Dissecting Arabidopsis phospholipid signaling using reverse genetics

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Chapter 1

**General introduction:**

**Phospholipid signaling in plants**

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1. Introduction

Living cells delimit themselves from their surroundings by a biological membrane. This biological membrane is essential to maintain chemical gradients between the intracellular and extracellular environment and leakage would quickly be followed by death. The structural integrity of the membrane is the result of the biophysical properties of its primary building blocks, the phospholipids. Phospholipids have two hydrophobic fatty acid tails and a hydrophilic headgroup, held together by a simple glyceryl backbone. When in aqueous solution, phospholipids spontaneously form a bilayer with their hydrophobic tails facing each other and their hydrophilic headgroups forming hydrogen bonds with the water molecules in the solution. The result is a barrier that is impermeable to large and charged molecules. In addition to preventing molecules to leave or enter, the membrane is also involved in communication between the outside world and the inside of the cell. These two functions are reflected by the composition of the lipid bilayer. The majority of the phospholipids have a structural role and their concentrations are relatively constant. However, a minority of the phospholipids rapidly turn over in response to various external stimuli. In mammals, the role of phospholipids in intracellular signaling is well described. For plants, evidence for such roles is emerging, although differences are also found. Plant phospholipid signaling is the subject of my thesis. In the first two sections of the general introduction, phospholipid biosynthesis will be described, as well as the different species of phospholipids and their mode of action. In the third section we will focus on the available techniques to study plant phospholipid signaling and our model organism, Arabidopsis thaliana will be introduced. In the final sections of this introduction, an overview will be given of several biological processes that have been associated with phospholipid signaling. These sections form a bridge to the four experimental chapters of this thesis. In the last chapter, selected findings are discussed within a broader context.
2. Phospholipidology

2.1 Phospholipid biosynthesis

Phospholipid biosynthesis takes predominantly place at the endoplasmic reticulum (ER) and starts by acylation of the non-lipid molecule glycerol-3-phosphate (G3P), resulting in lyso-phosphatidic acid (L-PA) (Fig. 1). A second acylation step yields phosphatidic acid (PA). PA is the branchpoint of two lipid biosynthetic pathways, leading to the formation of phosphatidylinositol (PI) or phosphatidylglycerol (PG) on the one hand and phosphatidylcholine (PC) or phosphatidylethanolamine on the other hand [1]. The committed step of the latter pathway is the dephosphorylation of PA to diacylglycerol (DAG) by PA-phosphatase (PAP). The next step is the addition of a headgroup onto DAG, forming either PC or PE. The other pathway starts by adding a cytidine monophosphate (CMP) moiety onto PA, forming CMP-PA. CMP can subsequently be exchanged for glycerol yielding PG or inositol, yielding PI. PI is a structural phospholipid that is abundantly present in the membranes of plant cells but is also the precursor of a class of molecules that are thought to have a signaling function. Chloroplasts and mitochondria have their own phospholipid biosynthesis but this is beyond the scope of this thesis.

2.2 Phospholipid signaling

In plants, PI can be phosphorylated at 2 positions of the inositol ring by specific PI-kinases (PIK) forming either PI3P or PI4P [2, 3]. PI3P levels are relatively low in plants (2-15% of the total PIP pool) and PI3P is predominantly localized on late endosomes and prevacuolar compartments [4-7]. PI4P has been found at the plasma membrane and the golgi apparatus in Arabidopsis. Interestingly, a tip-focussed gradient of PI4P was present at the plasma membrane in growing root hairs and a
massive accumulation of PI4P on the cell plate during cytokinesis was observed (Vermeer et al., submitted). Disruption of the two closely related Arabidopsis PI-4-OH kinases PI4Kβ1 and PI4Kβ2 resulted in aberrant root hairs and smaller plant size [8], suggesting an important role for PI4P during growth and development. PI4P can be phosphorylated to Pi(4,5)P2 by PIP-kinase activity [3]. When 32P-labeled, PIP2 levels of Arabidopsis are low, but after osmotic stress, PIP2 levels increase spectacularly [9]. In mammals, Pi(4,5)P2 is important for a variety of cellular events [10] and it is the precursor of three other signaling molecules, DAG, inositoltriphosphate (IP3) [11] and Pi(3,4,5)P3 [12], although the latter is only found in animals. In plants, Pi(4,5)P2 can be metabolized by two different enzymes, phospholipase C (PLC) and PIP2-phosphatases. When Pi(4,5)P2 is dephosphorylated, both PI4P and PI5P can be formed, depending on the specificity

Fig. 1. Phospholipid biosynthesis and signaling. See text for details.
of the PIP$_2$-phosphatase [13, 14]. PI5P constitutes only a minor fraction of the total PIP pool (3-8%) [15, 16] and its function and localization are still unknown. Some mammalian lipid kinases were reported to phosphorylate PI at the D-5 position \textit{in vitro}, but such an activity has not been described for plants [17, 18].

PLC acts by cleaving off the inositol headgroup from PI(4,5)P$_2$, thereby leaving a DAG molecule in the membrane and releasing water-soluble IP$_3$ into the cytosol [11]. In mammals, these molecules are important second messengers. IP$_3$ binds to IP$_3$-receptors on the ER where it triggers the release of Ca$^{2+}$ into the cytosol and DAG activates protein kinase C that in turn activates a protein phosphorylation cascade. These pathways are apparently not conserved in plants, as no IP$_3$-receptors, nor PKC homologues have been found. IP$_3$ can be dephosphorylated or further phosphorylated all the way up to IP$_6$ [19, 20]. Interestingly, the latter was shown to bind to the auxin receptor TIR1 [21]. At this point, it is not clear whether this binding serves a regulatory or structural role. DAG does not appear to have a signaling function in plants but it can be quickly phosphorylated by diacylglycerol kinase (DGK) to form PA [22]. PA can also be formed by the action of phospholipase D (PLD) that hydrolyzes PE and PC [23]. Thus, PA is not only a key intermediate of phospholipid biosynthesis [1], it is also the product of both the PLC/DGK- and PLD-pathway. Interestingly, PA has been proposed to have a signaling function in plants [24]. Activation of PLC/DGK and/or PLD pathway could potentially serve to control appropriate PA levels in different membranes. In the next section the downstream targets of PA and PIP$_2$ will be discussed.
3. Downstream targets of phospholipids

Phospholipid signaling in mammalian cells is a well-established field, whereas the equivalent in plant is very much in development. Therefore, more downstream targets of phospholipid second messengers have been identified in mammalian cells than in plants. To see what kind of mechanisms we can expect to find in plants, a few examples from the mammalian and the yeast literature will be discussed first.

3.1 Mammalian and yeast lipid-binding proteins

In addition of being a precursor of other signaling molecules [11], PIP\(_2\) has been shown to regulate a plethora of cellular events [10]. The predominant mechanism by which PIP\(_2\) exerts its effect is by binding to proteins via lipid-binding domains, of which the pleckstrin homology (PH) domain of human PLC\(_\alpha1\) is the best characterized [25]. Binding of PIP\(_2\) to proteins functions to spatially concentrate the target protein and/or to modify its activity [10]. The latter is exemplified by PLD, which requires PIP\(_2\) for activity [26] or by ion channels that are regulated by direct interactions with PIP\(_2\) [27, 28]. Recruitment of target proteins to the membrane can result in cytoskeletal rearrangements and membrane trafficking. Several proteins that bind to actin have been shown to bind to PIP\(_2\) as well. In this way, localized production of PIP\(_2\) can lead to changes in cell architecture [29, 30]. In a similar manner, Adaptor protein 180 has been shown to bind to PIP\(_2\) and clathrin simultaneously. PIP\(_2\) could orchestrate membrane trafficking events by serving as a docking site for the endocytotic machinery [31, 32]. Another interesting function of PIP\(_2\) is highlighted by its binding to the transcriptional regulator TUBBY. Upon PIP\(_2\)-hydrolysis, TUBBY is no longer retained at the membrane and translocates to the nucleus where it can regulate gene expression [33].
In animal cells, most of the PA involved in signaling seems to be derived from PLD [34, 35]. DGK also generates PA, but this is believed to predominantly attenuate DAG signaling [36, 37]. PLD activity is strictly regulated by several proteins [38] and by PIP₂ [26]. The PA produced by PLD is involved in mitogenic signaling, vesicular trafficking and the oxidative burst [24, 35]. PA formation might function by altering the biophysical properties of the membrane, potentially facilitating membrane trafficking events [39]. In addition, PA binds several signaling proteins and in some cases modifies their activities. The targets of PA include protein kinases and phosphatases, lipid kinases and other signaling proteins, such as small G-proteins and cAMP phosphodiesterase [24]. In contrast to PIP₂ binding proteins, no consensus PA-binding domain is known yet [40]. The best-known PA target is probably Raf-1 kinase. Raf-1 kinase is recruited to the membrane by binding to PA, where it is activated by other proteins [41, 42]. The in vivo relevance of this mechanism was shown by expression of a dominant negative Raf-1 kinase that could not bind PA, resulting in developmental abnormalities in zebrafish [43]. Another target of PA is a type I PIP kinase [44]. This target is of particular interest because PA stimulates its activity, resulting in the formation of PIP₂, which in turn stimulates PLD activity. Together, type I PIP kinase and PLD could form a positive feedback loop, potentially serving as a signal amplifier. Recently, growth factor signaling through receptor tyrosine kinases was shown to be dependent on PA-mediated membrane targeting of SOS (Son of sevenless) [45].

In the Yeast Saccharomyce cerevisiae, an interaction between PA and the transcriptional regulator Opi1p has been described that functions in a similar way as PIP₂ binding to TUBBY [33]. Opi1p was found to shuttle between the nucleus and the ER, depending on the PA concentration at the ER [46]. PA concentrations in Yeast are high when inositol concentrations are low. When inositol becomes available, it is rapidly incorporated in phospholipids at the expense of PA, causing Opi1p to dissociate from the ER and to enter the nucleus where it represses target
3.2 Plant lipid-binding proteins

Homology searches have revealed the presence of 53 genes that contain regions with similarity to PH domains in the Arabidopsis genome [47]. However, binding specificity of these PH domains should be determined experimentally, as not all PH domains bind to PIP$_2$ and some do not bind lipids at all. PDK1 (3-phosphoinositide dependent protein kinase 1) is the only Arabidopsis protein with a PH domain for which lipid binding specificity has been determined. By incubating a nitrocellulose membrane-fillyer on which various lipids were immobilized with recombinant PDK1, followed by immunoblotting (called lipid overlay) it was shown that PDK1 binds various phosphoinositides, including PIP$_2$ but also PA [48]. Interestingly, these two phospholipids were also found to stimulate PDK1 activity in vitro [49]. PDK1 can phosphorylate many but not all members of the large AGC family of protein kinases [50]. The AGC family consists of 39 members and is subdivided into 5 subfamilies [51], of which the AGC VIII subfamily is the best characterized. One of the PDK1 targets belonging to this subfamily is PINOID (PID) [52]. PID phosphorylates the auxin-efflux transporters PINFORMED1 (PIN1) and PIN2, and thereby regulates their proper cellular localization [53]. Mutations in the PIN1 gene lead to a pinformed phenotype due to disturbed polar auxin transport [54, 55]. pid mutants have a similar phenotype, hence the name pinoid [56-58]. PID is directly phosphorylated by PDK1, providing a link between polar auxin transport on the one hand and phospholipid signaling on the other [52] (Fig. 2). Moreover, PID was reported to localize to the cell periphery [51] and to bind to a variety of phosphoinositides and PA as judged by lipid overlay analysis [50].

genes, among which the $INO1$ gene which encodes for the rate limiting step in inositol formation.
Another member of the AGC VIII subfamily that is phosphorylated by PDK1 is AGC2-1. AGC2-1 is identical to OXI1, a gene that was found to be induced by oxidative stress [59]. Interestingly, phosphorylation of OXI1 was stimulated by PA [49], suggesting that PA can influence downstream signaling events. PDK1 activity could also be stimulated by xylanase [60], a fungal elicitor known to induce PA
formation in tomato cells [61]. This stimulating effect could be blocked by n-
butanol, a competitive inhibitor of PLD activity, but not by U73122, a PLC
inhibitor. OXI1 activity itself was also stimulated by xylanase, although to a lesser
extent than PDK1. Downstream of OXI1 is the protein kinase PTI1-2, which was
also activated by xylanase in Arabidopsis cells [60]. This activation was dependent
on PDK1 and OXI1, as deduced from silencing of PDK1 and OXI1 [60].
Interestingly, PTI1-2 resembles the tomato Pti kinase that has been implicated in the
hypersensitive response (HR), a strong defense response against pathogens. The
importance of OXI1 for defense against pathogens was demonstrated by enhanced
susceptibility of an ox1 mutant against the Hyaloperonospora parasitica strain
Emco5 [59]. In addition, ox1 showed less activation of MPK3 and MPK6, two map
kinases that have been associated with disease resistance signaling [62, 63].

Recently, CTR1 (Constitutive Triple Response) was found to bind PA [64]. CTR1
is an homologue of human Raf-1 kinase, and negatively regulates ethylene
responses. Since PA inhibited the kinase activity of CTR1, PA may be a positive
regulator of ethylene responses [65]. CTR1 is localized at the ER [66] but PA can
be formed by multiple pathways with different subcellular localizations. Therefore,
it is not clear how PA-mediated CTR1 inhibition would be regulated.

PA was reported to inhibit the protein phosphatase 2C (PP2C) ABI1 (ABA
insensitive). Because ABI1 is a negative regulator of ABA responses [67],
inhibition by PA would decreases ABI1 activity, hence positively regulating ABA
responses [68].

A proteomic screen for PA binding proteins resulted in the identification of
additional PA targets, including RCN1 (Roots Curl in NPA), SnRK2.10 [69] and
SnRK2.4 (C. Testerink, personal communication). The SnRK2 (Sucrose non
fermenting Related Kinase 2) family of protein kinases contains ten members, of

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which SnRK2.6/SRK2E/OST1 is the best characterized [70]. SnRK2.6 activity is stimulated by ABA [71] and snrk2.6 mutants are affected in stomatal closure [72]. All SnRK2 isoforms except SnRK2.9 are activated by hyperosmotic stress [73]. Consistently, transgenic lines that over- or underexpress SnRK2.8/SRK2c display enhanced and reduced salt tolerance respectively [74].

RCN1 was originally identified in a screen for altered responsiveness to an inhibitor of auxin efflux, naphthalphthalamic acid (NPA) [75, 76]. RCN1 is also called PP2AA1, because it is a member of the family of regulatory subunit A of PP2A [77]. RCN1 has also been associated with ABA [78] and ethylene responses [79]. Interestingly, RCN1 and CTR1 both interact with the PP2A catalytic subunit 1C in vitro [79]. RCN1 and its closest homologues PP2AA2 and PP2AA3 were shown to be important for embryo development [77]. Recently, these phosphatases were shown to regulate the phosphorylation status of PIN1 and PIN2, antagonizing PID [53]. Taken together, these data suggest a role for phospholipid signaling in modulating responses to various phytohormones and to pathogens (summarized in Fig. 2).
4. Techniques to study phospholipid signaling in plants

So far, phospholipid signaling has mainly been studied mostly by biochemical and pharmacological approaches, using various plant species. Genetic approaches are feasible when using the model plant Arabidopsis. Here, a brief overview of the available techniques is given.

4.1 Biochemical approaches

A straightforward way to study phospholipids is to extract them from a plant and quantitatively measure the different phospholipid species. Traditionally, the identification of phospholipids is performed after separation by thin layer chromatography (TLC) and/or HPLC. The introduction of mass spectrometry has increased the sensitivity and also allows the detection of different fatty acid species [80, 81]. Unfortunately, phosphoinositides cannot be detected by mass spectrometry yet, therefore limiting the use of this method to study phospholipid signaling [81].

Phosphoinositides can, however, be detected by isotopic labeling with $^{32}$PO$_4^{3-}$. Moreover, labeling studies can give information about the direction and rate of metabolic fluxes. Cell suspensions are suitable for these studies because they are more or less homogeneous and label in a much more synchronized manner. A detailed investigation of phospholipid turnover has been performed with the unicellular green alga *Chlamydomonas moewussi* [6]. When labeled with $^{32}$PO$_4^{3-}$, the ATP-pool is labeled first, followed by the phospholipids that are produced by lipid kinases. These include phosphorylation of PIP by PIK, PIP$_2$ by PIPK, PA by DGK, and diacylglycerol pyrophosphate [82] by PA-kinase. Later, the label enters the phospholipid biosynthetic pathway via G3P and via the biosynthesis of phosphorylated headgroups [1], ultimately leading to labeling of the structural phospholipids. These differences in labeling kinetics can used to distinguish
between DGK- and PLD-mediated PA formation. When labeling only for a short period of time, no structural phospholipids are labeled, therefore excluding PLD activity as the origin of $^{32}$P-PA [6]. In this thesis, relative amounts of labeled phospholipids were determined by dividing their radioactivity by the total amount of labeled phospholipids.

4.2 Pharmacological approaches

Pharmacological interference has been widely used to establish the contribution of phospholipid modifying enzymes to cellular responses. PLD activity can be conveniently monitored by the unique ability of PLD to use primary alcohols as acceptor for the phosphatidyl moiety. Under physiological conditions, water is transferred to the phosphatidyl moiety and PA is formed. When primary alcohols, such as 1-butanol are added, PLD can use them as acceptor as well, resulting in the formation of the artificial phospholipid phosphatidylbutanol (PBut). Since PBut has different chromatographic properties than PA, it can be easily separated and quantified, providing information about the relative contribution of PLD to PA formation. Because butanol is an alternative PLD substrate, it can be considered as a competitive inhibitor of PLD-mediated PA formation. However, butanol stimulates PLD activity as well, therefore raising the question whether PBut formation truly reflects PLD activity. In addition, butanol has been shown to interfere with the integrity of the cytoskeleton, presumably via PLD [83]. Therefore, the usage of butanol as an inhibitor of PLD activity is unadvisable when looking at long-term effects, as the collapse of the cytoskeleton is likely to have effects on overall cell biology.

PLC activity has been commonly manipulated by addition of the inhibitor U73122, although its effects are controversial. As a control, the inactive analogue U73343 is often used but several reports describe a similar effect observed with both
compounds, indicating that other processes are targeted than PLC [84-88]. In a comprehensive study, it was shown that some effects of U73122 are attributable to alkylation of various proteins [89]. PLC activity can be inhibited by neomycin as well. However, because neomycin binds to PIP₂, it potentially affects PLC-independent PIP₂-related processes [90].

In general, data obtained with inhibitors should be accompanied by data that support their specificity. In particular when long-term effects, such as gene expression, are studied, evidence based solely on inhibitors should be interpreted with care.

4.3 Genetically encoded phospholipid biosensors

An exciting development in the field of plant phospholipid signaling is the introduction of genetically encoded biosensors [91, 92]. These biosensors consist of a fluorescent protein fused to a protein domain that binds a phospholipid. Depending on the affinity of the sensor for the lipid and the concentration of the lipid in the membrane, the biosensor will either be cytosolic or bound to the lipid in the membrane. Translocation of the biosensor provides spatio-temporal information about the concentration of the phospholipid. In plants, biosensors have been successfully applied to study the localization of PI3P, PI4P, PI(4,5)P₂ and DAG by expressing respectively the FYVE domain of the FAPP protein (Vermeer et al., submitted), the PH domain of human PLCδ1 [93] and the C1a domain of PKCγ [94, 95] respectively. These biosensors have the potential to overcome most of the limitations that are associated with the traditional biochemical and pharmacological approaches. PI3P levels for instance, are low and difficult to distinguish from PI4P. Moreover, by extracting lipids, information about their spatial distribution is lost. By using fluorescent proteins with different spectral properties, multiple biosensors can be monitored simultaneously in a single living cell.
4.4 Forward and reverse genetics

Classical or forward genetic approaches depend on genetic variation, either naturally occurring or induced chemically, physically or biologically. When the genetic variation results in a phenotype, it is possible to link the phenotype to the responsible gene by map-based cloning [96]. The availability of the Arabidopsis genome sequence [97] has significantly shortened the time required for map-based cloning. Forward genetic approaches have proved to be extremely successful to identify genetic determinants for a large number of biological processes. However, no phospholipid-modifying enzyme has been identified by screening for phenotypes in disease resistance or salt tolerance.

The availability of the Arabidopsis genome sequence [97] has allowed the development of several reverse genetic tools for Arabidopsis. These tools are based on the generation of a large pool of mutagenized individuals by radiation [98], Agrobacterium tumefaciens-mediated transferred DNA (T-DNA) insertion [99, 100] or by transposon tagging. By screening pools of DNA with gene specific primers (for deletions) or an insertion specific primer in combination with a gene specific primer (for insertions), it is possible to identify mutations in any gene of interest. These tools have been developed further by high throughput sequencing of the junctions between T-DNA insertions and genomic DNA, resulting in gene-indexed insertion libraries [101, 102].

These insertion collections allow the systematic study of the genes encoding phospholipid-modifying enzymes. The Arabidopsis genome contains 9 \( PLC \) [3, 103], 7 \( DGK \) [22] and 12 \( PLD \) [23] genes. All \( PLC \) genes contain an EF-hand domain [104], followed by an X-, Y- and a C2-domain [3]. The EF-hand may serve a structural role by interacting with the C2 domain. The C2 domain may be important for membrane interaction and the X- and Y-domain are required for
catalytic activity. Arabidopsis PLC8 and PLC9 may be inactive, because substitutions are present in their catalytic domain. It is also doubtful whether PLC7 encodes a functional PLC because the only cDNA isolated contained an early stop codon, as a result of alternative splicing [103]. Of the remaining six PLCs, PLC1-5 have been shown to have hydrolytic activity towards PIP$_2$ in vitro [103].

The seven DGK genes all contain a DGK catalytic domain [22]. DGK1 and DGK2 contain an extra N-terminal domain, which is predicted to be a transmembrane helix and targets them to the ER upon overexpression [105]. Only DGK2 and DGK7 have been shown to have DGK activity in vitro [22, 106].

The Arabidopsis genome encodes 12 PLDs which can be divided into two subfamilies, based on the N-terminal lipid-binding domain. PLD$_{\alpha 1}$ and PLD$_{\alpha 2}$ contain a Phox- and a PH-domain, whereas the other 10 PLD genes contain a C2 domain. These PLD genes can be further subdivided based on sequence homology and in vitro activity requirements [23]. The predominant activity in plants originates from PLD$_{\alpha 1}$ [107] and PLD$_{\delta}$ [108]. The $\alpha$-class PLDs are active at acidic pH and require high concentrations of Ca$^{2+}$ [107]. The plasma membrane localized PLD$_{\delta}$ is stimulated by the addition of oleate, and also requires high Ca$^{2+}$ concentrations, although lower than the $\alpha$ class [109]. In the next sections, several biological processes in which phospholipid signaling is implicated will be described.
5. The Arabidopsis-Pseudomonas pathosystem

Plants have evolved multiple lines of defense against pathogens [110]. The first line of defense depends on the recognition of conserved parts (Pathogen-Associated Molecular Patterns; PAMPs) of the pathogen by specific receptors [111]. When these receptors are activated, downstream responses are triggered which lead to transcriptional reprogramming and ultimately to an effective defense response, also known as PAMP triggered immunity (PTI) [110]. A successful pathogen would have to change its PAMPs in such a way that they are no longer recognized, thus remaining undetected and evading the defense response. Plants, in turn, have responded over evolutionary time by changing their receptors. In this evolutionary arms race, pathogens are at a disadvantage because they have less ‘evolutionary freedom’ to change PAMPs without affecting their fitness, whereas changes in receptors are without major consequences for the plant.

As PAMPs allow limited evolutionary change, pathogens had to evolve drastic measures to counteract the alarm system of the plant. Such an adaptation is the bacterial type III secretion system (TTSS) that injects several bacterial proteins into the plant cell. The function of these effector proteins is to manipulate the plant to provide a more favourable habitat and importantly, suppress the plant defense responses. This process has been called effector-triggered susceptibility (ETS) [110, 112, 113].

The plant in turn has responded by evolving intracellular receptors that detect the presence of the effectors. However, the roles in this evolutionary arms race seem to be reverted now, because there are no restraints on the possible variation of pathogen effectors. Moreover, the pressure is on the plant to keep up with the novel adaptations of the pathogen. For this reason, it was proposed that a strategy in which the plant has an intracellular receptor for every effector, would always fail. Instead,
the plant should not detect the effectors directly but their virulence function. According to this ‘guard hypothesis’, the intracellular receptors should trigger a defense response, whenever an effector manipulates a plant protein. In this way, effector molecules can only escape detection by losing their virulence function. Substantial molecular evidence for the guard hypothesis has emerged over the last years and the most detailed example of the signal transduction pathways leading to this effector-triggered immunity (ETI) [110] will be discussed. Before that, an overview of the combined effects of PTI and ETS will be given. At the end of this section, the involvement of phospholipids in these responses will be discussed.

5.1 Basal disease resistance

Basal disease resistance can be viewed as PTI minus ETS. The paradigm for PTI is the detection of the *Pseudomonas syringae* protein flagellin by the Arabidopsis receptor kinase FLS2 [111]. Upon recognition of the flagellin-derived peptide flg22, FLS2 undergoes endocytosis [114] leading to the accumulation of salicylic acid (SA) [115], callose deposition [116], MAPK activation [116, 117] activation of WRKY transcription factors [62] and consequently transcriptional reprogramming [118].

Pathogenic bacteria inject approximately 20-30 effectors into the plant cell in order to suppress these defense response [119]. The dramatic effect that pathogen-derived effectors have on susceptibility of a plant is demonstrated by the inability of *Pseudomonas* mutants lacking a functional TTSS to cause disease. Virulence of these mutants can be rescued by *in planta* expression of effectors [116].

Plant defense responses against biotrophic pathogens such as *Pseudomonas syringae* and *Hyaloperonospora parasitica* are strongly dependent on SA [120] (Fig. 3). SA by itself is sufficient to induce strong defense responses by inducing
Fig. 3. PAMP-recognition leads to immunity and to PA formation

Flg22-perception by the receptor kinase FLS2 results in various downstream responses, including DGK-mediated PA formation and accumulation of SA. The latter is dependent on the signaling protein PAD4 and the biosynthetic enzyme SID2. SA accumulation results in the monomerization of NPR1, followed by its translocation into the nucleus. There, NPR1 interacts with various transcription factors that regulate the expression of pathogenesis related genes and subsequently PTI. The contribution of DGK-mediated PA formation to PTI has not been established yet.

defense gene expression such as \textit{PR1} and \textit{BGL2/PR2} [121]. Mutational analyses have led to the isolation of mutants with strong enhanced disease susceptibility phenotypes that are defective in SA accumulation or SA responsiveness [122, 123]. The \textit{pad4} mutant is defective in a regulatory gene that functions in a signal amplification loop that leads to the accumulation of SA in response to infection [124, 125]. \textit{sid2} mutants are defective in the \textit{ICS} (isochorismate synthase) gene that
is required for SA biosynthesis [126, 127]. SA accumulation is perceived by NPR1/SAI1/NIM1, a protein that was identified by screening for mutants that did not express \textit{pBGL2::GUS} in response to SA or its analogue INA [121, 128-130]. Uninduced, NPR1 forms homodimers via some of its cysteyl residues and resides in the cytosol. Accumulation of SA results in changes in the redox potential that breaks the sulfur bonds between cysteines, causing NPR1 to monomerize [131]. Monomeric NPR1 translocates into the nucleus [132], where it interacts with TGA transcription factors to regulate the transcription of numerous defense related genes, among which \textit{PRI} [133-136] (Fig. 3).

5.2 Effector-triggered immunity

ETI is the defense response that follows upon recognition of an effector. Strong ETI can lead to plant cell death, also known as hypersensitive response (HR). The most detailed example of ETI is the Arabidopsis RPM1-mediated immunity (Fig. 4). RPM1 can be activated by the Pseudomonas effector protein AvrRpm1. As recognition of AvrRpm1 results in a strong resistance response, AvrRpm1 is called an avirulence protein. Absence of either AvrRpm1 or RPM1 results in susceptibility (PTI-ETS). RPM1 is a CC-NB-LRR (Coiled-Coil Nucleotide Binding Leucine-Rich-Repeats) Resistance (R) protein [137, 138] that resides at the plasma membrane [139]. AvrRpm1 is a small protein that undergoes N-terminal myristoylation upon its delivery in the plant cell [140]. As predicted by the guard hypothesis, RPM1 does not interact directly with AvrRpm1. Instead, both RPM1 and AvrRpm1 interact with RIN4 (RPM1 interacting 4) [141]. AvrRpm1 induces the phosphorylation of RIN4, which triggers RPM1 activation. As expected, AvrRpm1 enhances the virulence of pseudomonas on \textit{rpm1} plants [142]. In fact, RIN4 is also targeted by AvrRpt2, although this perturbation is not detected by RPM1 but by another R protein, RPS2 [143, 144]. AvrRpt2 is a cysteine protease [145] that is activated in the plant and cleaves RIN4 which leads to activation of RPS2 [146].
Fig. 4. Recognition of AvrRpm1 by RPM1 leads to immunity and PA formation. Several Pseudomonas effector proteins, including AvrRpm1 are injected into the plant cell and cause virulence (ETS). AvrRpm1 induces the phosphorylation of RIN4, which betrays its presence to RPM1. RPM1 activation leads to a strong resistance response (ETI). One of the downstream responses of RPM1 activation is PLD-mediated PA formation but its contribution to ETI has not been established yet.

In a near-saturating screen for mutants that fail to respond to conditional expression of AvrRpm1, many (95) rpm1 alleles were found [147]. Unexpectedly, the remaining mutants fell in two complementation groups only. The largest one (eight alleles) was found to be allelic to pbs2 (avrPphB susceptible) and rar1. The other complementation group were shown to be mutated in HSP90.2 [148]. pbs2 and rar1 were identified in screens for loss of RPS5 mediated resistance [149] and for loss of
resistance against the avirulent *H. parasitica* isolate Noco2 respectively [149, 150]. Additional testing revealed that RAR1 was required for the function of many but not all R proteins. It was found that RPM1 protein levels were reduced in *rar1* mutants, indicating that RAR1 is important for R protein stability or accumulation [151]. Reduced RPM1 levels were also observed in the *hsp90.2* alleles, although resistance mediated by other R proteins was not affected [148]. The *hsp90.2* alleles carried rare (4, opposed to 95 *rpm1* alleles) missense mutations in the conserved ATPase domain of HSP90.2. In summary, both RAR1 and HSP90.2 function in maintaining appropriate levels of RPM1. Despite the high extent of saturation, no downstream components of RPM1 signaling were identified in this screen. Apparently, these components are either essential for viability or act redundantly.

Downstream events of RPM1 activation have been studied mainly by biochemical methods. These include Ca2+ elevations [152], the production of reactive oxygen species [153] and the formation of PA [154] (Fig. 4).

### 5.3 Pathogen-induced changes in phospholipid composition

Early studies showed that suspension-cultured soybean treated with the elicitor polygalacturonic acid responds with a transient increase in IP3, which was accompanied by a decrease in PIP2 [155]. These responses could be blocked by neomycin, suggesting that they were mediated by PLC. A decrease in 32P-PIP2 labeling was also observed when tomato cells were treated with the bacterial PAMP flg22, suggesting activation of PLC [156]. Moreover, an increase in the formation of PA in response to flg22 was observed, which was shown to be derived from DGK activity, using a differential 32P-labeling protocol (Fig. ). No contribution of PLD to flg22-induced PA formation could be detected as judged by the absence of PLD-mediated transphosphatidylation of propanol. These results suggest that PAMPs activate a PLC/DGK pathway in plants.
The involvement of phospholipid signaling in ETI has been studied using tobacco BY-2 cells that heterologously express the tomato resistance protein Cf-4. When treated with the *Cladosporium fulvum* avirulence protein Avr4, these cells responded by producing PA [157]. Using the short-labeling strategy, DGK was shown to be involved, whereas no PLD-mediated transphosphatidylation was observed. The formation of PA could be inhibited by U73122, indicating that PLC-mediated DAG formation might precede DGK-mediated PA-formation. The avirulence protein xylanase also induces PA-formation in tomato cells [156]. In this case, both DGK and PLD activity contributed to PA formation. The involvement of PLD was further supported by experiments in which tomato *PLDβ1* was silenced [61]. These transgenic cell-lines displayed less xylanase-induced PA formation. Not only do these genetic experiments complement data obtained by biochemical methods, they also can inform us about individual members of the large families of genes involved in phospholipid signaling. This resolution is hard to obtain with biochemical methods alone.

Recently, the study of phospholipid signaling during ETI was extended to Arabidopsis. Using transgenic plants that conditionally express AvrRpm1, it was shown that RPM1-activation results in PA formation [154] (Fig. 4). The formation of PA was accompanied by the disappearance of the PLD substrates PC and PE. A role for PLD during this PA response was further supported by reduced PA formation in the presence of 1-propanol, which acted as a competitive inhibitor in this assay (explained in 3.2). This study demonstrates that Avr-induced phospholipid signaling can be studied using whole Arabidopsis plants.
6. Salt stress signaling in Arabidopsis

Forward genetic screens have identified three sos (salt overly sensitive) loci that significantly contribute to salt tolerance in Arabidopsis [158, 159]. SOS1 encodes a plasma membrane localized Na⁺/H⁺-antiporter that presumably functions to keep cytoplasmic Na⁺-concentrations low [160]. SOS1 is phosphorylated by the protein kinase SOS2 [161] which is dependent on its interaction with the membrane-localized Ca²⁺-binding protein SOS3 [162, 163]. SOS2 belongs to the large SnRK family of protein kinases. At least one other SnRK protein, the SnRK2.8/SRK2c isoform is involved in salt tolerance [74]. Interestingly, SnRK2.4 and SnRK2.10 were identified in a proteomic screen for PA-binding proteins [69] (Christa Testerink, personal communication). Moreover, SOS2 and other SnRK isoforms were found to interact with the protein phosphatases, ABI2 and/or ABI1 [164]. ABI1 (and possibly ABI2) binds to PA [68], suggesting that these phospholipids serve to spatially concentrate signaling proteins and their binding partners.

Although the major determinants of Arabidopsis salt tolerance have been identified, it is still not clear how salt stress is actually perceived. Possibly, salt stress alters the physical properties of membranes, which are sensed and translated into a downstream response. Several studies have shown that NaCl induces a rapid change in the composition of phospholipids, including the accumulation of PIP₂ [9, 165] and PA [166]. The NaCl-induced formation of PA in tomato cells was attributed to DGK activity based on experiments using the differential labeling protocol (explained in 3.2) [166]. In Arabidopsis cells and seedlings, the NaCl-induced formation of PIP₂ is accompanied by IP₃. [165, 167]. When treated with U73122, NaCl triggered less IP₃ formation while PIP₂ accumulated to even higher levels, suggesting that PLC was activated by NaCl [167]. NaCl-induced PIP₂ accumulation was further demonstrated by the translocation of cytosolic PHPLC1::GFP (explained in 3.4) to the plasma membrane [93]. Possibly, PIP₂ and/or PA function by
recruiting or activating cytosolic proteins. E.g., the phospholipid binding proteins PDK1, SnRK2 and ABI1 could function in such a signal signalosome complex. Alternatively, enhanced formation of PIP₂ might influence the activity of ion channels such as SOS1, which has an important role in salt tolerance.
7. Auxin

The phytohormone auxin regulates a wide variety of responses such as organogenesis and tropic growth [168-170]. A central question in auxin biology has been how one molecule can induce such a diversity of responses. Key to answering this question is the observation that auxin is actively distributed, leading to localized concentrations of auxin. The majority of auxin is synthesized in apical tissues and transported via the phloem to basal tissues. Here, every cell expresses a unique set of polarly localized auxin influx and efflux carriers, ensuring a directional movement of auxin from cell to cell. The resulting local patterns of auxin distribution are translated into the expression of a large number of target genes [21, 171-173].

7.1 Auxin influx

Auxin influx is mediated by members of the LAX (Like AUX1) family of transmembrane amino acid permeases [174-176]. The LAX family consists of 4 members of which AUX1 was the first to be identified [177]. AUX1 is expressed in the phloem, columella, the lateral root cap and epidermal cells of the root apex [178]. aux1 mutants were isolated based on their resistance to the inhibitory effects of auxin on root growth. In addition, aux1 mutants are agravitropic [179] and develop less lateral roots [180]. Consistent with a role as auxin influx carrier, aux1 mutants can be rescued by the membrane permeable auxin analogue NAA, whereas the membrane impermeable auxin analogue 2,4-D cannot [181]. In some cells, AUX1 has a polar localization and cycles between the plasma membrane and endosomes [182]. Recently, the ER protein AXR4 has been shown to be required for the correct intracellular localization of AUX1 [183], although the molecular basis for this requirement is not clear.
Like aux1, lax3 mutants form less lateral roots while aux1 lax3 double mutant hardly develop lateral roots at all [184]. Lateral roots develop from pericycle cells that surround the vasculature as a single cell layer. It is likely that AUX/LAX-mediated auxin unloading from the phloem is necessary to reach the required intracellular auxin concentration to determine the faith of the pericycle cells.

### 7.2 Auxin efflux

Auxin efflux is mediated by members of the MDR/PGP family [185] and the PIN family [186]. *pgp1* and *pgp19* mutants have auxin related phenotypes, which may be partially explained by physical interactions between PGP and PIN proteins [187]. The PIN family consist of 8 members. *pin1* was isolated based on its pinformed phenotype as caused by reduced acropetal auxin transport [54]. Both PIN2 [188, 189] and PIN3 [190] function in tropisms while PIN4 [191] and PIN7 [192] function in root patterning and embryogenesis respectively. Loss of multiple PIN genes causes severe developmental phenotypes [192, 193].

PIN proteins have an asymmetrical subcellular localization which is established and maintained by their constant cycling between the plasma membrane and endosomes. The cycling of PIN proteins may allow their rapid relocalization and hence altering the direction of auxin flow. PIN3 for instance, relocalizes laterally upon gravity stimulation [190]. PIN cycling is regulated by the ARF GEF protein (guanine nucleotide exchange factor) GNOM [194, 195] which is sensitive to inhibition by BFA [196]. BFA treatment results in the accumulation of endosomes into large multivesicular bodies, which is a useful tool to study the cycling of PIN proteins.

Interestingly, different PIN proteins can have a different polar localization within the same cell, as exemplified by a basal localization of PIN1 and apical localization
of PIN2 in epidermal cells. As PIN1 expressed from the PIN2 promoter is still basically localized in these cells, the polarity-determining information must reside within the protein itself [197]. It was shown that the phosphorylation status of PIN1 determines its subcellular localization and is antagonistically controlled by the regulatory subunit of protein phosphatase 2a, RCN1 and the protein kinase PINOID (PID) [53]. PID is phosphorylated by PDK1 [52]. As both PDK1 [48] and RCN1 [69] bind PIP$_2$ and/or PA, these lipids could be involved in the pathway that determines PIN polarity.
8. Outline of this thesis

Plant phospholipid signaling has been studied mainly biochemically. Changes in the levels of PIP\(_2\) and PA have been observed in response to biotic and abiotic stresses and several proteins that bind PA or PIP\(_2\) have been discovered (Fig. 2). However, the significance of phospholipid signaling for the tolerance of plants to these stress conditions has not been adequately addressed. Arabidopsis contains 9 PLC, 7 DGK and 12 PLD genes. We took a reverse genetic approach to test the requirement of these genes for tolerance against biotic and abiotic stresses. PA induction has been observed during PAMP- and effector-triggered immunity (PTI and ETI). During PTI, PA appears to be exclusively derived from DGK (Fig. 3) whereas both DGK and PLD contributed to ETI-associated PA formation (Fig. 4). Chapter 2 describes the phenotype of a dgk5 T-DNA mutant, which had reduced resistance against virulent Pseudomonas. dgk5 had completely lost the ability to express the marker gene PR1. Surprisingly, this could not be rescued by SA treatment, suggesting that DGK5 functions downstream of SA accumulation. In chapter 3, pld\textalpha{}1 and pld\textdelta{} T-DNA mutant alleles are described that display a reduced HR when challenged with avirulent Pseudomonas. Loss of both PLD\textalpha{}1 and PLD\textdelta{} resulted in a reduced ability to restrict bacterial growth. Moreover, at least PLD\textdelta{} was found to contribute to AvrRpm1-induced PA formation. In chapter 4, the contribution of PLC to NaCl tolerance was explored by phenotyping plc3, plc6 and plc9 single, double and triple mutants. In chapter 5, evidence is presented for a role of PLC3 in lateral root formation. Subsequent experiments suggested that PLC3 is involved in cellular influx of auxin. Co-expression and co-localization of PLC3 with known auxin influx carriers is consistent with this hypothesis. In chapter 6, I will summarize the impact of these findings on the current knowledge of plant Arabidopsis signaling systems.
References


40. Chapter 1


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