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Chapter 2

Plant PA signaling via diacylglycerol kinase

Steven A. Arisz, Christa Testerink and Teun Munnik

Abstract

Accumulating evidence suggests that phosphatidic acid (PA) plays a pivotal role in the plant's response to environmental signals. Besides phospholipase D (PLD) activity, PA can also be generated by diacylglycerol kinase (DGK). To establish which metabolic route is activated, a differential ^{32}P-radiolabelling protocol can be used. Based on this, and more recently on reverse-genetic approaches, DGK has taken center stage, next to PLD, as a generator of PA in biotic and abiotic stress responses. The DAG substrate is generally thought to be derived from PI-PLC activity. The model plant system *Arabidopsis thaliana* has 7 DGK isozymes, two of which, *At*DGK1 and *At*DGK2, resemble mammalian DGKε, containing a conserved kinase domain, a transmembrane domain and two C1 domains. The other ones have a much simpler structure, lacking the C1 domains, not matched in animals. Several protein targets have now been discovered that bind PA. Whether the PA molecules engaged in these interactions come from PLD or DGK remains to be elucidated.

Introduction

Phosphatidic acid (PA) has emerged as an important lipid second messenger in plants, being involved in a wide range of biotic (*e.g.* pathogens) and abiotic (*e.g.* osmotic, temperature) stress responses. Two signaling pathways are predominantly held responsible for its formation. PA is directly formed via activation of phospholipase D (PLD), and indirectly, via activation of a PLC pathway, which generates diacylglycerol (DAG) that is phosphorylated to PA via DAG kinase (DGK). Plant PLD signaling will be reviewed by our colleague, Dr. X. Wang. Here, the role of DGK in plant PA signaling responses is discussed.

Structure and classification

DGKs are members of a unique and conserved family of lipid kinases that catalyze the phosphorylation of DAG into PA. In mammalian cells, 10 different DGKs can be distinguished, which have been classified into 5 different subtypes (Figure 1; [1]). *Saccharomyces cerevisiae* was initially thought to lack a DGK, until recently the lab of George Carman discovered a novel type, consuming CTP rather than the common ATP [2]. In the genome of the model plant *Arabidopsis thaliana*, 7 *DGK* genes are present which fall into 3 phylogenetic clusters (Figure 1; [3]). Cluster I most closely resembles the DGKe type, which represents the most basic DGK in mammalian cells, containing the conserved catalytic DGK kinase domain, two C1-type domains, which are cystein-rich domains thought to be responsible for binding the substrate DAG, and a predicted trans-membrane helix which targets this DGK to the membrane [4]. Cluster II and III *AtDGK*s lack the latter two domains and only exhibit the conserved kinase domain (Fig. 1). Some DGKs of cluster III are distinguished by alternative splicing variants, containing a calmodulin-binding domain (CBD) [3,5]. The rice genome encodes 8 *DGKs*, falling into the same clusters as described for *Arabidopsis*.

The requirement of C1 domains for DAG binding and for catalytic activity of mammalian DGKs has been a matter of dispute. The absence of these domains from two clusters of plant DGKs suggests that they are not required for activity. In fact, *At*DGK7, which falls in cluster III, was shown to have DGK activity [6]. The C1 domains of DGK may bind to protein partners rather than DAG, which would provide a mechanism for the spatial and functional coupling to other enzymatic activities [7]. Information on the 3-D structures of DGKs will help to establish the sites of DAG binding and mechanisms of activity regulation.

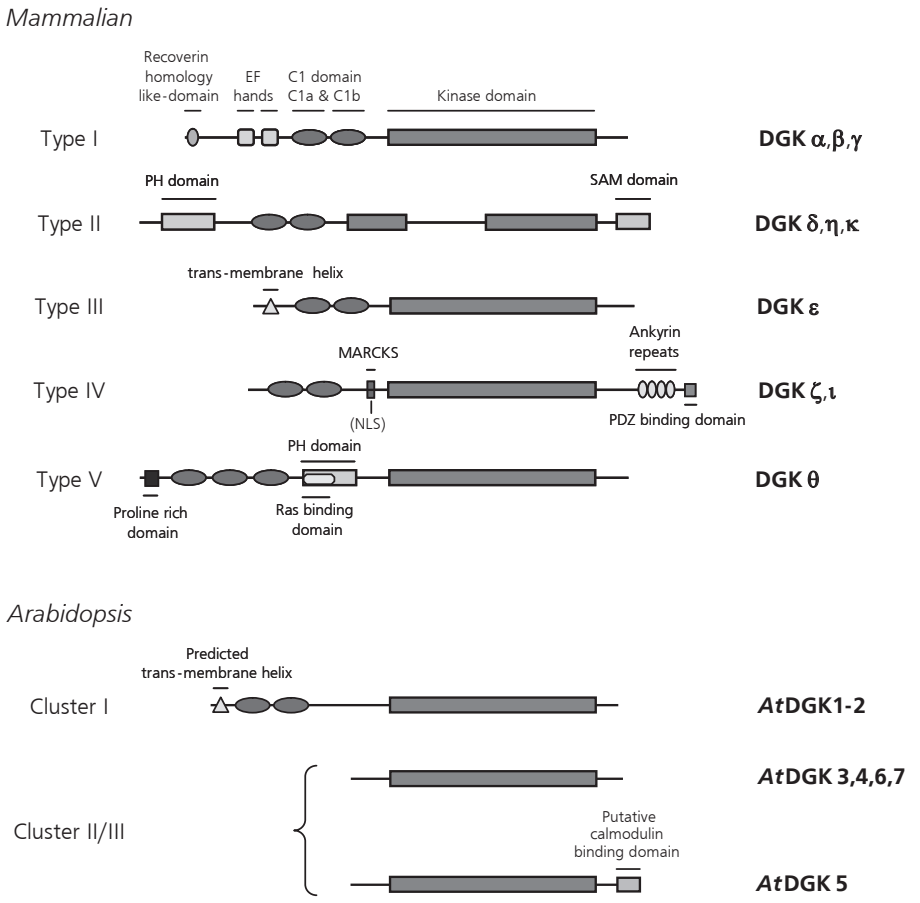

Figure 1. Protein structures of members of the DGK family of mammals and *Arabidopsis thaliana*. Characteristic regulatory domains of typical DGK isoforms are indicated. The DGK clusters of A. *thaliana* are solely based on amino acid homology. *At*DGK3, 4 and 7 are in cluster II, *At*DGK5 and 6 are in cluster III [3]. The putative carboxyterminal calmodulin-binding domain is in a splice variant of *At*DGK5, and is similar to the structure of *Sl*CBDGK in tomato. In addition to the domains indicated, there are two putative Ca^{2+} binding EF hand domains between the C1 domains and the catalytic domain in *At*DGK1 [16]. Abbreviations: MARCKS, myristoylated alanine-rich C-kinase substrate, NLS, nuclear localization sequence, PH, pleckstrin homology, SAM, sterile α-motif. Adapted with permission from [1].

Localization

DGK activity has been characterized biochemically in roots and shoots of wheat [8] and in suspension-cultures of tobacco [9] and *Catharanthus roseus* [10-13]. Activity was predominantly found at the plasma membrane [8,9,12-14], while some was associated with the cytoskeleton [15], nucleus [16] and chloroplast [17]. A DGK from *Catharanthus* was purified as an intrinsic membrane protein, with a molecular mass of 51 kDa (i.e. a cluster II/III DGK). It required phospholipids or deoxycholate and divalent cations for activity, with

Mg^{2+} being the most effective. Negatively charged lipids such as PI and PG stimulated the activity [12, 14], in contrast to the wheat enzyme which responded more to PPIs [8].
Mammalian DGK activity is generally regulated through signaling-dependent recruitment to specific membranes, mediated by defined structural domains (Fig. 1). Plant DGKs are likely to be regulated differently, since they hardly contain such domains (Fig. 1). Nevertheless, a DGK from tomato, *Sl*CBDGK1, has been shown to have a CBD, which would allows its Ca^{2+}-dependent membrane binding [5]. From the same locus, an alternative splice product gives rise to *Sl*DGK1, which lacks the CBD and the Ca^{2+}-dependent membrane localisation. While a Ca^{2+}-dependent membrane localization is known for mammalian DGKs, the calmodulin-dependent mechanism seems a unique to plants.
*At*DGK5 is the *Arabidopsis* homolog of tomato *Sl*DGK1 and also has two splice variants, one of which may contain the CBD [3]. This could have interesting implications in terms of physiological function, because a cytosolic rise in Ca^{2+} is a common element in biotic and abiotic stress signaling pathways.
Mammalian type I DGKs are regulated by Ca^{2+} through EF-hand domains (Fig. 1). Ca^{2+}-binding results in a conformational change, which potentially modulates enzyme activity and/or exposes other domains. *At*DGK1 has two putative EF hands between the C1 domains and the catalytic domain [18], but empirical validation of their presence is still missing.
A recent study established that an amino-terminal transmembrane domain in *At*DGK1 and *At*DGK2 was both sufficient and necessary to sequester them to the ER [4]. Transient expression of chimaeric constructs of green fluorescent protein (GFP), fused to truncated *At*DGK1 and *At*DGK2 containing the transmembrane domain, revealed ER localization. Deletion of the N-terminal region resulted in the loss of membrane localization and in this case *At*DGK2-associated fluorescence was restricted to the nucleoplasm, while GFP-*At*DGK1 was cytosolic. Amino acid-sequence analysis by the *pfscan* program (http://plantst.genomics.purdue.edu) identified putative bipartite nuclear localization signals (NLS) in between the two C1 domains of cluster I *At*DGKs (not shown). Overexpression of the hydrophobic region fused to yellow FP in *Arabidopsis*, showed localization to the ER in leaves and roots, but not the plasma membrane. Concurring with the prevalence of biochemical activity, the results suggested that *At*DGK1 and *At*DGK2 are permanently localized to membranes, primarily at the ER, although we have to keep in mind that the parts of the protein outside the hydrophobic stretch, which may also affect localization, were not considered in these experiments. The detection of DGK activity in the nucleus and Golgi apparatus (ibid.) is particularly interesting in the light of studies in mammals and yeast, which have indicated functions for DAG and PA in nuclear signaling [19] and vesicular transport between the ER and Golgi [20-22].
Since most of the *in vitro* DGK activity resides at the plasma membrane, the smaller, cluster II- and III- DGKs most likely account for this. Indeed, a GFP fusion of *At*DGK5 was found in the plasma membrane when expressed in *Arabidopsis* (B. van Schooten & T. Munnik, unpublished) and earlier, a 51-kDa DGK from *Catharanthus* was purified

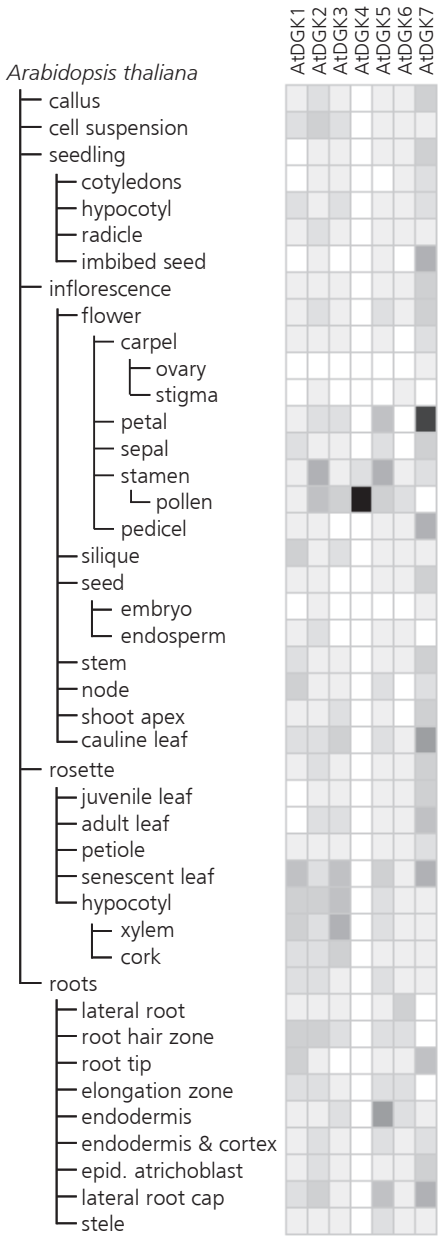

Figure 2. Expression pattern of *Arabidopsis* DGK genes in planta. Dark-grey represents the highest expression, white represents the lowest. Data were retrieved from www.genevestigator.ethz.ch

as an intrinsic plasma membrane protein [14]. Since these smaller DGKs lack the C1- and hydrophobic domains, this raises the question of how they are anchored to the plasma membrane and how they find their substrate DAG.

Northern blot analysis of *AtDGK1* showed transcripts in roots, shoots and flowers [18] while *AtDGK2* mRNA was mostly abundant in cauline leaves, flowers, siliques, roots and rosette leaves [3]. Promoter-GUS fusions of *AtDGK2* were used to study its expression during various developmental stages [3]. Interestingly, the GUS-staining pattern in cotyledons shifted in the first week after germination from an even distribution towards a concentration in the vascular bundles. Similarly, a shift from the root-shoot junction to the root tip was observed. RT-PCR analysis of *AtDGK7* transcripts showed expression in flowers, young seedlings (2 wk) and cauline leaves [6]. Using the expression data retrieved by the *Genevestigator* software package (www.genevestigator.ethz.ch), tissue-specific expression patterns of various *AtDGK* genes can be observed (Figure. 2).

Distinguishing between DGK- and PLD-generated PA

Both PLD and DGK can generate a PA response in plant stress signaling, so how do we know which activity is involved? Normally, plant PA levels are 1-2 % of total phospholipids, depending on the plant and tissue. This is usually measured

chemically by TLC, GC or HPLC methods, which only reveal relatively big changes in mass rather than subtle, localized, alterations in PA synthesis and/or turnover that occur in response to cellular stimulation. The majority of the PA probably even reflects the pool that functions as precursor for the biosynthesis of structural phospholipids at the ER, which is made via two consecutive acylations of glycerol 3-phosphate and lyso-PA, respectively, via the Kennedy pathway. Nonetheless, subtle PA responses can readily be picked-up by pre-labelling plant material with inorganic ^{32}P-orthophosphate ($^{32}P_i$). An example is shown in Figure 3, using the unicellular green alga, *Chlamydomonas*. Cells rapidly take up the label and incorporate it into their phospholipids. First, the minor signaling lipids PIP, PIP_2 and PA become labelled because they are formed through direct phosphorylation using ATP, which is one of the first compounds to become labelled upon $^{32}P_i$-incubation. Moreover, these lipids turn-over much faster than structural phospholipids (*e.g.* see PE, PI, PG in Fig. 3B), and, as a consequence, the latter are labelled much slower. E.g., it takes 6 days for the structural phospholipids to reach isotopic equilibrium, as opposed to a few minutes for the minor signaling lipids [23-25].

PLD activity can easily be monitored because the enzyme has a unique *transphosphatidylation* ability. In a two-step reaction, PLD first removes the headgroup (e.g choline or ethanolamine) and then transfers the phosphatidyl moiety to a nucleophillic acceptor, which is water under normal conditions, resulting in the formation of PA. However, PLD can also transfer the phosphatidyl moiety to primary alcohols, such a 1-butanol, resulting in the formation of phosphatidylbutanol (PBut), and this lipid is normally not present in cells but is readily synthesized *in vivo,* when cells are co-incubated with low concentrations (0.1-0.5%) of 1-butanol (Fig. 3A and 3C). Thus, when cells are prelabelled with $^{32}P_i$, the formation of ^{32}P-PBut can be taken as a relative measure for PLD activity *in vivo* [26, 27]. Note, that the PBut will only be radioactive when the cells are prelabelled long enough to ensure labelling of PLD's substrate, *i.e.* PE, which usually takes hours rather than min (Fig. 3A and 3B). Obviously, this also holds for the PA that would normally be coming through the PLD pathway (PA_{PLD}). This is in sharp contrast with the PA generated via the DGK pathway (PA_{DGK}) as the latter is synthesized through phosphorylation of DAG using ATP. Thus, when cells are only shortly pre-labelled, a stimulus-dependent increase in ^{32}P-PA can only occur through the DGK pathway and not via PLD. This so-called *'Differential labelling protocol',* originally described for neutrophils [28], was validated for plants 11 years ago [25, 85] and has since been frequently used to distinguish between both pathways in a variety of plant systems [27, 29].

Another, complementary approach, applying the analysis of molecular species by GC-MS or LC-MS, uses the unique fatty acyl compositions of phospholipid classes to establish precursor-product relations. Thus, the PA that is produced via PLC/DGK matches the polyphosphoinositides in their fatty acid composition [23, 27, 30]. Likewise, PLD-generated PA inherits the composition of the PLD substrate, e.g. PC and PE [23, 31] which can again

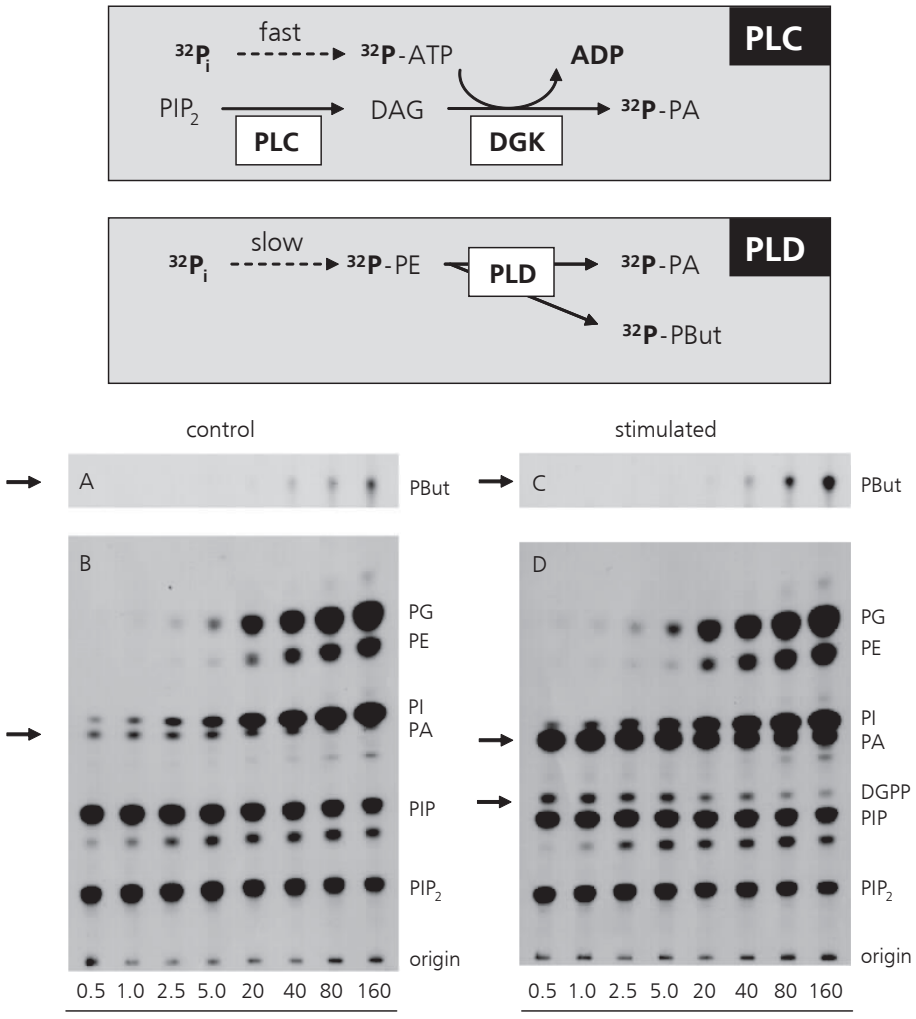

Figure 3. Differential ^{32}P-labelling protocol to distinguish between PA generated via a PLC/DGK- or PLD pathway. *Chlamydomonas* cells were metabolically prelabelled with ^{32}P-orthophosphate for the times indicated and subsequently treated for 1 min with either buffer (*control*; panel A, B) or 1 µM mastoparan (*stimulated*; panel C, D), both in the presence of 0.1 % n-butanol. Lipids were then extracted, split into half, and either seperated on EtAc TLC (A, C), to separate the PLD-catalyzed phosphatidylbutanol (PBut), or Alkaline TLC (B, D), to visualize the rest of the phospholipids, including PA and its phosphorylated product, diacylglycerolpyrophosphate (DGPP). The point is that all cells are treated for the same time period (1 min) but that the PBut will only be radioactive when its substrate (i.e. PE) becomes labelled (> 40 min). The same holds of course for the PA that would be coming through the PLD pathway. In contrast, when PA is generated via DGK, then the label comes from ATP and since this is labelled within seconds, a massive radioactive PA response can be witnessed if a DGK was involved. Since PA kinase also uses ATP, a similar response in DGPP can be picked up (panel D). Since the specific radioactivity of ^{32}P-ATP decreases in time, the 1-min response decreases concomitantly. The combined assay is a relative measure and evidence for PA coming through a DGK and/or PLD pathway.

be validated by analyzing the fatty acid composition of PBut [23]. Hence, the molecular species of PA is indicative of its metabolic origin.

The activities of PLC and DGK, and the downstream responses, are often probed using inhibitors, *viz.* R59022 to inhibit DGK, and U73122 and neomycin to inhibit PLC. R59022 was found to be more effective against the cluster I *At*DGK2 than against cluster II *At*DGK7 [6]. Nonetheless, the reliability and specificity of some of these compounds have been questioned [25].

Historically, DGK has always been linked to the PI-PLC pathway, phosphorylating the DAG that is generated through hydrolysis of PIP_2. However, higher plants have very little PIP_2, and a canonical IP_3/Ca^{2+}- and PKC pathway is missing [32, 34]. Nevertheless, there is plenty of PI4P in the plasma membrane [33] and *Arabidopsis* contains 9 potential PI-PLCs to take this lipid as a substrate. *In vitro*, plant PLCs hydrolyse PI4P and PIP_2 equally well [25].

Interestingly, *Arabidopsis* also contains 5 bacterial-type PLCs. The latter are non-specific and hydrolyse structural phospholipids like PC and PE. Recently, an *Arabidopsis* KO mutant of a non-specific phospholipase C (NPC5) was shown to be required for 50% of the digalactosyldiacylglycerol (DGDG) accumulation in leaves during phosphate limitation [35]. This lipid conversion, however, does not involve DGK activity. Hence, we suspect that the bacterial PLCs are involved in lipid metabolism rather than cell signaling.

Galactolipids constitute approximately 70% of the total lipids as opposed to 20% phospholipids in a typical leaf extract, yet there does not seem to be a lot of free DAG [36]. This is in huge contrast to the floral organs of *Petunia hybrida*, where large amounts of DAG were found. In stamens and pistils, DAG is supplied from PC by non-specific PLC activity and *de novo,* via the Kennedy pathway and PA phosphatase, whereas in petals, a two-step catalytic pathway via PLD and PA phosphatase predominates. Again, these data are likely to reflect metabolism (e.g. fuel for volatile emission) rather than signaling.

Expressing the DAG biosensor YFP-$C1_{PKC\gamma}$ in tobacco BY-2 cells or *Arabidopsis* seedlings, revealed no membrane labelling, with all fluorescence ending up in the cytosol (J.E.M. Vermeer & T. Munnik, unpublished). Together, these data imply that the DAG levels in the cytosolic leaflet of membranes are very low, or even absent, and that in response to stimulation, a PI-PLC together with DGK generates a PA for signaling, and the NPC (or PLD/PAP) a DAG for metabolism.

DGK-specific PA responses

Biotic stress

The PLC/DGK pathway seems generally activated in pathogenic responses, resulting in a rapid accumulation of PA during exposure to elicitors or pathogenic infection by bacteria, fungi or oomycetes [37]. Specific host-pathogen interactions in cells/tissues of

tobacco and *Arabidopsis*, but also general elicitors typically induce a biphasic PA response [38-41]. Several of these studies showed that the first, rapid, phase (mins) involves a PLC/DGK pathway while the second phase (hours) involves a PLD. The concurrent biphasic accumulation of reactive oxygen species (ROS), is believed to be downstream from PA formation. In xylanase-treated tomato cells, the gaseous hormone nitric oxide (NO) was projected upstream of the first phase of PA-dependent ROS accumulation [42]. ROS formation and the oxidative burst often prelude the hypersensitive response (HR), which includes programmed-cell death of the infected and adjacent cells, biosynthesis of antimicrobial phytoalexins, and expression of pathogenesis-related (PR) genes. PA may interfere directly with oxidative stress signaling through interaction with a protein kinase pathway (see below) [29].

Benzothiadiazole (BTH) is a biologically active structural analogue of salicylic acid (SA) which induces resistance against pathogens. A rice DGK gene, *OsBIDK1*, was recently found to be transcriptionally induced by BTH or infection with the blast fungus *Magnaporthe grisea*. Moreover, tobacco plants, constitutively overexpressing *OsBIDK1,* displayed enhanced resistance against tobacco mosaic virus (TMV) and *Phytophtora parasitica* infection [43]. Along the same lines, an *Arabidopsis dgk5* knock-out mutant was found to be impaired in its basal resistance to a virulent *Pseudomonas syringae* strain (S.C.M. van Wees, B. van Schooten, J.E.M. Vermeer, S. van der Ent, C. Testerink, M.A. Haring and T. Munnik, unpublished). Moreover, the transcriptional induction of *PR1* by the pathogen or SA was almost completely abolished. These results provide the first genetic evidence that PA signaling through DGK is instrumental to the plant's defence response to pathogens. Interestingly, two genes that are required for *de novo* glycerolipid biosynthesis (via PA), have also been implicated in SA-dependent resistance signaling, i.e. *NHO1* and *SFD1*, encoding a glycerol kinase and a dihydroxyacetone phosphate reductase, respectively [44-47].

Abiotic stress

Salinity stress generates a PA response within seconds to minutes of NaCl application. This seems universal as many different plant systems show this, including green algae like *Chlamydomonas* and *Dunaliella*, suspension-cultured cells of tomato, tobacco, *Arabidopsis*, tobacco pollen tubes, and seedlings and leaves of *Arabidopsis*, rice and tomato [48-53]. Often, concomitant increases in the levels of DGPP and PIP_2 were reported.

Osmotic stress, in the form of drought or by treatment with mannitol or PEG, also generates PA via DGK although these responses look less severe than NaCl treatments and are usually slower. The down-stream responses for this PA increase are still unknown but in a screen for PA-binding proteins, two interesting protein kinases were found [54] which are known to be activated by osmotic stress and belong to the SNF-related

protein kinase 2 (SnRK2) family [55-57]. Whether PA is involved in their activation and/or recruitment is still unknown.

Cold-shock treatment (0 °C) of *Arabidopsis* suspension-cultured cells rapidly triggered PA formation [30]. During the first 10 min, DGK prevailed, accounting for approx. 80% of the total PA response, whereas later PLD activity dominates. In a transcriptome analysis of *Arabidopsis* seedlings, an 'early' (3 h) and long-lasting induction of *AtDGK1* was found, followed by a late induction (24 h) of *AtDGK2* [58]. The two genes seem to depend on the upstream transcription factor, ICE1 (inducer of CBF expression 1), which regulates many cold-induced genes, as their expression was differentially affected by the *ice1* mutation [58, 59]. These data suggest that the two structurally similar DGKs are regulated separately and have distinct functions in the cold response.

Vergnolle et al. [60] investigated the transcriptome of *Arabidopsis* suspension cells in response to cold stress, and used putative inhibitors of PLC and PLD (i.e. U73122 and 0.9 % 1-butanol, respectively), to implicate either pathway. As such, two groups of cold-regulated genes were discerned: 58 genes that were regulated via PLC and 87 genes via PLD. Only a few genes were downstream of both routes.

Freezing stress is different from cold in that it causes a dramatic drop in external water potential due to ice formation. Freezing injury triggers massive PA formation, mainly through PLD activity, which is unleashed when membranes get disrupted [53], although PLD could also play a role in the acclimation response [61]. Nonetheless, molecular species analysis of the fatty acids of *Arabidopsis* lipids after incubation of plants at -8 °C for 2 h, revealed the formation of a minor fraction of PA with a 34:6 fatty acyl structure (total carbon atoms : double bonds), which is typical of plastidial MGDG [31]. It was inferred that this galactolipid had been broken down to DAG, which was subsequently phosphorylated to PA, implying a DGK activity. Interestingly, isolated chloroplasts contain DGK activity [17] and PA can activate a specific MGDG synthase [62].

Although both PLC/DGK and PLD give rise to stimulus-triggered PA increase, differences in *DGK-*, *PLC-* and *PLD* gene expression, the regulation of their enzymatic activity and downstream responses suggest a functional separation of the two pathways. Apart from PA formation, the PLC/DGK pathway, of course, also entails DAG- and inositolpolyphosphate formation and the removal of PIP and PIP_2. Also, the fatty acyl composition of the resulting PA's are distinct. Future studies will have to evaluate the implications of these differences.

PA signaling and targets

PA formation has a profound effect on membrane curvature and surface charge [22, 63]. PA's small anionic phosphomonoester headgroup resides very close to the hydrophobic interior of the lipid bilayer, and this is different from other anionic phospholipids. Moreover,

hydrogen bonding increases the negative charge of PA, explaining why it can form strong interactions with target proteins, which has recently been proposed as the *Electrostatic/ hydrogen bond switch model* [64, 65]. The combined effects are likely to be crucial for specific PA responses [64, 66-69]. An excellent example of how PA formation affects vesicular trafficking was recently revealed by the observation that PA, in cooperation with BARS, induces membrane curvature to promote COPI vesicle formation [66].

Major progress has been made in identifying molecular targets for PA. Mammalian targets include protein kinases and phosphatases, and various proteins involved in vesicular trafficking [29, 70]. Also in plants, several PA-binding proteins have been identified (Table 1). Those characterized include the protein kinases CTR1 [71] and PDK1 [72] and the protein phosphatase ABI1 [73]. PDK1 is stimulated by PA whereas CTR1 and ABI1 are both inhibited. CTR1 and ABI1 are negative regulators in ethylene and ABA signaling, respectively. Through PDK1, PA enhances the activity of another AGC2 kinase, OXI1 [72], which mediates responses to ROS in root hair development and pathogens [72, 74]. PA has been shown to promote actin polymerization, through binding and inhibition of the actin capping protein, *At*CP [75]. Other proteins with affinity for PA include RCN1, a protein phosphatase 2A regulatory subunit that is involved in ABA, ethylene and auxin signaling [54], the aforementioned SnRK2 protein kinases that are activated by osmotic

Table 1. Plant PA target proteins

Protein	Function	Effect of PA	Lipid specificity	Method	Ref.
*Zm*CPK11	Calcium-dependent protein kinase	Activation	Anionic phospholipids	*In vitro* activity	[76]
*At*PDK1	Phosphoinositide-dependent protein kinase	Activation	Several PPIs and PA	Lipid beads, lipid overlay, liposomes, *in vivo* activity	[72, 79]
TGD2	PA transport into the chloroplast	Binding	PA	Lipid overlay	[78]
*At*CTR1	Raf/MLK-like protein kinase	Inhibition	PA>>PS	Lipid beads, liposomes, *in vitro* activity	[71]
PEPC	Phosphoenol pyruvate carboxylase	Inhibition	Anionic phospholipids	Lipid beads, *in vitro* activity *	[54]
AGD7	Arf GTPase activating protein (GAP)	Activation	PA	Lipid overlay, *in vitro* activation of GAP activity	[77]
ABI1	Protein phosphatase 2C	Inhibition	PA	Lipid overlay, ITC, *in vivo* ABA responses through ABI1	[73, 80]
*At*CP	Actin capping protein	Inhibition	PA, PIP_2	Lipid overlay, *in vitro* activity, intrinsic tryptophan fluorescence quenching	[75]

Abbreviations: ITC: Isothermal Titration Calorimetry, MLK: Mixed-lineage kinase, PPI: phosphorylated phosphatidylinositol. *Testerink, unpublished data.

stress [54], the calcium-dependent protein kinase *Zm*CPK11 [76], the ArfGAP AGD7 [77] and TGD2, a protein implicated in PA transport into the plastid [78] (see Table 1). However, a general PA-binding domain still remains obscure. While ABI1 has been shown to bind PA_{PLD} [73, 80], in most other cases it is still unclear which PA-generating pathway is involved [27, 29, 34, 71, 72, 79].

Perspectives

Clearly, PA is an important second messenger in plants, and DGK is one of the crucial contributors. Individual knock-out mutants in *Arabidopsis*, so far, have resulted in minor defects (Munnik lab, unpublished), which is likely due to redundancy within the large gene family of DGKs. As such, double- and even triple mutants will be required to determine which DGK is involved in which process and to investigate downstream gene expression responses. Secondly, it will be highly important to develop a PA-biosensor, using a specific PA-binding domain fused to a fluorescent protein, similar to what has been described for PI3P [51,81], PI4P [33], $PI(4,5)P_2$ [82-84], PS [70] and DAG [83,84]. Such tools will tell us where and when PA is generated, in response to the different stimuli. Co-expression of PA targets in another fluorescent color may further reveal where and how PA signaling works.

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