weed by most people. However, it has been extensively used in plant research, for good reasons. With a relatively small diploid genome, self-pollination and production of many seeds, Arabidopsis is good for genetic alteration and experimentation; it also grows relatively quick and is inexpensive (Somerville and Koornneef, 2002). There are several wild type strains (accessions) that have been found and usually named after the place of discovery. The most used accession, Col-0, was first used in the 1950s in Columbia, USA. A plant of this accession was also the first plant ever to be genetically sequenced. Additionally, there are readily available collections of Arabidopsis lines with *Agrobacterium tumefaciens* transfer DNA (T-DNA) insertions that together spans the whole genome (O'Malley and Ecker, 2010). This makes it relatively easy to find and order mutants for genes of interest. As with all model organisms, the results are hopefully transferable to other species, but may also be unique to the specific organism studied.

Arabidopsis, although now found world-wide, is native throughout the Eurasian continent and North Africa (Weigel, 2012). Differences in environmental conditions and geographical distances have resulted in subdivisions of species, known as ecotypes (Page and Grossniklaus, 2002). Plants from such ecotypes have been collected and isolated for laboratory use and are, due to inbreeding, practically homozygous. Such lines are called accessions (Page and Grossniklaus, 2002), and that is the term used in this thesis as well.

Lipids - extraction and analysis

There are many methods for extraction, separation and analysis of lipids. Modern technologies such as mass spectrometry (MS) coupled to gas chromatography (GC) and liquid chromatography (LC) has revolutionized the field of lipid research. These methods make it simpler and quicker to analyze large amounts of samples and detect more substances. Such advances in analysis, and gained knowledge about the involvement of lipids in disease and as key regulators of cellular functions, are reasons for why the interest in lipid research and lipidomics has grown significantly during the last decade. Analysis can be either targeted or non-targeted. Targeted analysis means that a more specific method will be used, designed for the lipids of interest. This usually results in higher sensitivity

(Li et al., 2014). The most important development in analysis is mass spectrometry methods where relatively small samples can be analyzed without the need for pre-separation (Welti and Wang, 2004). To analyze complex lipids before these techniques became available often required large amounts of extract, pre-separation of lipid classes and resulted in bad resolution. Newer methods, such as tandem mass spectrometry (LC-MS/MS) performed with a triple quadrupole (QQQ) allow analysis of the precursor ion as well as the product ions after fragmentation. This is possible since the instrument has two mass analyzers that work in tandem (hence the name). Specified scanning for precursor and product ions (multiple reaction monitoring, MRM) allow better specificity and resolution. A method based on LC-MS/MS technique was developed by Nilsson et al. (2014) and is used in this thesis (with different variations) for analysis of complex lipids. Further information on the methods used can be found in the papers.

Freeze thawing – more wounding than naturally occurring?

In the lab, tissue damage is usually caused by insect herbivores, microorganisms or by mechanical means. To study what happens in the tissues during and after damage, the tissues needs to be damaged in a reproducible way. Different methods have been used in studies, like wounding tissues with a hemostat (pean). Another method used in the papers in this thesis is to simply freeze the tissues in liquid nitrogen and then allow the tissue to thaw at room temperature. This will emulate massive wounding and makes it possible to test several samples at the same time, quickly and reproducible. In Arabidopsis, the amount of produced free and bound OPDA after freeze-thawing the tissues has been shown to be comparable to those produced after wounding with hemostat (Paper I). It is worth to note that some compounds are only produced in living, intact cells, and may not be found after freeze-thawing. For instance, there is no JA produced as a result of freeze-thawing (Glauser et al., 2009; Johansson et al., 2015).

Induction of HR – bacterial pathogens or ectopic expression of avirulence genes

Hypersensitive response (HR) can be induced by the use of avirulent bacterial pathogens that will trigger ETI. Although this works, and is often used (Paper IV, Paper V, Kourtchenko et al., 2007; Johansson et al., 2015), the use of living pathogens introduces some potential experimental variability depending on the bacterial viability. It will also lead to more unspecific defense responses, like both MTI and ETI. An alternative and more specific method is to express an effector protein *in planta* as a

response to some external stimuli. This makes it possible to control the initiation of HR, and is what the dexamethasone (DEX) system does. DEX is a glucocorticoid, which is taken up into the tissues upon contact, and will bind to a glucocorticoid receptor. This in turn frees a transcription factor that initiates the transcription of the desired protein (in this case a bacterial effector). The DEX system used in the work presented in this thesis has the bacterial effector AvrRpm1 from *P. syringae*, which is expressed as response to DEX exposure. This leads to ETI/HR through the recognition of AvrRpm1 by the R protein RPM1 (Grant et al., 1995). This method allows high reproducibility even with many samples that would be difficult to manage with living bacteria. One drawback with this method is the time consuming process needed to put the system into new lines by crossing and screening.

Testing the defenses – fungal penetration testing

To measure the penetration resistance against fungal pathogens, plants were infected with spores from the biotrophic model fungi *Blumeria graminis* f. sp. *hordei* (*Bgh*) or *Erysiphe pisi* f. sp. *pisi* (*Ep*). *Bgh* was cultivated on barley (*Hordeum vulgare*, Barbro variety), and *Ep* on pea (*Pisum sativum*, Kelvedon wonder variety), both susceptible to infection from respective pathogen. The *Bgh* spores were spread over Arabidopsis plants positioned inside a settling tower. This is basically a cardboard box with a cut-out hole where the infected barley plants were shaken. After dispersing spores inside the box the Arabidopsis plants were left until the spores had settled on the leaves. For infection of Arabidopsis with *Ep*, the spores from infected pea leaves were transferred to Arabidopsis leaves with a small brush. This is a more suitable method for infection, since the *Ep* spores are firmly attached to the pea leaves.

After spreading the spores on the Arabidopsis plants, the spores were allowed to germinate and attempt to infect. The infected material was then stained with trypan blue to count and score the infection success versus failures. Since trypan blue is only able to pass through the membrane of dead cells and will not stain living cells, it is an indicator for cell death. This method depends on living fungal spores, and thus the results can vary somewhat depending on their vitality. As a result of this, the amount of successful penetration attempts for *Bgh* on wild type Col-0 is usually between 10-20%. The relative amount of succeeded penetration attempts can then be determined in the mutants of interest.

4 SYNTHESIS OF COMPLEX OXYLIPINS IN PLANTS

Occurrence of complex oxylipins in the plant kingdom

In Arabidopsis, the (dn)OPDA is mainly found bound to galactolipids. Lipid bound (dn)OPDA, originally identified in Arabidopsis, has only been found in a few other species (Figure 2 in Paper III). In Paper III, species representing major groups of land plants were investigated for production of head group acylated galactolipids (discussed in chapter 5). These plants were also screened for glycerolipid-bound (dn)OPDA. However, no lipid bound (dn)OPDA was found in any of the species tested regardless of freeze-thawing or not (Figure 2 in Paper III). Arabidopsis is a member of the Brassicaceae family, and that plants more related to Arabidopsis would be more likely to produce similar complex oxylipins. To investigate this, lipids were extracted from leaf tissue from *Brassicaceae* plants, several of the Arabidopsis genus, with and without freeze-thawing. OPDA-containing lipids were found in all tested species of the Arabidopsis genus, and in some of the other species in the *Brassicaceae* family. A summary of the findings in this and other studies, as well as a phylogeny of plant species is shown in Figure 2 in Paper III. Arabidopsides have previously been reported in *Melissa officinalis* (Zabranska et al., 2012), *Cirsium arvense* (Hartley et al., 2015), *Ipomoea tricolor* (Ohashi et al., 2005) and a limited number of plant species in the *Brassicaceae* family (Böttcher and Weiler, 2007; Kourtchenko et al., 2007).

Other oxylipins besides (dn)OPDA have been isolated in plants, and some of them have been found esterified to complex lipids. For instance, colneleic acid esterified to phosphatidylinositol in potato tubers (Fauconnier et al., 2003) and etherolenic acid bound to MGDG (linolipin A and B) and DGDG (linolipin C and D) (Chechetkin et al., 2013) found in flax are examples of such complex oxylipins.

In theory, there are two ways that complex oxylipins could be formed. The traditional theory has been that free intermediate fatty acids could be converted into oxylipins and then esterified to complex lipids. The alternative is that the oxylipins could form directly from fatty acids that stay esterified to the complex lipids during the process (Figure 7). The Arabidopsis *act1* mutant is inhibited in an acyltransferase step of glycerolipid synthesis in the plastid. This result in inhibition of complex lipids formed through the prokaryote pathway after wounding. Interestingly, synthesis of arabidopsides is not inhibited to the same extent, which indicates that they could be synthesized from fatty acid

hydroperoxides bound to the glycerol backbone (Kourtchenko et al., 2007). There have however been no experimental evidence for *in situ* formation of (dn)OPDA from fatty acids that stay bound to the glycerolipids. This was investigated in Arabidopsis for arabidopsides (Paper I) and in flax for linolipins.

Synthesis of arabidopsides

For arabidopsides to form from free fatty acids, these free fatty acids must first be cleaved from the glycerol backbone by lipases through hydrolysis. After enzymatic modification by LOX, AOS and AOC, the formed (dn)OPDA needs to be transferred back to the glycerol backbone to form arabidopsides. This process would likely lead to an increase in free trienoic fatty acids and probably free OPDA as a result of freeze-thawing. This was tested in Paper I, but no such increase of free trienoic fatty acids was found after freeze-thawing. In fact, the amount of free trienoic fatty acids stayed at relatively low levels while lipids with esterified (dn)OPDA accumulated quickly (Paper I). This indicates that the pathway where the fatty acids stay esterified to the glycerol is more likely than the pathway with free fatty acid intermediates. Since free fatty acid intermediates could cycle between esterified and free form, this needed further study.

To investigate if there is any hydrolysis occurring during arabidopside accumulation, leaves were allowed to take up ^{18}O-labeled water before freeze-thawing and lipid extraction. If there were free fatty acid intermediates, there would be incorporation of ^{18}O into the freed intermediates (Figure 7). The results of the labeling experiment show that the ^{18}O was not incorporated into the formed (dn)OPDA, which further demonstrate that the fatty acids remain esterified to the glycerol backbone while they are enzymatically converted into (dn)OPDA (Figure 3 in Paper I).

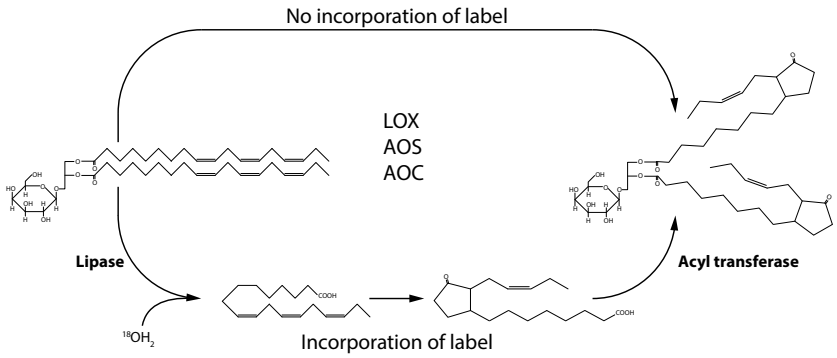


Figure 7. Alternative pathways of fatty acid conversion to the oxylipin (dn)OPDA.

Even if the fatty acids remain esterified when (dn)OPDA is formed, this gives no information on whether all steps in synthesis are enzyme dependent. The unstable allene oxide formed by AOS could spontaneously cyclize, forming a racemic mixture of 9R,13R-OPDA and 9S,13S-OPDA. Alternatively it could be used as substrate for AOC, which makes optically pure 9S,13S-OPDA (Hofmann et al., 2006). To test which of these pathways is used in arabidopside production, the galactolipid fraction from Col-0 was extracted and hydrolyzed after freeze-thawing. After methylation the fatty acids were separated by chiral-phase HPLC (Figure 4 in Paper II). The formed (dn)OPDA was only found in 9S,13S form, which shows that they are enzymatically produced by AOC directly from lipid bound fatty acids.

These results indicate that the synthesis of arabidopsides in Arabidopsis is strictly enzyme dependent and also can be done directly from lipid bound fatty acids, without free intermediates. The quick synthesis and accumulation of high amounts of arabidopsides further strengthens this hypothesis.

Accumulation of arabidopsides

There are several Arabidopsis accessions, and the genetic variations between these accessions can be used to identify genes responsible for a certain trait (Weigel, 2012), such as regulation of arabidopside production. In the Arabidopsis Col-0 accession, lipid bound dn(OPDA) quickly accumulates to high concentration as a response to freeze-thawing (Paper I) and as a response to bacterial effectors (Andersson et al., 2006; Kourtchenko et al., 2007; Vu et al., 2012). This quick accumulation was explored in Paper II, where the natural variation in ability to produce

arabidopsides and acyl-MGDG was investigated in 14 Arabidopsis wild type accessions (including Col-0). The results showed that the steady state amount of MGDG was quite similar between the accessions, and that 93-98% was lost after freezing in liquid nitrogen and thawing in room temperature for one hour (Figure 1A in Paper II). However, there was a significant variation in production of arabidopside A, B and the OPDA head group acylated species arabidopside E and G (Figure 1B and C in Paper II) between accessions. The greatest difference in arabidopside and acyl-MGDG production was found between Col-0 and C24.

Since arabidopsides are formed quickly after tissue disruption it seems logical that the enzymes needed for formation are already in place before wounding. This would implicate that differences between accessions in expression of the enzymes responsible for (dn)OPDA synthesis could result in the observed differences in arabidopside production. Expression of *LOX2*, *AOS* and all four *AOC* genes in Col-0, C24 and L*er* (since L*er* displayed arabidopside production between that of Col-0 and C24) was measured with quantitative real time PCR (qPCR) (Figure 5 in Paper II). This generated similar expression profiles for the six tested genes, which indicated that transcriptional regulation of these genes was not the major reason for the observed variation of arabidopside accumulation between these accessions.

To explore genetic factors that determine the ability to synthesize arabidopsides, Col-0 and C24 was crossed to obtain a mapping population. F2 plants were analyzed in regards of arabidopside A, B, E and G after freeze-thawing. A large variation in ability to accumulate arabidopsides was found (Figure 6A in Paper II). A PCR-based quantitative trait loci (QTL) analysis was used to find loci that determine this variation in accumulation of arabidopsides. One significant QTL was found on chromosome 4. PCR-guided fine mapping further pinpointed the QTL to a 67 kbp region with 21 genes. One of these genes was *HPL1*.

The Col-0 accession has a deletion (10 nucleotides) in the first exon of the *HPL1* gene which makes the protein non-functional (Duan et al., 2005). A non-functional *HPL1* gene could explain why Col-0 produces more arabidopsides, since AOS would have more available substrate (Figure 5). Additionally, Arabidopsis HPL has been shown to be able to use glycerolipid bound hydroperoxides as substrate (Nakashima et al., 2013). However, both C24 and L*er* have functioning *HPL1* alleles, and L*er* accumulates much more arabidopsides than C24. Therefore the expression of the *HPL1* gene in C24 and L*er* was determined by qPCR. The results showed that the expression in C24 was an approximately 80-fold higher

than that of L*er* (Figure 7 in Paper II). This difference in expression seems to explain the difference in arabidopside accumulation.

To test if lack of *HPL1* in Col-0 was the cause of arabidopside accumulation, the full genomic sequence of C24 *HPL1* was cloned and transformed into Col-0 plants via *A. tumefaciens*. Compared to wild type Col-0 the measurements of OPDA esters in the T_2 generation did not differ from wild type Col-0, and the expression of *HPL1* was only slightly elevated. This was rather unexpected, but could be the result of trans-acting elements specific for the accession. To ensure high transcription of *HPL1*, Col-0 was transformed with a C24 *HPL1* cDNA construct fused with the CaMV 35S promoter. The resulting plants with high expression of C24 *HPL1* produced less lipid-bound OPDA compared to wild type Col-0 (Figure 8 in Paper II), and the measured amount of formed OPDA esters was inversely proportional to the level of *HPL1* transcripts (Figure 8C in Paper II).

These results show that Arabidopsis HPL1 and AOS compete for the acyl hydroperoxide fatty acids. Arabidopside production was found to vary between the tested accessions, and this is likely the result of differential expression of *HPL1*.

Synthesis and accumulation of linolipins in flax

Freezing and then thawing tissues to induce damage has proven to be a very good method of inducing formation of (dn)OPDA containing lipids in Arabidopsis, where there is a quick formation of such lipids after freeze-thaw induced tissue damage (Paper I and Paper II). It has also been reported as a method that induces the formation of linolipins in flax (Chechetkin et al., 2013). However, it seems that freeze-thawing without prior stimuli may not be the best method to induce production of linolipins. Accumulation of etherolenic acid containing lipids as a result of freeze-thawing was much slower and resulted in much lower concentrations in our experiments compared to OPDA-containing lipids in Arabidopsis (Paper I).

In Paper II we show that there is competition for substrate between AOS and HPL in Arabidopsis and that the relative activity of these two enzymes determines arabidopside accumulation after freeze-thaw wounding. Since the Arabidopsis Col-0 ecotype has a non-functional HPL protein, comparing accumulation of arabidopsides in Col-0 with linolipins in flax may not be relevant. Presumably, there is similar competition between the enzymes of the oxylipin pathway in flax and other plant

species. This means that there could be competition between DES, AOS and HPL (Figure 5), resulting in different concentrations of accumulated oxylipins depending on additional factors, such as biotic and abiotic stress.

The amount of linolipins produced after freeze-thaw wounding in flax was found to vary greatly between experiments. Tissue damage and light stress have been shown to induce LOX transcription and activity in Arabidopsis (Rossel et al., 2007; Zhao et al., 2014), and presumably do the same in flax. This would provide more substrate available to the enzymes of the oxylipin pathway. However, exposing the plants to high intensity light during 72 h and/or inducing damage to the flax leaves with a pean (hemostatic clamp) 72 h before collecting and freeze-thawing did not increase formation of linolipins (not shown). There was no difference in the ability to produce linolipins in the wounded tissue itself or tissue adjacent to the damage. Instead, the maximum amount of linolipins acquired was from plants that were infected with gnats and probably other insect herbivores from an early age and until freeze-thaw and extraction. In these plants accumulation of (ω5Z)-etherolenic acid (EA) in the leaf tissues peak at about 200 nmol per gram fresh tissue after thawing for 30 minutes (Figure 8).

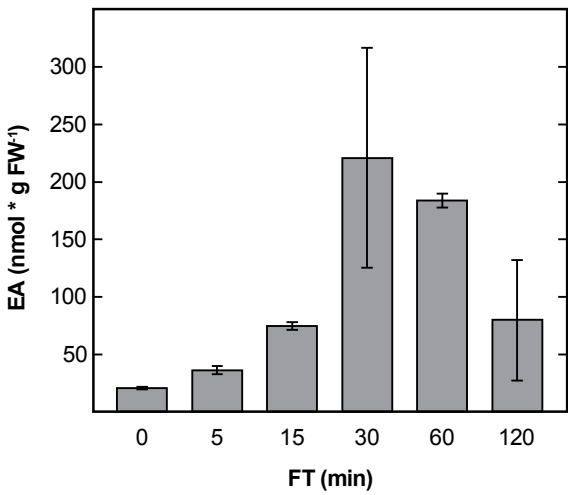


Figure 8. The amount of etherolenic acid (EA) increases after freeze-thawing and seem to be peak at around 30 minutes.

The influence of herbivore insects on the plants ability to form linolipins was tested by spraying plants with insecticide during the growth period, which removed both the insects and freeze-thawing induced formation of

linolipins. Interestingly, similar results have been obtained in tobacco, where transcript for a 9-DES (NtDES1) responsible for production of colneleic and colnelenic acid was not found in any tissue in healthy unwounded or wounded plants. Inoculation with an oomycete (an incompatible race of *Phytophthora parasitica* var. *nicotianae*) in a stem assay resulted in local accumulation of NtDES1 transcript after 12 h (Fammartino et al., 2007).

Field-grown flax was collected from the Gothenburg botanical garden and leaf tissue was subjected to freeze-thawing followed by lipid extraction. The field-grown flax was shorter and had somewhat smaller leaves compared to flax grown in growth chambers, probably due to the strong sunlight and exposure to wind and rain. Additionally, these plants were most likely under natural stress from herbivorous insects, fungi and other pathogens. The field-grown plant material accumulated about twice the amount of EA esters after freeze-thawing compared to the flax grown in controlled growth chambers. For these reasons, field-grown flax was used to investigate the linolipin synthesis pathway. Experiments with ^{18}O-labeled water, like previously done in Arabidopsis (Paper I), were performed to test if the label would be incorporated into the formed glycerolipid bound etherolenic acid or not.

Preliminary results indicate that the enzymatic synthesis of EA in flax occurs on fatty acids esterified to complex lipids (Figure 9). If 18:3 would have passed through a free intermediate, the amount of ^{18}O would be more similar to that of 18:2 at zero time. This suggest that synthesis of linolipins in flax is, at least in part, is done similar to synthesis of arabidopsides in Arabidopsis.

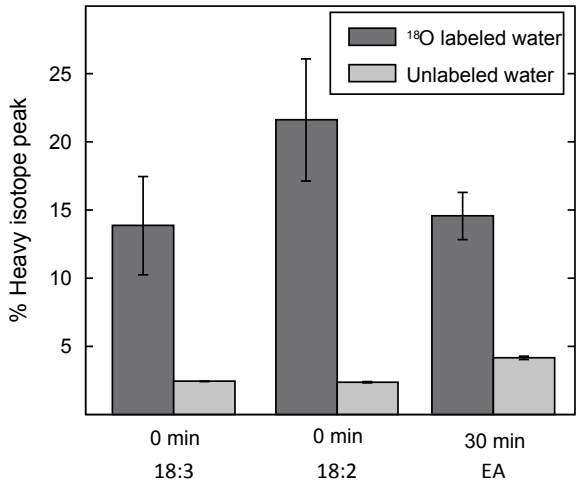


Figure 9. Stalks from flax plants were fed ^{18}O-labeled or unlabeled water for 42 hours. The amount of unlabeled molecular ion and labeled (+2 m/z) molecular ion was measured and used to calculate the labeled to unlabeled ratio of methyl esters. Grey bars represent unlabeled and black bars labeled water.

Taken together, the amount and type of oxylipins formed in Arabidopsis and flax seem to depend on the expression levels of LOX and enzymes in the CYP74 family. Changes in expression/activity of these enzymes due to abiotic/biotic stress, age or other factors can result in different oxylipin profiles. This indicates that the synthesis of oxylipins can depend on factors that may be difficult to manage and measure. This could potentially explain why some studies report finding complex oxylipins in plant species that cannot be found in other studies. The failure to detect linolipins in flax after removal of insects is an example of this. Further studies are needed to determine if the fatty acids remain esterified to the glycerol backbone while they are enzymatically converted into EA.

5 GENERALITY AND SYNTHESIS OF ACYLATED MGDG

Occurrence of acyl-MGDG in the plant kingdom

As previously stated, head group acylated galactolipids have previously been identified in some plant species. However, it has not been established how common they are in the plant kingdom or what enzyme is responsible for the acyltransferase activity. To investigate how common these are, material from representative species was collected from the Gothenburg botanical garden for analysis (Paper III). The material from all of the tested plants was found to contain acyl-MGDG both before and after inducing damage by freeze-thawing, although with big variation in accumulated amounts in the latter case (Table 1 in Paper III). These results suggest that formation of head group acylation is common, if not omnipresent, in the plant kingdom.

Identification of a putative MGDG acyl transferase from oat

To find the enzyme responsible for the head group acyl transfer some common and easily cultivated 18:3 (plants with mostly 18:3,18:3-MGDG) plants were screened for high enzyme activity. The advantage with 18:3 plants is that they have almost no 16:3 containing MGDG. Using readily available spinach MGDG (containing large amounts of 16:3) as substrate will result in acyl-MGDG that differs from that of an 18:3 plant. Oat (*Avena sativa*) was among the plants that accumulated high amounts of acyl-MGDG, together with barley (*Hordeum vulgare*), pea (*Pisum sativum*) and white mustard (*Sinapis alba*). Oat was chosen for isolation of the enzyme since the amounts of accumulated acyl-MGDG were high, and we had access to an oat transcript sequence database.

Extracting and testing an active enzyme fraction from oat by application of exogenous lipids extracts from spinach resulted in a measurable formation of acyl-MGDG. Although both 16:3 and 18:3 were found attached to the head group, there were much more transfer of 18:3 (Figure 4 in Paper III). This indicates that the enzyme have some specificity towards 18:3, compared to 16:3. After further purifying the active fraction, trypsin degradation followed by tandem mass spectrometry was used to identify the polypeptides, which were then used to search an oat transcript sequence database. This resulted in a list of 285 polypeptides, where one was annotated as “phospholipase a1-ii δ-like”.

A homologue MGDG acyl transferase in Arabidopsis

The amino acid for the sequence was used with BLAST against the Arabidopsis genome (TAIR10, www.arabidopsis.org). The resulting list of hits had a highly similar gene, At2g42690, which is described as an "alpha/beta-Hydrolases superfamily protein". The cloned coding sequence from Arabidopsis was expressed in *E. coli* as a His-tagged GST-fusion protein that was able to produce acyl-MGDG from spinach MGDG. As in oat, the enzyme seems to be more efficient at transferring 18:3 to the head group of MGDG. The name acylated galactolipid associated phospholipase 1 (AGAP1, Paper III) was proposed for this enzyme.

With the *in vitro* function of AGAP1 demonstrated the next step was to investigate if the enzyme had the same activity *in vivo*. For this, two T-DNA insertion lines, *agap1-1* and *agap1-2* were obtained and tested by extracting lipids from leaf tissue directly or at five or 60 min after freeze-thawing (Figure 8b in Paper III). Accumulation of acylated (dn)OPDA-containing MGDG and non-oxidized acyl-MGDG in the mutant lines was only a fraction compared to that of the wild type after 60 min. The content of the acyl arabidopsides in fresh tissue from the mutant lines was around 10% of that found in the wild type. Production of head group acylated OPDA-containing DGDG (Ibrahim et al., 2011) and PG (Nilsson et al., 2014) also failed in the mutant lines (Figure 8C in Paper III).

The results show that the AGAP1 enzyme is responsible for formation of most of the acyl-MGDG in Arabidopsis, both containing (dn)OPDA and the non-oxidized form. Considering that the *agap1* mutants still produced some acyl-MGDG there could be other genes with overlapping functions. Interestingly, despite the substantial loss of acyl-MGDG production there were no visible differences between the *agap1* mutants and wild type Col-0 during growth in standard conditions.

Phylogenetic analysis of AGAP1 and homologous sequences

Including AGAP1, there are twelve members in the PLA_1 family found in *Arabidopsis thaliana*: At1g30370, At2g44810, At4g16820, At1g51440, At1g06800, At2g30550, At1g05800, At2g31690, At4g18550, At2g31100, At1g06250, and AGAP1 (At2g42690). These have been put into three classes (Class I, II and III) based on similarities in sequence and N-terminal properties (Ishiguro et al., 2001). Genome sequences of representative land plants in datasets from JGI Phytozome (www.phytozome.org) were searched for homologs of the 12 PLA_1

members, resulting in a phylogenetic tree (Figure 5 in Paper III). T-DNA lines for the other genes with the highest sequence similarity to AGAP1 (At2g31100, At1g06250 and At4g18550) have been isolated, but none of these differed from wild type in appearance or amount of produced acyl-MGDG. Considering the low amount of acyl-MGDG formed in the *agap1* mutants, the putative contribution of one or more of these AGAP1-like enzymes could be relatively small. Crossing these with the *agap1-1* mutant has been done in the hopes of finding mutants that completely lack the ability to produce acyl-MGDG. Perhaps such plants, with no acyl-MGDG, would have some phenotype that is not found in *agap1* mutants alone. An interesting question remaining to be answered is the relevance of the formed acyl-MGDG. What is the purpose of acylated galactolipids? The small amount of acyl-MGDG formed in the *agap1* mutants may be sufficient for the supposed need of the plants. Alternatively, there is also the possibility that production of acyl-MGDG is nothing more than a by-product, possibly from stress-induced alteration of enzymatic function. However, since damage induces acyl transferase activity in a wide variety of plant species, this seems less likely. Further studies will hopefully give an answer to these questions.

Localization of AGAP1

AGAP1 with eCFP fused to the C-terminal, controlled by a 35S-promotor was made and used for ectopic expression in the leaves of tobacco (*Nicotiana benthamiana*) plants by transformation with *Agrobacterium tumefaciens*. There was no CFP signal found in chloroplasts from epidermal cells or in isolated mesophyll protoplasts. The signal was instead found in the cytoplasm in the investigated cells (Figure 7 in Paper III). AGAP1 has previously been localized to the cytosol in Arabidopsis (Lo et al., 2004), and since AGAP1 seems to be missing a transit peptide, this seems likely. During the fractionation of oat tissue, the activity was pelletable (at 100 000 g_{max}), which points to a membrane localization. The transacylation activity has been localized to the chloroplast envelope in previous work by Heinz et al. (1978). The combined results points to a cytosol and outer envelope membrane localization.

To further investigate the localization of AGAP1, an Arabidopsis AGAP-CFP mutant could be used, with the same construct as used for expression in tobacco.

6 HR/ETI AND THE OXYLIPIN PATHWAY

Delayed onset of HR in LOX2 mutant plants

The hypersensitive response (HR) is a part of the ETI response. In Arabidopsis, large amounts of arabidopsides are formed during HR (Paper I; Andersson et al., 2006; Kourtchenko et al., 2007; Zoeller et al., 2012). Oxygenation of polyunsaturated fatty acids by LOX2 is the first step in the jasmonate pathway where AOS and AOC use the produced hydroperoxides to form (dn)OPDA. As shown in Paper IV, the *lox2-1* loss of function mutant (Glauser et al., 2009) was found to have a delayed HR induced cell death compared to wild type. To investigate all the steps in this synthesis chain, the *dde2-2* mutant line was used, which has a non-functional allele of AOS (von Malek et al., 2002). Additionally, a triple mutant for the fatty acid desaturases FAD3, FAD7 and FAD8 (*fad378*) (McConn and Browse, 1996), that has no trienoic fatty acids was also included. These three lines were tested by infiltrating the *Pseudomonas syringae* pv. *tomato* (*Pst*) bacterial effector AvrRpm1 into leaf tissue, left floating on water in wells. By then measuring the change in conductivity of the water, the amount of ion leakage caused by cell death was evaluated (Figure 1A in Paper IV). Both *dde2-2* and *fad378* had a lower ion leakage compared to the wild type Col-0 tissue. However, *lox2-1* was significantly lower than all the other lines at 3, 4 and 5 hours post infection (hpi) but then increased to the same level as *dde2-2* and *fad378* at 6 hpi. *P. syringae* expressing the effector proteins AvrRpt2 or AvrRps4 were also tested by infiltration, with similar results (Figure 4A and B in Paper IV). Acylated galactolipids are induced during HR (Andersson et al., 2006), and to see if they could be involved in HR, ion leakage experiments were done with the *agap1-1* mutant (Paper III). Contrary to the *lox2-1* phenotype, there was a slight increase in ion leakage with AvrRpm1 but no significant change using AvrRpt2.

Mutant lines with DEX-constructs, expressing the bacterial effector protein AvrRpm1 (DEX:AvrRpm1) *in planta* were used for further ion leakage experiments. The results for the LOX2 mutant (DEX:AvrRpm1/*lox2-1*) are consistent with those from the experiments using live bacteria (Figure 3 in Paper IV). Like shown in Nilsson et al. (2014), there was a slight, but not significant decrease of ion leakage in the AOS mutant (DEX:AvrRpm1/*dde2-2*) compared to the wild type Col-0 tissue. Interestingly, the ion leakage of *lox2-1* equals that of Col-0 and *dde2-2* at 6 h, and is significantly higher 8 h post induction of AvrRpm1 expression (Figure 3 in Paper IV).

The cell death induced during HR has been connected to changes like membrane rupture and organelle disruption (Coll et al., 2011). By investigating the chloroplasts with electron microscopy, the delay in cell death seen in the *lox2-1* mutant could be connected to a decreased chloroplast disruption in the mutant compared to clearly visible chloroplast disruption in wild type tissue 2 hpi with *Pst* (Figure 2 in Paper IV). Interestingly, regardless of these differences, *lox2-1* had the same ability to restrict the growth of *Pst*:AvrRpm1 as wild type Col-0 (Figure 5 in Paper IV).

LOX2 could normally form hydroperoxides from the fatty acids of the lipid membrane, and perhaps disrupting the membrane structure and lead to leakage of reactive oxygen species (ROS). LOX2 could perhaps also activate enzymes that lead to production of ROS or removal of the photosystem II protection against them. In the *lox2-1* mutant, membrane disruption may be performed by other processes, like other LOXs, which could lead to a slower disruption of the membrane, and a delay in ROS leakage and initiation of HR induced cell death. There is a LOX2 dependent decrease of 18:3 during the HR, indicating formation of fatty acid hydroperoxides. However, what complex lipids these hydroperoxides are bound to remains to be identified. The fatty acid hydroperoxides and keto derivates produced by LOX2 could also be damaging to the chloroplast and photosystems, but since the Col-0 (as mentioned in chapter 4) has a non-functional HPL, there is no competition for the formed hydroperoxides, and activity of LOX2 and AOS would be the only regulator for the amount of hydroperoxides available.

Since the non-functional AOS mutant *dde2-2* had a slightly lower ion leakage compared to wild type Col-0, involvement of the hydroperoxides would seem less likely and rather strengthens the role of direct LOX2 function and perhaps the production of ketodienes and ketotrienes in the HR induced cell death. When performing ion-leakage experiments in complete darkness, the wild type plant also exhibits a delay in HR (Figure 8 in Paper IV). This is probably due to the slower production of ROS in darkness. Perhaps that the prolonged buildup of ROS in the mutant, that would be caused by slower membrane disruption, also explains the stronger ion leakage exhibited by the *lox2-1* mutant after 6 h (Figure 3 in Paper IV). Induction of α-DOX1 expression and activity has been demonstrated in Arabidopsis leaves, where a 10-fold increase of activity was seen as response to infiltration with *Pst* AvrRpm1 (De Leon et al., 2002). It could be interesting to investigate if there are any connections between LOX2 and α-DOX1 during HR.

7 LIPID TRANSFER PROTEINS AND PENETRATION RESISTANCE

Transcriptional response

Microarray data indicated that some of the glycophosphatidylinositol (GPI) anchored LTPs (LTPGs) could be involved in resistance against fungal pathogens (Figure 10). Like most of the LTPs, LTPGs have a signal peptide that targets them to the secretory pathway, and they are found in the apoplastic space. Lipid transfer proteins (LTPs) have been reported to inhibit growth of bacterial and fungal pathogens *in vitro* (Yeats and Rose, 2008). Since many biotrophic fungal pathogens infect by penetrating the cell wall and creating feeding structures that push down into the plasma membrane, the LTPG molecules will be at the site of penetration and infection, and thus seemed like interesting candidates for penetration resistance proteins. Previous studies have identified 34 LTPGs from Arabidopsis and demonstrated that they can be placed into three expression modules (Edstam et al., 2013).

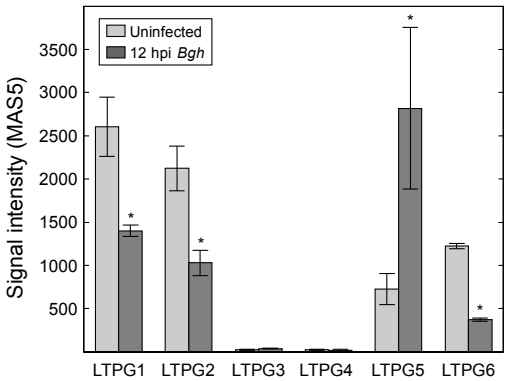


Figure 10. Transcriptional GEO data for the indicated LTPG genes were extracted for wild type non-inoculated or 12 hours after inoculation with Bgh (experiment GSE12856) on Col-0. An asterisk denotes statistically significant difference between non-inoculated and inoculated (error bars are standard deviation).

Initially *LTPG1* was identified as an interesting candidate gene in the microarray screening. The other five *LTPG* genes were found on The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). After investigating microarray data for all six genes, *LTPG1*, *LTPG2* and *LTPG6* were found to be of interest, since their expression was significantly reduced after infection with *Bgh*. These LTPGs are placed in the so called AtI group by Edstam et al. (2013), and are thus involved in

formation of cuticular wax. *LTPG5* was also selected, since the expression was found to be significantly increased in response to infection with *Bgh*. *LTPG3* and *LTPG4* did not show any change in expression after infection with *Bgh* and were selected to see if any found penetration phenotype followed the results from the microarray data.

LTPGs in penetration resistance

To test if the selected proteins were involved in penetration resistance against powdery mildews, knock-out/down mutants for the six selected LTPG were obtained. These mutants were then tested by infecting them with *Bgh* or *Erysiphe pisi* (*Ep*) spores from host barley or pea plants to quantify the amount of successful penetrations and cell death. The results show that the *ltpg1*, *2*, *5* and *6* mutants were significantly impaired in stopping the penetrating hyphae from the infecting *Bgh* spores (Figure 1A in Paper V) compared to wild type Col-0 plants. Interestingly, there was no such difference found in the mutant plants infected with *Ep* (Figure 1B in Paper V). This indicates that some of the LTPGs are involved in protection against specific fungal pathogens, like *Bgh*, but not against others, like *Ep*.

It is interesting that loss of both LTPG1 and LTPG2 resulted in the same loss of penetration resistance as loss of either protein alone (Figure 1C in Paper V). Perhaps this is the result of a threshold effect, where a certain concentration of these LTPGs could be needed for an effect on penetration resistance. Lowering the protein concentration further would then not change the penetration resistance.

Investigation of wax composition

Very-long-chain aldehydes have been shown to promote germination and differentiation of *Bgh* conidia (Hansjakob et al., 2010), as well as being important for pre-penetration processes of the powdery mildews (Hansjakob et al., 2011; Weis et al., 2014). Changes to the composition of the cuticular wax layer could be a reason for the penetration phenotype found in the LTPGs. But when analyzing the cuticular wax composition of the LTPG mutants, no connection was found between penetration resistance and the cuticular wax composition of the LTPG mutant lines. Additionally, no penetration phenotype was found for the *cer4* mutant plants that lack long chain alcohols (Figure 3A in Paper V) or the *cer1* mutant plants, which have a greatly reduced wax load (Figure 3B in Paper V). These results indicate that the composition of the cuticular wax is not a reason for the penetration resistance.

LTPG1 localization during powdery mildew infection

To investigate the localization of LTPG1 during infection with *Bgh* and *Ep*, a construct of YFP and LTPG1 in *ltpg1-2* background was used (YFP-LTPG1) (Debono et al., 2009). Infection with both *Bgh* and *Ep* resulted in YFP-LTPG1 localization to the papillae (Figure 4 in Paper V). The accumulation of YFP-LTPG1 around the papilla could be the result of changes in the geometry of the apoplastic space. When the penetration peg pushes down on the cell wall, there will be a local change in cell wall shape that could lead to a widening between the plasma membrane and the cell wall around the site of attempted penetration. This area of increased apoplastic space could lead to automatic accumulation of LTPG1 according to the "balloon in a box model" suggested by Ambrose et al. (2013). Investigation of the localization of the other five LTPGs during penetration resistance would be interesting. One hypothesis about the observed penetration phenotype is that these LTPGs could bind to the fungal membrane when the penetration peg is pushing down through the cell wall, where after the LTPGs could be part of a signal transduction for plasma membrane MAMP receptors. The LTPGs could also facilitate transport of substances to the site of penetration, and be needed to form a fully functioning/protecting papilla.

8 CONCLUSIONS

Formation of complex oxylipins in Arabidopsis and flax

We show that in *Arabidopsis thaliana* there is a quick formation and accumulation of (dn)OPDA containing lipids after tissue damage (Paper I and Paper II). The fatty acids remain bound to the galactolipids while they are converted into (dn)OPDA, and all the steps in synthesis are enzyme dependent (Paper I and Paper II). HPL competes with AOS for lipid bound hydroperoxy fatty acids, and levels as well as spatial distribution of these enzymes determine the further fate of hydroperoxides formed from lipoxygenation (Paper II). OPDA-containing galactolipids were only found in a few plant genera (Paper III). Furthermore, the genes that are involved in formation of acylated arabidopsides were identified (Paper II and Paper III). Preliminary findings in flax (*Linum usitatissimum*) suggest that enzymatic synthesis of linolipins in flax might be performed directly from the bound fatty acids.

Formation of head group acylated complex lipids

Non-oxidized acyl-MGDG was detected in all tested plant species, and seems to be widely distributed in the plant kingdom (Paper III). An enzyme responsible for head group acylation of galactolipids MGDG and DGDG and phosphatidylglycerol was identified and named Acylated Galactolipid Associated Phospholipase 1 (AGAP1) (Paper III).

LOX and the hypersensitive response

LOX2 is needed for quick initiation of HR in Arabidopsis, but AOS is not (Paper IV). The reason for this effect on HR is not clear, but could be the result of LOX2 induced membrane degradation followed by a ROS response or LOX2 products driving HR.

Involvement of LTPGs in penetration resistance against *Bgh*

Some of the LTPGs are involved in pre-penetration resistance against the powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*), but not against *Erysiphe pisi* (*Ep*) (Paper V). The mechanisms behind this penetration phenotype are unclear, but it may be related to transport of antimicrobial or structural compounds to the papilla. It could also be direct antipathogenic properties of the LTPGs or that they function by transferring MAMPs or other signals to receptors to mediate defense. Whatever the cause, this further strengthens the role of LTPs in pathogen defense.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Växter har varit en viktig del av människors utveckling sedan åtminstone den yngre stenåldern, för mer än 12000 år sedan, då människors livsstil ändrades från den tidigare nomadiska till en allt mer bofast. Allt eftersom befolkningsmängen i världen ökar så ökar också efterfrågan på växter, som människoföda, foder till djur, naturfiber, produktion av vegetabiliska oljor, med mera. Samtidigt ställs allt högre krav på att växterna ska vara odlade med så få ”kemiska tillsatser” och så ”naturligt” som möjligt. En av svårigheterna med att framställa tillräckliga skördar är förluster på grund av bakterier, virus och växtätande insekter och djur. Ett centralt problem för växter är att de är förankrade där de lever, och inte kan flytta sig till bättre platser om de utsätts för stress, såsom vattenbrist eller för att undvika växtätare och mikroorganismer.

Ämnen kallade prostaglandiner bildas i kroppen vid inflammation. Dessa hör till en grupp ämnen som kallas oxylipiner, på grund av att de produceras via oxidation (tillsättande av syre) av fettmolekyler (lipider). Växter producerar också oxylipiner, främst kopplade till försvar mot patogener, men de återfinns också bundna i membranlipider. I artiklarna 1-3 undersöker vi hur dessa bildas och vad som styr hur mycket som bildas och hur vanligt förekommande några av dessa är i växtriket.

I naturen finns mängder av bakterier, svampar och virus, som kan infektera växter och leda till sjukdom och död. Trots detta så är de flesta av växterna man ser utomhus friska och tillsynes opåverkade av den aggressiva omgivning de faktiskt lever i. Detta beror på att varje växtcell är utrustad med ett immunförsvar som kan känna av angrepp och aktivera försvarsmekanismer i form av giftiga substanser och strukturella förstärkningar för att motarbeta mikroorganismer. Ett mer specialiserat försvar är hypersensitiv respons (HR), som aktiveras om växten känner av speciella proteiner som den invaderande patogenen använder för att försöka bekämpa växtens immunsystem. Detta leder till att cellen offrar sig och dör. Poängen är att den döda cellen inte längre går att infektera och kan inte heller sprida sjukdom till friska celler i växten. När cellerna initierar denna programmerade celldöd så läcker de joner. Genom att infektera vävnad som ligger i vatten med bakterier för att sätta igång HR, kan läckaget mätas och användas för att jämföra skillnader mellan växter som saknar vissa proteiner för att se om dessa proteiner är inblandade i HR. Detta gör vi i artikel fyra, där vi undersöker om det finns kopplingar mellan HR och oxylipin-bildande enzymer.

Det finns speciella proteiner som bland annat fungerar som transportörer av lipider i växter, så kallade lipid transfer proteins (LTP), som också kopplats till försvarsmekanismer. Flertalet av dessa LTPs finns utanför cellernas membran, där svampar som kan infektera cellerna försöker ta sig in. Vi visar i artikel fem att vissa av dessa LTPs är inblandade i att öka skyddet mot en sådan invaderande svamp.

Tyvärr har flertalet av dessa proteiner också visats ligga bakom många matallergier, och eftersom de är mycket tåliga och både kan hettas upp och färdas genom matsmältningssystemet oskadda, kan de orsaka allvarliga allergiska reaktioner hos människor. Genom historien har människor odlat växter och mer eller mindre medvetet förändrat grödorna. Genom selektion av de växter som för oss såg bra ut, gav stor avkastning och smakade bra, har den naturliga selektionen pressats tillbaka för att våra preferenser ska styra grödornas utveckling. Det utvecklas ständigt mer effektiva metoder att ändra i växtgenomet till vår fördel, och det kanske är en lockande tanke att helt enkelt ta bort de substanser i växterna som kan orsaka allergier eller andra problem hos människor. Eller varför inte omvandla växter till levande fett/oljefabriker för industrin? Men samtidigt är det viktigt att betänka hur ändringarna påverkar växternas förmåga att försvara sig mot patogener och växtätande insekter och djur.

De arbeten som ingår i denna avhandling fyller några kunskapsluckor, och förhoppningsvis kan de bidra med fler frågor för framtida forskning och kunskap.

10 ACKNOWLEDGMENTS

När jag började läsa biologi, för sisådär hundra år sedan, så hade jag ingen aning om att jag skulle träffa så många underbara människor på Göteborgs Universitet (min fru exempelvis). Hur som helst så finns det ingen chans att jag kan få ner mina känslor för er alla här. Såklart kommer jag att klanta till det och glömma någon, och ber därför proaktivt om ursäkt och ber er läsa längst ner på sidan.

Min handledare **Mats Andersson**. Som Francesco en gång sa: det var inte mycket till papper innan Mats fick det (angående PLD-pappret). Det var verkligen inte mycket till någonting innan du fick det i dina händer. Ditt engagemang och driv är fantastiskt. Tack för att du gett mig utrymme för allt från personliga kriser till tokiga idéer men ändå kunnat ryta till när det behövts! **Henrik A**, min bonushandledare och ventil för överstressade dagar. Tack för all hjälp och för att du tålmodigt lyssnat! **Anders Nilsson** och **Oskar Johansson**. Ni två är helt otroliga. Jag har aldrig träffat två personer som är så drivna och kunniga. Jag kan bara tacka och bocka för ert engagemang och all hjälp. Utan er skulle denna avhandling inte kunna skrivas. Ett extra tack för att ni läst avhandlingen och kommit med mycket värdefull feedback! **Anders T**, jag vet inte vad jag gjort vissa dagar om jag inte kunnat gå och gnälla lite hos dig! Tack för allt stöd och hjälp på lab, det har alltid uppskattats! Även om du fick mig att skriva om hela avhandlingen… Doh!

Emelie L, som det varit en fröjd att dela känslor och kontor med. Tack för ditt stöd och alla bra argumentationer, och tack för att du är rak nog att kunna be någon (ehm) hålla käften vid behov. Du är helt underbar! **Karin J** och **Jenny E**, ni är fantastiska. Som alltid får en att känna sig bättre vad man än må ha för problem. Jag hoppas att jag kunnat bidra med en gnutta glädje tillbaks! Jag kan nog vara skyldig Karin en lunch för den delen. **Lisa A**, som är en av mina äldsta vänner i Göteborg och en av få som har sett mig ”dansa”. Jag hoppas du inte tänker utpressa mig med det. **Per S**, den snällaste personen söder om Treriksröset. Tack för alla samtal och givande stunder. Ibland är humor det nördigaste som finns, och du och jag är helt klart någon sorts separation av tvillingar med defekt humor. May the force be with you. **Sven**, utan dig skulle ingenting fungera på Botaniska. Vad de än betalar dig så är du värd mer. **Björn**, **Henrik N**, **Ola**, **Åsa**, **Britt**, **Åslög**, **Jenny**, **Ellen** och alla andra underbara människor som jag överröstat under många luncher, jag kommer att sakna er! Thanks also to **Andrei**, **Somnath**, **Olga** and **Bernard** for all the fun discussions.

Min fru **Lotta** och mina barn **Wilma** och **Simon**, tack för att ni gör livet värt att leva. Till mina biologiska syskon **Pauline** och **Erik** och mina bonussyskon **Frida**, **Helena** och ”lilla” **Carina**, tack för att ni finns! **Laila** och **Lars-Rune**, sedan länge har jag sett er som föräldrar och vänner. Utan er hade jag aldrig hamnat på västkusten och mitt liv hade varit helt annorlunda. Det finns inga ord som räcker till, så jag får väl helt enkelt säga tack för allt!

Till (alla) er jag glömt att nämna, och som känner sig lite ledsna över det: hör av er så tar vi en öl eller två. Det är bättre än lite text på en sida i en avhandling som ändå ingen kommer att läsa.

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