Plant phosphatidic acid metabolism in response to environmental stress

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Chapter 1

Introduction

Biochemistry and physiology of phosphatidic acid metabolism in plants

Phospholipids have long been recognized as structural elements of membranes, which form semipermeable barriers delimiting all cells and intracellular compartments. The last two decades however, phospholipids have come into focus because some of them are formed under specific environmental conditions and act as intracellular signals or signal precursors. Such lipid second messengers (LSMs) are versatile regulators of metabolic and developmental processes, and can initiate responses to environmental stresses. Whereas LSMs are present in minute amounts, their levels increase rapidly in response to stimuli. Over the last decade, evidence has been accumulating, arguing that phosphatidic acid (PA) is a LSM and in plants PA is rapidly formed in response to abiotic as well as biotic stresses. However, PA metabolism is complex because of the multiplicity of its origins, being also a central intermediate in *de novo* synthesis and breakdown of phospholipids, glycolipids and triacylglycerols.

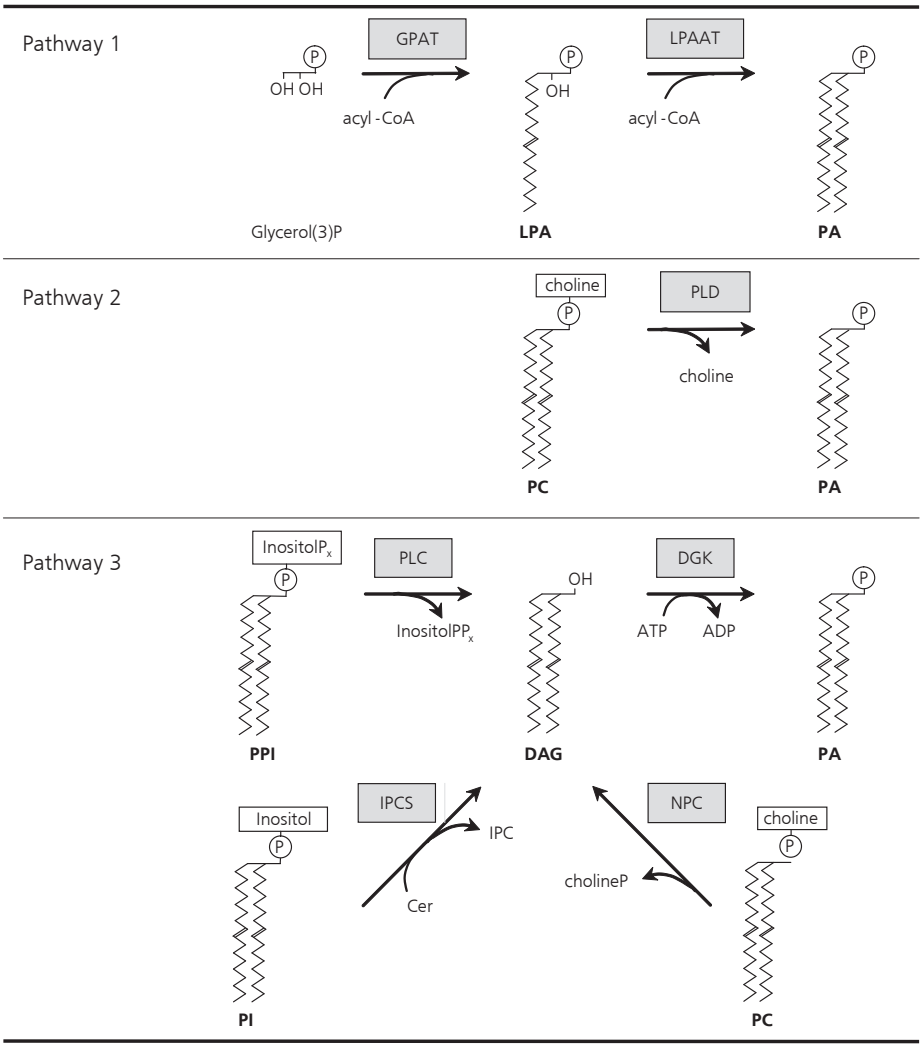
The question of its metabolic origin is crucial to understanding the diverse functions of PA. This chapter therefore introduces the pathways of PA metabolism, including methods to experimentally dissect them, and PA's specific relevance to glycolipid biosynthesis. Moreover, its emerging roles as LSM in the regulation of stress responses will be considered. The two plant model systems used in this thesis will be introduced in the final section.

1. Pathways of PA formation

1.1. PA as a product of lysophosphatidic acid acyl transferase (LPAAT): de novo glycerolipid synthesis

In plants, PA is formed as a precursor to glycerolipids through the sequential acylations of glycerol-3-phosphate (GroP) to lysophosphatidic acid (LPA), and of LPA to PA, by the activities of glycerol-3-phosphate *sn*-1-acyltransferase (GPAT) and lysophosphatidic acid *sn*-2-acyltransferase (LPAAT), respectively (Fig. 1, pathway 1, [1]). The fatty acid substrates, predominantly 16:0 (palmitic acid) and 18:1 (oleic acid), are synthesized *de novo* in the chloroplast stroma, and either used directly for the production of chloroplast lipids by a pathway in the plastid (the 'prokaryotic pathway'), or exported to the cytoplasm as acyl-CoA esters, which are incorporated into lipids by enzymes in the ER (the 'eukaryotic pathway'). The two sites of *de novo* lipid synthesis utilize different isozymes of GPAT and LPAAT. The distinct activities of GPAT4 and GPAT6, required for cutin biosynthesis, esterify acyl groups to the *sn*-2 position of GroP and, in addition, display phosphatase activity giving rise to (*sn*-2)monoacylglycerol rather than LPA [2].

Figure 1. Pathways of PA formation



Importantly, LPAATs, but not GPATs, distinguish accurately between the length of fatty acid substrates: whereas plastidial LPAAT generally uses only 16:0 as substrate, LPAAT that is bound to the ER membrane (as well as the mitochondrial isozyme) selectively uses 18:1. Hence, the plastidial and extraplastidial pathways produce different molecular species of PA which are used for the synthesis of complex phospholipids and glycolipids. Recent studies suggest that *de novo* synthesized fatty acids may also be directly introduced into PC through acylation of lyso-PC. The highly active acyl editing mechanism appears to bypass PA as an intermediate [3]. PC and galactolipids are substrates for fatty acid desaturases that introduce additional double bonds. For the synthesis of the structural

phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at the ER, PA is dephosphorylated by phosphatidic acid phosphatase (PAP) to diacylglycerol (DAG), which reacts with the nucleotide-activated headgroups, CDP-choline and CDP-ethanolamine (Fig. 2). Alternatively, the synthesis of phosphatidylinositol (PI) and phosphatidylglycerol (PG) uses nucleotide-activated PA, CMP-PA (also called CDP-DAG), that reacts with *myo*-inositol or GroP (Fig. 2). In contrast to the other phospholipids, PG is also formed in the plastid, where prokaryotic CMP-PA is its precursor. The *sn*-2-(16:0) acyl chain of plastidial PG is desaturated to the characteristic 16:1(Δ3-*trans*) acyl chain. This molecular species is essential for function of the photosystem II complex [4].

PA is not only precursor to complex phospholipids but also to the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG, Fig. 3), which are the main components of chloroplast membranes and together comprise ~75% of total membrane lipids in leaves [5]. Similar to PC synthesis, albeit localized to the plastid, PA is first dephosphorylated by PAP (LPP in Fig. 3) to generate DAG, which reacts with the activated headgroup, i.c. UDP-galactose, producing MGDG. DGDG is synthesized through the addition of a second galactose by a galactosyltransferase. Importantly, the PA segment that serves as precursor to galactolipids can also be derived from the eukaryotic pathway, due to its redirection to the chloroplast. The volume of this PA flux, and hence the contribution of the ER pathway to galactolipid synthesis, differs largely between plant species. As both pathways generate different PA molecular species, the resulting galactolipid species are different as well: prokaryotic MGDG is characterized by *sn*-2-linked 16:3, whereas eukaryotic species have *sn*-2-linked 18:3. Hence the contribution of either pathway to total MGDG is reflected in the molecular species.

1.2. PA as a product of phospholipase D (PLD)

PLD hydrolyzes structural phospholipids such as PE and PC to generate PA and the respective hydrophillic headgroup, i.e. ethanolamine or choline (Fig 1, pathway 2). The activity is stimulated in plants exposed to a variety of stress conditions, e.g. osmotic stress, drought, chilling, wounding and pathogenic interactions [6, 7]. PLD is genomically represented in *Arabidopsis* by 12 isozymes, which reflects its large diversity in expression, localization and function. Two PLD proteins, encoded by AtPLDζ1 and AtPLDζ2, are structurally similar to the mammalian and yeast PLDs, containing a combined N-terminal, lipid-binding, PX-PH domain, whereas the remaining ten, i.e. PLDα1-3, PLDβ1-2, PLDγ1-3, PLDδ and PLDε contain a C2 domain. PX/PH and C2 domains have been implicated in inducible translocation to membranes through their lipid-binding potential.

The special ability of PLD to generate artificial phosphatidylalcohols in the presence of primary alcohols, such as *n*-butanol, has been widely used to study the involvement of PLD in cellular responses *in vivo* [8];Chapter 4). The physiological significance of this, so-called, transphosphatidylation is unknown.

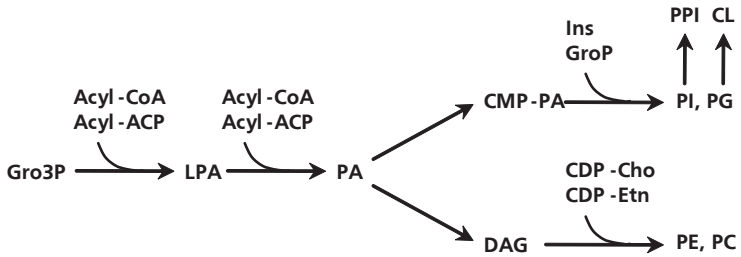


Figure 2. Phospholipid *de novo* synthesis pathways. Two successive acylations of glycerol3phosphate (Gro3P) generate PA which is used for phospholipid synthesis via two routes. PI, PG and their derivatives are formed from an activated form of PA, CMP-PA, whereas PE and PC have its dephosphorylation product, DAG, as precursor. The polyphosphoinositides (PPI) are formed through the activity of lipid kinases and phosphatases.

1.3. PA as a product of diacylglycerol kinase (DGK)

The activity of DGK, phosphorylating DAG to PA (Fig. 1, pathway 3), has also been implicated in responses to environmental stresses, giving rise to rapid (within minutes) increases in PA [9]. The signal-dependent formation of DAG, e.g. through the induced activity of phospholipase C (PLC) on polyphosphoinositides (PPIs), is immediately followed by its phosphorylation, which restricts DAG accumulation and promotes a rise in PA. In *Arabidopsis*, DGK is represented by 7 genes, which are subdivided in 3 clusters [10, 11]. Cluster I resembles the mammalian DGKε type, as its members, *At*DGK1 and *At*DGK2, contain not only the conserved catalytic domain, but also two cystein-rich C1 type domains, which may be involved in membrane binding, and a transmembrane helix. The remaining DGKs are smaller, lacking the conserved structural domains of mammalian DGK. The activity is mainly associated with the plasma membrane, but also internal membranes, e.g. the ER.

Recent advances have highlighted another source of DAG in plants, namely PC hydrolysis by bacterial type PLCs, called non-specific PLCs (NPC, Fig. 3), of which one is, for example, activated under conditions of phosphate limitation [12, 13].

2. Methods to differentiate between metabolic origins of PA

PA is, like other lipids believed to function in signal transduction, e.g. including PPIs, and the related metabolites LPA and DGPP, present in relatively low amounts. Steady state PA makes up 0.5-2.0 mole% of the total phospholipids in *Arabidopsis*, which is likely to represent the metabolic pools at the ER and the chloroplast. Nonetheless, under stimulatory conditions, PA levels increase by 1.4 to 3.0-fold, depending on the

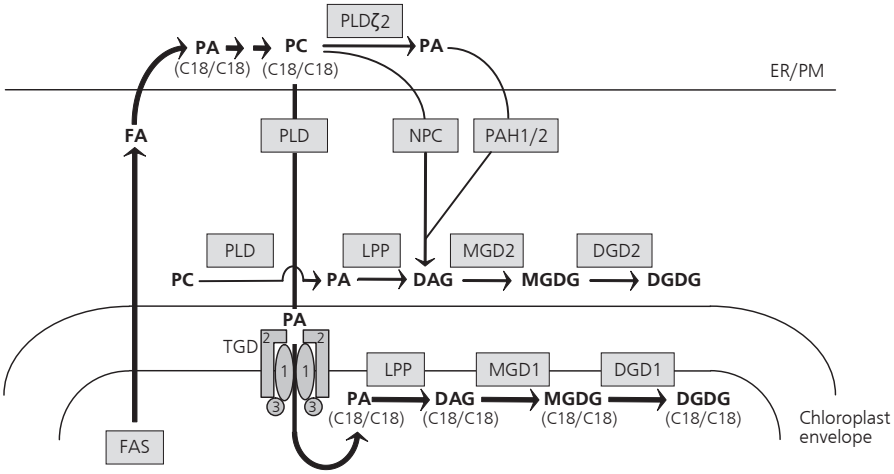


Figure 3. Pathways of eukaryotic phospho- and galactolipid synthesis under non-starved (thick arrows) and P-starved (thin arrows) conditions. *De novo* synthesized fatty acids (FA) by the FA synthesis complex (FAS) are used for ER –localized synthesis of PC and other phospholipids at the ER. For eukaryotic MGDG synthesis, PC may be hydrolyzed by PLD to PA which is imported into the plastid through the TGD complex, to serve as substrate for MGDG synthesis by MGD1. The MGDG species are characterized by two C18 fatty acids, reflecting the composition of its precursors, PA and PC, that are synthesized at the ER. During P-starvation, other pathways, involving PC-hydrolyzing NPCs, PAP (PAH) and PLD function to provide PA and DAG as substrate for DGDG synthesis in the outer chloroplast membrane through MGD2 and DGD2. Abbreviations are explained in the text.

type, intensitiy, and duration of the stimulus. Thus, to perform detailed analysis of PA's constituents, sensitive analysis of this minor fraction is required. This is achieved essentially by two approaches, each providing different information on PA metabolism. The first is based on mass analysis, whilst the second relies on *in vivo* ^{32}P-orthophosphate ($^{32}P_i$) radiolabelling and lipid turnover.

2.1. Analysis of PA based on mass detection

The analysis of PA by HPLC-MS or GC(-MS) has been applied to quantitatively study the formation of PA and the compositions of its fatty acids. This information can provide strong evidence of PA's metabolic precursors under specific conditions, since PA inherits their fatty acid part. As fatty acid compositions differ between lipid classes, they can be used as 'fingerprints' to trace PA's precursors, thus providing evidence of its metabolic pathway. For example, based on the similarity between the fatty acid compositions of PA and the PPIs in cold-treated *Arabidopsis*, the PLC-DGK pathway was implicated in the response [14]. Similarly, the activity and substrate selectivity of the PLD pathway has been established under KCl-induced stress in *Chlamydomonas moewusii* (Chapter 4, [15]). The analysis of lipid mass and structure has also been applicated to investigate the consequences of knock-out and overexpression of *PLD*, *DGK, PLC* genes.

2.2. Analysis of PA based on metabolic ^{32}P-radiolabelling

A common method to study phospholipid metabolism makes use of the uptake and incorporation of $^{32}P_i$ into organic molecules in a great variety of cells and tissues. When, after a period of $^{32}P_i$-prelabelling, a stress treatment is given, changes in the levels of $^{32}P_i$-lipids can be observed. Their quantities are easily determined by phosphoimaging of total lipid extracts separated by thin-layer chromatography (TLC) into different phospholipid classes. Insights into the factors that determine the kinetics of labelling of individual phospholipids have greatly helped to map out PA metabolism. Thus, a distinction can be made between ^{32}P-PA responses which can be detected using only a brief pre-labelling time (1 to 20 minutes), visualizing DGK activity, and responses that require a long pre-labelling time (hours-days), suggesting PLD activity. How the distinct labelling kinetics can be indicative of the metabolic pathway involved, is discussed in Chapter 2. A ^{32}P-radiolabelling approach provides information on the flux within metabolic pathways, not on the actual lipid quantities, and is therefore complementary to GC- and MS-based analyses.

3. The metabolic fate of PA

While PA is the product of multiple pathways, it is also a precursor to various lipids (Figure 4). Under conditions where PA functions as LSM, their formation reflects signal attenuation. Similar to the PA generating pathways, it is a challenge to unravell the PA metabolizing pathways and identify the responsible enzymes. The activity of some of these enzymes leads to the accumulation of lipids which are only detected under stress conditions.

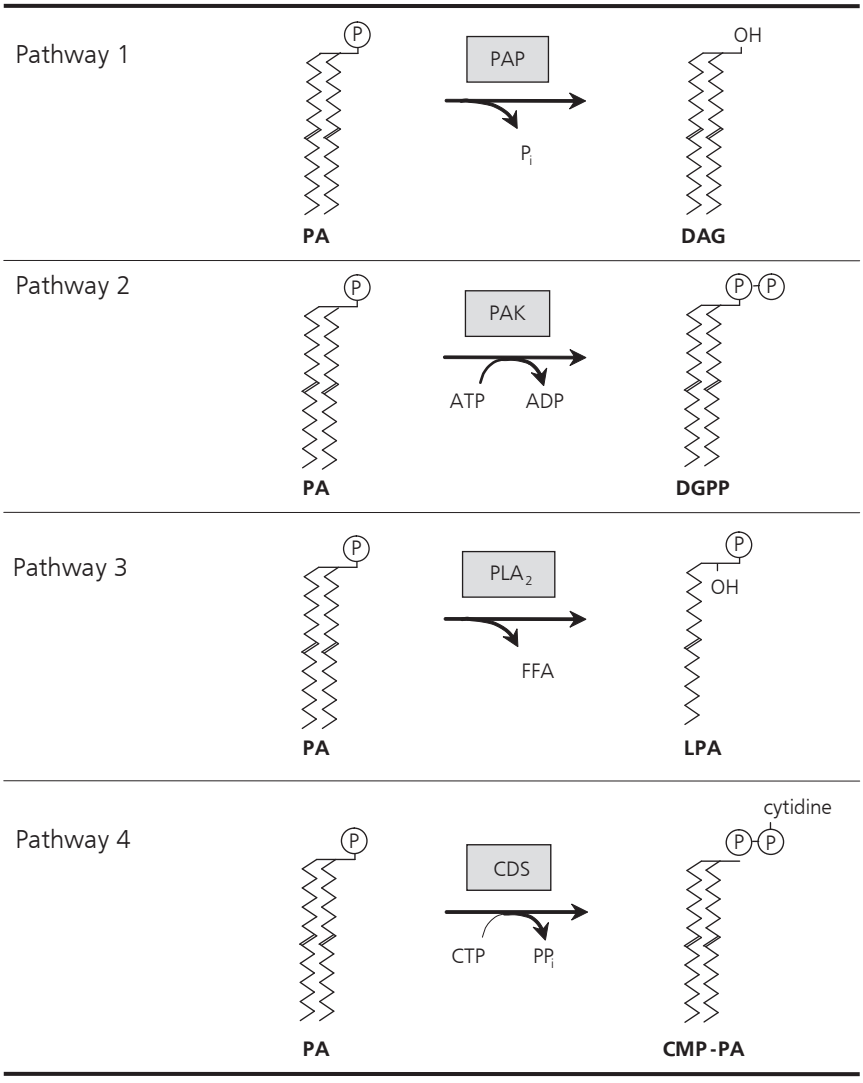
3.1. PA dephosphorylation by PAP

The activity of PAP, which hydrolyzes PA to generate DAG, is pivotal in both chloroplast- and ER-localized glycerolipid synthesis (Fig. 4, pathway 1). The DAG produced is used for the synthesis of plastidial glycolipids (Fig. 3) as well as the extraplastidial phospholipids PC and PE (Fig. 2). PAP can also function to control the level of PA as LSM. While the *Arabidopsis* genome harbours at least nine PAP encoding genes, little is known about there functions. PAPs are subdivided into two groups, *viz*. the lipins, which are soluble enzymes that function in lipid biosynthesis [13], and lipid-phosphate phosphatases (LPPs), which are transmembrane proteins, capable of hydrolyzing not only PA, but also sphingosine-1-phosphate, ceramide-1-phosphate, LPA and diacylglycerolpyrophosphate (DGPP) *in vitro* [16]. The *Arabidopsis* genome contains LPP genes which belong either to the 'eukaryotic LPPs' (4 genes), homologs of mammalian LPP, or to the 'prokaryotic LPPs' (5 genes), homologous to cyanobacterial LPPs. LPPα2, belonging to the first group, appears to function in ABA signalling during seed germination, as the *lpp2-1* mutant displayed enhanced PA levels and ABA hypersensitivity, resulting in decreased germination rates [17].

3.2. PA phosphorylation by PAK

The transient accumulation of DGPP through the phosphorylation of PA has been described for plants treated with abiotic as well as biotic stresses, whereas it does not occur in mammals (Fig. 4, pathway 2). To date, no genes encoding PAK activity have been cloned, yet the activity was partially purified from *Catharanthus roseus* membranes, and suggested to be an integral membrane protein, prevalent at the plasma membrane [18]. *In vivo* DGPP formation was first characterized in *Chlamydomonas*, where it is generated upon osmotic stress. Interestingly, whereas PA was shown to increase in response to a

F**igure 4**. The metabolic fate of PA



wide range of hyperosmotic salt concentrations, DGPP was only generated within a limited window of concentrations, suggesting that the metabolic fate of PA was determined by the level of stress [19]. It has been suggested that the enzymes catalyzing phosphorylation (PAK) and deacylation (PLA, see below) compete for the same PA substrate [20].

3.3. PA deacylation by phospholipase A (PLA)

PLA catalyzes the hydrolysis of phospholipids into lysophospholipids and free fatty acids, either at the *sn*-1 (PLA_1) or *sn*-2 position (PLA_2) of the glycerol backbone (Fig. 2.3.). In *Arabidopsis*, three families are distinguished: 4 small secretory sPLAs, 10 patatin-like pPLAs and 14 lipase-like PLA_1s [21]. Although little is known about the substrate they use, one gene, *AtSRG2*, was found to encode a putative PA-specific PLA_1 activity [22]. Interestingly, the knock-out mutant was defective in shoot gravitropism.
Osmotic stimulation of *Chlamydomonas* was shown to trigger the transient accumulation of LPA, following a rapid rise in PA. Various lines of evidence suggest an involvement of PLA_2 ([20]; this thesis, Chapter 5).

3.4. PA use for the synthesis of CDP-DAG by CDP-DAG synthase (CDS)

PA is substrate to CMP-PA synthase (CDS) to form CMP-PA as precursor in the biosynthetic pathways of PI, PG and cardiolipin (Fig 4, pathway 4). In the inner chloroplast envelope membrane, CMP-PA is used for the synthesis of PG and in mitochondria for the synthesis of PG and cardiolipin (CL, Fig. 2). However, most CDS activity resides in microsomal membrane fractions, and demonstrated to be present in plasma membrane preparations from *Catharanthus roseus* [23]. Two CDS-encoding genes have been cloned, *StCDS* and *AtCDS1*, from potato and *Arabidopsis* respectively [24]. In mammals and *Drosophila* CDS has been implicated in lipid signalling, as it is required for the regeneration of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) which is hydrolyzed by PLC [25]. In plants, there is no evidence for such function.

4. Eukaryotic PA species as precursors in galacto-lipid synthesis

The finding that eukaryotic ER-based lipid synthesis is responsible for (part of) plastidial synthesis of galactolipids, raised the question how their precursor PA (or DAG) is generated, and transported into the chloroplast.

4.1. PA import into the chloroplast via the TGD1/2/3 transporter

Arabidopsis mutants affected in ER- to plastid lipid trafficking have been isolated which lead to the identification of three proteins, TGD1, -2 and -3, forming a complex in the

plastid envelope, that is required for the import of PA for galactolipid synthesis (Figure 3; [26]). This complex is similar to bacterial ABC transporters, containing a permease, TGD1, an ATP-ase, TGD3, and a substrate-binder, TGD2 [27]. The latter protein binds specifically to PA, and a C-terminal sequence of 25 amino acids was responsible [28]. All *tgd* mutants displayed an unusual accumulation of trigalactosyldiacylglycerol and triacylglycerol (TAG). Moreover, consistent with a function in the supply of PA as substrate for galactolipid synthesis, they showed enhanced PA levels at the ER and outer chloroplast envelope membrane, and decreases in galactolipids derived from the ER pathway. Following the import of ER-derived PA into the plastid, it is dephosphorylated by LPPγ, a prokaryotic type of PAP [29], to generate DAG as substrate for MGDG synthesis (Fig. 3).

4.2. PA as precursor for galactolipids during phosphate starvation

When plants experience limited availability of phosphate (P_i), phospholipids are used as a resource and replaced by galactolipids, particularly DGDG, which is formed in the outer chloroplast envelope membrane by DGD2 (Fig. 3; [30]). Several enzymes have been found that are required for this remodelling, some of which may also support eukaryotic galactolipid synthesis under non-starved conditions.

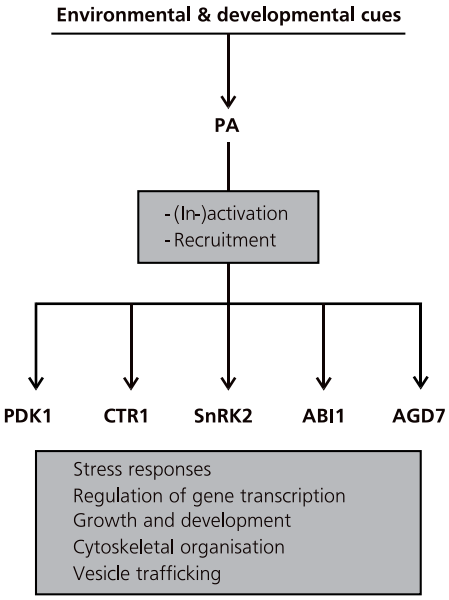
Two members of the NPC-family of phospholipases, NPC4 and NPC5, are transcriptionally induced during P_i-starvation [12]. While NPC4 is a cytosolic protein, thought to hydrolyze PC at the plasma membrane during P_i-starvation [31], NPC5 is localized to the plastidial outer envelope membrane to generate DAG for galactolipid synthesis [12]. Whereas the latter pathway was found in leaves and does not generate PA, another route, suggested to have a function in P_i-starved roots, involves a PLDζ2 at the plasma membrane or ER [32]. The formed PA may be substrate for PAH1 and PAH2, two lipin-type PAPs, which have been implicated in eukaryotic galactolipid synthesis and are also induced during P_i-deprivation [13, 17].

5. PA as a phospholipid signal

Evidence of PA's function in stress-induced signal transduction is emerging from studies demonstrating that it is formed within seconds-minutes and can bind enzymes and modify their activity [33, 34]. Similar to PPIs, PA can provide a membrane docking site to recruit cytosolic target proteins. This function relies on the unique biophysical and chemical properties of PA, as it has a negative charge which increases upon binding to proteins via hydrogen bonds, and the electrostatic interaction is thought to support the lipid-protein association [35].

A list of potential PA interactors in plant biology, summarized in Fig. 5, includes: the protein phosphatase ABI1 [36], the protein kinases CTR1 [37], PDK1 [38] and SnRK2 [39], and the ARF-GAP AGD7 [40]. Several other proteins show *in vitro* PA binding, such as

Figure 5.



Arabidopsis dehydrin1 (DHN1, [41]) and wheat phosphoethanolamine methyltransferases (*Ta*PEAMT1/2, [42]). A chrystal structure of a PA-binding domain has not been resolved yet but some motifs have been reported [37].

PA has also been implicated in vesicle trafficking and cytoskeletal organisation. PLD associates with microtubules [43, 44], and with the actin cytoskeleton [45]. PA promotes actin polymerisation, through binding and inhibition of actin-capping protein (*At*CP, [46]).

6. *Chlamydomonas* and *Arabidopsis*: two distinct plant models to study phospholipid metabolism

Although there is a large degree of conservation of the pathways of glycerolipid metabolism and signalling within the plant kingdom, and even compared with animals and fungi, there are also significant differences. Many of these differences are apparent when two evolutionary distinct organisms, the unicellular green alga *Chlamydomonas* and the seed plant *Arabidopsis thaliana*, are considered. The first belongs to the Chlorophytes, containing the green algae, and the second to the phylogenetic branch containing the land plants; they share a common ancestor 1.1 * 10 ^{9} years ago [47].

Interestingly, the study of phospholipid- and Ca^{2+}-based signal transduction in green algae has revealed many similarities to animal signalling, often involving homologous components that are not present in land plants [48]. For example, *Chlamydomonas*, in

contrast to land plants, contains an inositol(1,4,5)trisphosphate (IP_3) receptor, which, upon ligand binding, mediates the influx of Ca^{2+}. IP_3 can be generated through the PLC-mediated breakdown of $PI(4,5)P_2$, a ubiquitous signalling route in animals [33]. Another distinctive feature of green algae is the presence of TRP-family channels, also found in animals and yeast, whose function can directly be regulated by PPI. Many TRP channels function in sensory transduction and a yeast homologue is a mechanosensitive Ca^{2+} channel in the vacuolar membrane which is activated upon hyperosmotic shock [49].

Glycerolipid species and metabolism in *Chlamydomonas* displays several features not shared with *Arabidopsis*. *Chlamydomonas* is a typical 16:3 plant, having MGDG species with an *sn*-2-linked 16-carbon fatty acid, whereas *Arabidopsis* shows galactolipids of mixed origin. This implies that the alga relies on the chloroplast for glycolipid synthesis and does not need a mechanism for import of eukaryotic PA/DAG species to the plastid. As this process in *Arabidopsis* is based on PC metabolism, the lack of PC in *Chlamydomonas* may reflect the absence of ER-derived MGDG synthesis. Instead of PC, it contains the zwitterionic betaine lipid diacylglyceryltrimethylhomoserine (DGTS, Chapter 3).

Chlamydomonas has been succesfully used to study phospholipid signalling, which has lead to the identification of several components. For example, the PPI [50], IP_3 [51], the signal-dependent formation of DGPP [52], PI3P [53], PI5P [54] and $PI(3,5)P_2$ [55] and PLD-catalyzed formation of PA, and, in the presence of n-butanol, phosphatidylbutanol, used as activity marker for PLD [8]. Importantly, the uniformity in the sampling of great numbers of these unicellular organisms allowed for a very detailed study of the kinetics of ^{32}P-labelling of phospholipids, under control and stimulatory conditions.

On the other hand, *Arabidopsis thaliana*, has taken center stage as a seed plant model in plant physiology. Its genome was sequenced first and soon mutants and transgenic lines became available. In particular, the use of T-DNA insertion lines raised the possibility to implicate proteins and their metabolic products in particular physiological functions, e.g. in lipid metabolism [56]. For *Chlamydomonas*, molecular genetic techniques of transformation and silencing have also been developed [57], and the *C. reinhardtii* genome has recently been sequenced [58], on a par with *Arabidopsis*.

We have used both plant models to study the rapid increases in PA in response to abiotic stress, in particular hypersalinity and cold. Based on analysis of fatty acid and molecular species compositions, and ^{32}P-labelling of phospholipids, the metabolic origin of induced PA responses was investigated. Chapter 5 exemplifies the effective use of these complementary approaches in the analysis a novel potential phospholipid signal in *Chlamydomonas*, LPA.

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