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REVIEW PAPER

Molecular, cellular, and physiological responses to phosphatidic acid formation in plants

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Abstract

Phosphatidic acid (PA) is an essential phospholipid involved in membrane biosynthesis and signal transduction in all eukaryotes. This review focuses on its role as lipid second messenger during plant stress, metabolism, and development. The contribution of different individual isoforms of enzymes that generate and break down PA will be discussed and the downstream responses highlighted, with particular focus on proteins that bind PA. Through characterization of several of these PA targets, a molecular and genetic basis for PA's role in plant stress and development is emerging.

Key words: DGK, diacylglycerol kinase, phosphatidic acid, phospholipase, phospholipid metabolism, phospholipid signalling, plant development, plant stress, PLC, PLD.

Introduction

Cellular membranes are composed of a wide range of different lipids, including sphingo-, neutral-, glyco-, and phospholipids, all with unique biophysical properties. While the majority of these lipids have a structural role, a few have direct signal-transducing properties. Hallmarks of such signalling lipids are their low abundance and rapid turnover. In response to environmental cues or endogenous signals, their synthesis is transiently increased so that they can activate downstream signalling pathways, leading to specific cellular events and physiological responses. In eukaryotes, typical signalling lipids include phosphatidylinositol lipids (polyphosphoinositides; PPIs), certain lyso-phospholipids, diacylglycerol (DAG), and phosphatidic acid (PA) (Meijer and Munnik, 2003; Wang, 2004; Munnik and Testerink, 2009; Xue *et al.*, 2009; Munnik and Vermeer, 2010). PA has emerged as a key molecule in cellular signalling and trafficking in several eukaryotes, including yeast, insects, mammals, and plants (Donaldson, 2009; Li *et al.*, 2009; Raghu *et al.*, 2009; Testerink and Munnik, 2005).

In plants, PA can be formed via different pathways. It is directly formed by the action of phospholipase D (PLD), which hydrolyses structural phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (Fig. 1). Plant PLDs come in two different flavours, the plant-specific, C2-domain-containing, α, β, δ, ϵ, and γ isoforms, and the PX- and PH-domain-containing ζ isoforms, the latter being conserved in all eukaryotes (Qin and Wang, 2002; Bargmann and Munnik, 2006; Li *et al.*, 2009).

PA can also be formed through the combined action of phospholipase C (PLC) and diacylglycerol kinase (DGK) (Fig.1; Arisz *et al.*, 2009; Munnik and Testerink, 2009). Two types of PLC enzyme have been identified: those that take PPIs as substrate, the PI-PLCs, and those that hydrolyse structural phospholipids, termed NPCs (for non-specific PLCs). In both cases, DAG is the product, which is then further phosphorylated to PA by DGK (Arisz *et al.*, 2009). PA derived from the DGK pathway can be distinguished from PLD-derived PA, based on its fatty acid composition and differential $^{32}P_i$-labelling characteristics (Arisz *et al.*, 2009).

Abbreviations: ABA, abscisic acid; ACBP, acyl-CoA-binding protein; DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; ER, endoplasmic reticulum; KO, knock-out; LPP, lipid phosphate phosphatase; NPC, non-specific phospholipase; PA, phosphatidic acid; ROS, reactive oxygen species; PPI, polyphosphoinositide; PM, plasma membrane; PLD, phospholipase D; PLC, phospholipase C; PEPC, phosphoenolpyruvate carboxylase; PC, phosphatidylcholine; PAMP, pathogen-associated molecular pattern; PA, phosphatidic acid; OE, overexpression.

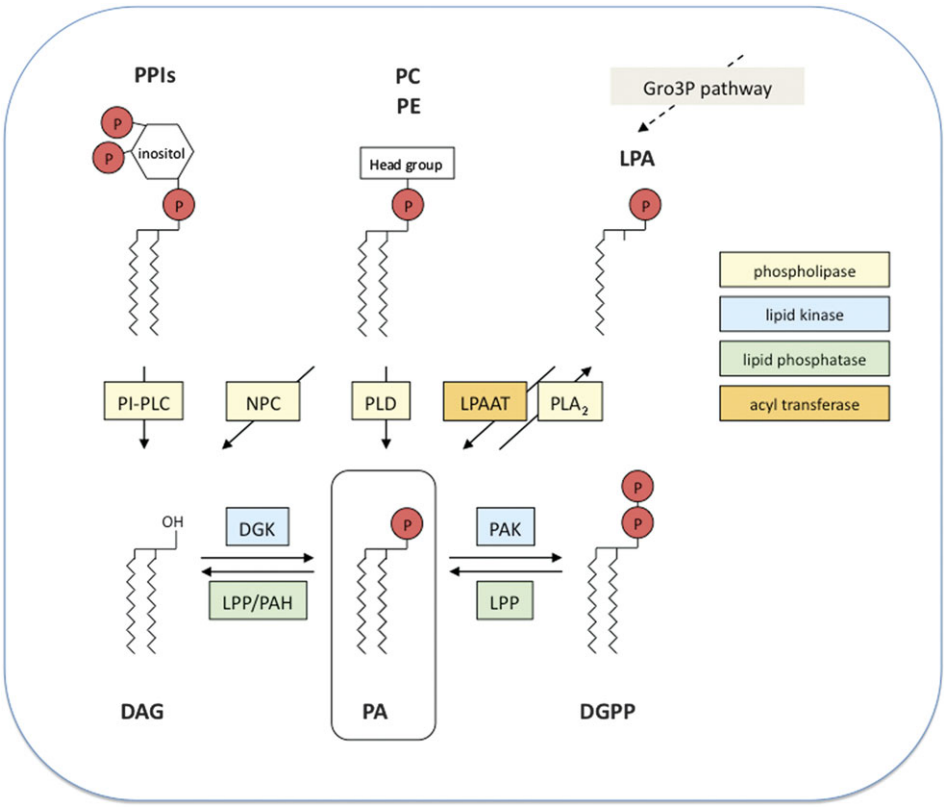

Fig. 1. Overview of PA formation and degradation pathways in plants, showing both 'signalling' and 'lipid metabolism' routes. Adapted from general eukaryotic pathways presented in Kooijman and Testerink (2010). Abbreviations: PE, phosphatidylethanolamine; LPA, lyso-PA; Gro3P, glycerol 3-phosphate; PI-PLC, PPI-hydrolysing phospholipase C; LPAAT, LPA acyltransferase; PLA_2, phospholipase A_2; DGPP, DAG pyrophosphate. Other abbreviations are given in the Abbreviations section.

The formation of signalling lipids in response to a stimulus is typically transient. Therefore, the enzymes that break them down are likely to be equally important for their signalling function as those involved in their synthesis. Enzymes that dephosphorylate PA include lipid phosphate phosphatases (LPPs) and PA hydrolases (lipins; PAHs). On the other hand, PA can be phosphorylated to diacylglycerol pyrophosphate (DGPP) by PA kinase (PAK) (van Schooten *et al.*, 2006) or metabolized to lyso-PA (LPA) through PLA_2 activity (Meijer *et al.*, 2001; Arisz, 2010).

Adding further to the complexity, PA is not only a signalling lipid, it is also an important intermediate in lipid biosynthesis (Ohlrogge and Browse, 1995). In plants, PA is formed by lysophosphatidyl acyltransferases from the Gro3P pathway-derived LPA pool (Fig. 1), at both the endoplasmic reticulum (ER) (Kim *et al.*, 2005) and the chloroplast (Kim and Huang, 2004; Yu *et al.*, 2004), where it functions as a precursor for phospho- and galactolipids, respectively (Athenstaedt and Daum, 1999; Arisz, 2010).

The 2005 review by the present authors concluded that over the years, PA had been firmly established as a plant lipid second messenger by then, but that a number of important questions still remained (Testerink and Munnik, 2005). These included: where is PA formed in the cell in response to its many stimuli; how is specificity achieved; and what does it take for a protein to bind PA? Unfortunately, most of these questions are still unanswered and remain the major challenges in the field. Nonetheless, great progress has been made in elucidating the contribution of individual isoforms of enzymes that generate or attenuate PA. The *Arabidopsis* genome already contains 12 PLDs, 9 PI-PLCs, 6 NPCs, and 7 DGKs, underlining the importance of regulating the formation of PA (Munnik and Testerink, 2009). The use of *Arabidopsis* knock-out (KO) mutants has revealed specific phenotypes for several of them in development and various stress responses, including those to pathogens and osmotic stress (see below). Moreover, substantial progress has been made in the identification and characterization of PA target proteins. Recent data and models, which have significantly increased our understanding of how PA interacts with proteins and how PA formation can induce downstream responses, will be discussed.

Plant stress responses

PA notably plays a role in plant stress signalling. Over the past decade, almost every environmental cue has been found to trigger a rapid (seconds–minutes) PA response. These include salinity, cold, drought, heat, wounding, and pathogen attack, through activation of either PLD, the PLC/DGK pathway, or both (Laxalt and Munnik, 2002; Testerink and Munnik, 2005; Arisz *et al.*, 2009; Li *et al.*,

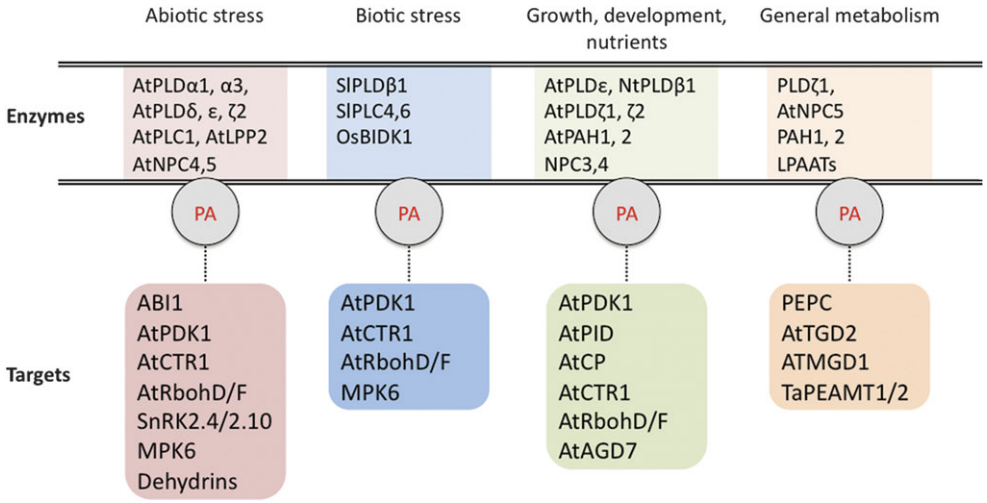

Fig. 2. Summary of pathways in plant stress, metabolism, and development involving PA. PA synthesizing and metabolizing enzymes are indicated for those cases where genetic data support their function in a pathway. Abiotic stress includes drought, freezing, cold, salinity, wounding, and responses to the stress hormones ABA and ethylene. Biotic stress includes senescence/cell death. Please note that not all listed target proteins are specific for PA, some of them also bind PPIs and/or other anionic phospholipids. Only proteins that have been shown to bind PA *in vitro* using at least two independent lipid-binding/activity methods or those for which *in vivo* evidence has been presented have been listed. For references, see text.

2009; Mishkind *et al.*, 2009). In accordance, various genetic data support a role for PA in these stress responses and meanwhile, several PA target proteins known to be involved in biotic and/or abiotic stress signalling have been identified (Fig. 2).

Osmotic stress and abscisic acid signalling

Osmotic stress, in the form of salinity or drought, triggers the fast and transient formation of PA in both green algae and higher plants, including *Chlamydomonas*, *Dunaliella*, *Arabidopsis*, tomato, tobacco, alfalfa, and rice (Einspahr *et al.*, 1988; Munnik *et al.*, 2000; Katagiri *et al.*, 2001; Meijer *et al.*, 2002; Arisz *et al.*, 2009; Bargmann *et al.*, 2009*b*; Hong *et al.*, 2010). In general, both PLC/DGK and PLD pathways are activated, with the notable exceptions of tobacco pollen tubes and rice leaves where salinity stress was found to inhibit PLD activity (Zonia and Munnik, 2004; Darwish *et al.*, 2009). Why some PLDs are activated while others are inhibited is not clear, but may involve tissue-specific expression of certain PLD isoforms or their regulators (e.g. affecting activity, membrane localization).

The contribution of individual plant-specific (C2-domain-containing) PLDs in salt and osmotic stress signalling has recently been reviewed (Hong *et al.*, 2010) and is only briefly summarized here. In *Arabidopsis*, especially the α- and δ-types have been shown to contribute to PA formation and salinity tolerance. Root growth of *pldα3* KO mutants or *pldα1/δ* double KOs is supersensitive to salt (Hong *et al.*, 2008*a*; Bargmann *et al.*, 2009*b*). *pld*ε KO mutant seedlings also exhibit reduced primary root growth under hyper-osmotic stress conditions (Hong *et al.*, 2009) but in this case, the phenotype is thought to be the result of a role of PLDε in nutrient signalling (see below). Besides its role in osmotic stress responses, the PLDα1 enzyme has also been implicated in cold, frost, and wound stress signalling (Bargmann *et al.*, 2009*a*; Hong *et al.*, 2010) and appears to act primarily by promoting responses to the stress hormone abscisic acid (ABA), especially in stomata (Mishra *et al.*, 2006).

Based on differential $^{32}P_i$-labelling experiments (Arisz *et al.*, 2009), also PLC/DGK pathways have been shown to be activated by salinity (Munnik *et al.*, 2000; Arisz, 2010). So far, no genetic evidence has been reported for individual contributions of DGKs. One of the PI-PLCs, *AtPLC1*, was shown to be induced in response to salinity and drought (Hirayama *et al.*, 1995) and to be required for ABA-induced inhibition of germination and gene expression (Sanchez and Chua, 2001).

More recently, an *Arabidopsis* NPC isoform, NPC4, was shown to modulate responses to ABA and to promote salt and drought tolerance (Peters *et al.*, 2010). *npc4* KO mutants displayed decreased responses to ABA in seed germination, root growth, and stomatal closure. Since addition of either DAG or PA to the growth medium could complement the *npc4* phenotype in roots, but DAG in the presence of a DGK inhibitor could not, it was concluded that PA is the active molecule in restoring the ABA response (Peters *et al.*, 2010). In accordance with the proposed positive role for PA in ABA responses, *lpp2* KO mutants, which accumulate higher levels of PA, are hypersensitive to ABA inhibition of germination (Katagiri *et al.*, 2005). It will now be interesting to establish the relative contributions and possible interactions between PLDα1 activity (Mishra *et al.*, 2006) and both PLC pathways in modulating ABA responses.

The identification of several ABA signalling proteins as potential PA targets has shed light on the molecular mechanism by which PA could mediate ABA responses.

PA was shown to interact with and inhibit the activity of ABI1, a protein phosphatase that negatively regulates ABA responses (Zhang *et al.*, 2004; Mishra *et al.*, 2006). More recently, the NADPH oxidase isoforms RbohD and RbohF were found to bind PA (Zhang *et al.*, 2009). PA stimulated NADPH oxidase activity both *in vitro* and in guard cell protoplasts. The RbohD PA-binding site was mapped to a region between its N-terminus and two EF hands. Site-directed mutagenesis of four positively charged residues in this domain abolished binding to PA. Transient expression of this non-PA-binding RbohD indicated that the PA–RbohD interaction is required for ABA-induced reactive oxygen species (ROS) generation and stomatal closure (Zhang *et al.*, 2009). From this work, a signalling pathway in guard cells is emerging in which PA is mainly generated through PLDα1 activity and positively regulates ABA responses through the promotion of ROS production and by inhibiting ABI1 protein phosphatase activity (Fig. 2).

Although less is known about PA's mode of action in salinity responses, several proteins involved in salt stress signalling are potential PA targets. In a proteomics screen for PA-binding proteins in *Arabidopsis*, an SnRK2 protein kinase was identified (Testerink *et al.*, 2004). The SnRK2s are generally involved in osmotic stress signalling in plants. The identified isoform belongs to a subclass that is activated by osmotic stress, but not by ABA (Boudsocq *et al.*, 2004). Another recently identified PA target is the MAPK isoform MPK6 (Yu *et al.*, 2010), which is activated by both abiotic and biotic stress, and is involved in stress signalling as well as in development (Colcombet and Hirt, 2008). MPK6 activation in response to salinity stress was shown to be dependent on PLDα1-generated PA formation. PA was found to bind recombinant MPK6 *in vitro*, and to stimulate MPK6 activity which was immunoprecipitated from *Arabidopsis* leaf extracts (Yu *et al.*, 2010).

Besides osmotic stress, salinity also involves an ionic component and induces signalling pathways that regulate ion transport to maintain ion homeostasis. (Zhu, 2002; Munns and Tester, 2008; Bertorello and Zhu, 2009). The Na^+/H^+ exchanger SOS1 plays a significant role by transporting Na^+ ions out of the cell upon exposure of roots to salt. Yu *et al.* (2010) found that MPK6 can phosphorylate SOS1 *in vitro* and that this activity can be stimulated by adding salt or PA. This raises the interesting possibility that salt-induced PA formation could impact on the SOS signalling pathway through modulation of MPK6 kinase activity.

Freezing/cold/wounding

Cold and frost induce the formation of PA through both PLD and DGK pathways (Ruelland *et al.*, 2002; Arisz *et al.*, 2009; Li *et al.*, 2009). Gene expression of *Arabidopsis* DGK1 and 2 isoforms has been shown to be induced by cold (Gomez-Merino *et al.*, 2004; Lee *et al.*, 2005), but again, no genetic evidence has been reported for their role in cold responses (Arisz, 2010). Intriguingly, PLDα1 and δ mutants were shown to exhibit opposite phenotypes during freezing stress. It seems that the formation of PA through the activity of the highly abundant PLDα1 is detrimental to cell membranes exposed to freezing or prolonged drought (Welti *et al.*, 2002; Devaiah *et al.*, 2007; Hong *et al.*, 2008*b*), whereas PA formed by the action of PLDδ helps the plant to acclimate to these stresses (Katagiri *et al.*, 2001; Welti *et al.*, 2002; Li *et al.*, 2004).

Acyl-CoA-binding proteins (ACBPs) belong to a family that share a conserved acyl-CoA-binding domain and have been implicated in lipid metabolism and repair of the membrane bilayers (Xiao and Chye, 2009). ACBP1 over-expression (OE) in *Arabidopsis* was shown to result in an increased PA/PC ratio and decreased freezing tolerance (Du *et al.*, 2010), while OE of ACBP6 increased freezing tolerance (Chen *et al.*, 2008). The observed effects were suggested to be mediated by PLD action, as ACBP6 OE plants show upregulation of *PLDδ* expression, while *PLDα1* expression is increased in ACBP1 OE plants, which is in accordance with the opposite roles of PLDα1 and δ reported before. The difference could be caused either by concentration of the lipid formed, or by their specific location. While PLDα1 is present in several internal membranes and the cytosol (Fan *et al.*, 1999), PLDδ seems to be located only in the plasma membrane (PM) (Li *et al.*, 2004). Interestingly, the ACBP1 isoform itself was shown to bind PA (Du *et al.*, 2010). Other potential targets related to cold and freezing tolerance are the dehydrin family of proteins. Because of their structure, they are thought to protect against freezing damage to membranes. Maize DHN1 was shown to bind several anionic phospholipids, including PA, via its K-segment domain (Koag *et al.*, 2003, 2009).

Responses to pathogens

Plants can sense the presence of their pathogens by recognizing certain pathogen-derived molecules. These can be general for many pathogens, in which case they are called pathogen-associated molecular patterns (PAMPs), which are recognized by plant receptors. To circumvent the resulting PAMP-triggered immunity, some pathogens also produce host-specific elicitors, called effectors (Jones and Dangl, 2006). Although recognition of PAMPs and effectors differs, in both cases it leads to the activation of very similar signalling pathways inducing plant defence responses (Boller and Felix, 2009).

Over the years, PA has been shown to accumulate in response to several PAMPs, including xylanase, flagellin, *N*-acetylchitooligosaccharide, and chitosan in tomato, alfalfa, and rice cells (Van der Luit *et al.*, 2000; Den Hartog *et al.*, 2003; Bargmann *et al.*, 2006; Raho *et al.*, 2011). Also specific effectors, such as *Cladosporium fulvum* Avr4 (de Jong *et al.*, 2004) and *Pseudomonas syringae* AvrRpm1 and AvrRpt2 (Andersson *et al.*, 2006) trigger PA responses in their hosts.

Recently, the first genetic evidence for a role of PLCs was reported by Vossen *et al.* (2010). Silencing of *SlPLC* isoforms 4 or 6 in tomato revealed that both were required for full resistance to infection by *C. fulvum*. Subsequently, it

was shown that while *SlPLC6* is required for the response to several pathogens, including *P. syringae* and *Verticillium dahliae*, *SlPLC4* seemed to be specific to *C. fulvum* Avr4-induced hypersensitive response, mediated by tomato Cf4 (Vossen *et al.*, 2010). In rice, expression of the DGK isform OsBIDK1 was shown to be induced by infection with *Magnaporthe grisae*. OE of the rice isoform in tobacco resulted in enhanced resistance to tobacco mosaic virus and *Phytophthora parasitica* infection (Zhang *et al.*, 2008).

In accordance with this, direct application of PA to leaves has been shown to induce pathogen-related gene expression and cell death (Park *et al.*, 2004; Andersson *et al.*, 2006). Although it is not clear how PA is taken up, in which membrane or cell it ends up, or whether it is even further metabolized, the data are all consistent with a positive role for PA in mediating responses to pathogens. In tomato cells, xylanase induces a PA response, which involves a PLDβ (Laxalt *et al.*, 2001). Silencing of this specific isoform, however, made these cells hyperreactive to xylanase (Bargmann *et al.*, 2006). These data would support a role for PA in the internalization of xylanase or its receptor EIX via receptor-mediated endocytosis (Ron and Avni, 2004).

The *Arabidopsis* ecotype Pi-0 is resistant to *Pseudomonas* infection due to a natural loss-of-function mutation in a conserved α/β-hydrolase, SOBER1 (Cunnac *et al.*, 2007). Recently, SOBER1 was shown to have PLA_2 activity, and PA was found to accumulate in the *sober1-1* mutant background in response to the AvrBsT elicitor (Kirik and Mudgett, 2009). Although the molecular mechanism behind PA accumulation in the *sober1-1* plants remains unclear, and other lipid mediators may be involved, this work is again consistent with a positive role for PA in plant defence.

How PA exerts these effects is still an open question. Several protein targets related to plant defence signalling have been identified. These include the PDK1 and MPK6 protein kinases and the RbohD and RbohF NADPH oxidases, which are also known to be involved in biotic stress responses (Rentel *et al.*, 2004; Torres and Dangl, 2005; Anthony *et al.*, 2006; Colcombet and Hirt, 2008). PA formation has also been implicated in ethylene signalling (Fan *et al.*, 1997), potentially via inhibition of the negative regulator CTR1 (Testerink *et al.*, 2007, 2008).

Plant growth and development

Plant growth and development not only follow strict developmental programmes, but are also subjected to regulation by various signalling networks that constantly monitor the environment. For example, gravity, nutrient and water availability, but also biotic stimuli affect development and direction of root growth (Malamy, 2005; Nibau *et al.*, 2008; Takahashi *et al.*, 2009). PA has recently been implicated to play a role in the growth modulation of roots and pollen tubes.

Auxin, phospholipid signals, and root development

The plant hormone auxin plays a central role in the regulation of flexible growth responses. Its mode of action requires the formation of gradients throughout the plant body, which depend on active cell-to-cell polar auxin transport. This process is largely controlled by the PIN-FORMED (PIN) protein family of auxin efflux transporters (Friml *et al.*, 2003) whose polar localization in the cell directs the flow of auxin. During their continuous recycling in the cell (Kleine-Vehn and Friml, 2008), PIN proteins are sorted to either the apical or basal PM, depending on phosphorylation by the PID AGC-type protein kinase and dephosphorylation by the PP2A phosphatases (Michniewicz *et al.*, 2007).

Reverse genetic data support a role for PA in *Arabidopsis* root development and gravitropism through the action of its two conserved (PX- and PH-domain-containing) ζ-type of PLDs (Fig. 2). In mammals and yeast, homologues of these PLDs are involved in membrane trafficking and are essential for membrane fusion in yeast sporulation and in endocytosis of membrane proteins (Roth, 2008; Donaldson, 2009). The *Arabidopsis PLDζ1* gene was identified as a direct target of the GLABRA2 transcription factor, which is a key determinant in root hair patterning (Ohashi *et al.*, 2003). Inducible expression of *PLDζ1* showed that it plays a role in root formation. *pldζ2* KO mutants on the other hand, displayed decreased sensitivity to auxin and a reduced root gravitropic response (Li and Xue, 2007). PLDζ2 OE and PA application resulted in enhanced vesicle trafficking of PIN2, as judged by their effect on reducing the presence of PIN2 in BFA compartments (induced by the exocytosis inhibitor, brefeldin A). This suggests a role for PLDζ2 and PA in the cycling of PIN2 protein and polar auxin transport, although the normal physiological circumstances under which PLDζ2 would regulate PIN2 recycling remain to be established (Li and Xue, 2007). Interestingly, *pldζ2* KO mutants were also shown to be impaired in root hydrotropism, which is the directional growth of a root towards moisture under drought conditions (Taniguchi *et al.*, 2010). By suppression of gravitropism, the drought-induced PLDζ2 protein is thought to accelerate the hydrotropic response.

PLD and PA might also be involved in polar auxin transport through regulation of PIN phosphorylation. Like the majority of AGC kinases, PID can be activated by the master regulator of AGC kinases, PDK1 (Zegzouti *et al.*, 2006*a*). PDK1 is activated through direct interaction with PA and PIP_2 (Anthony *et al.*, 2004), providing a possible link between lipid responses and PID phosphorylation. In addition, PID itself was shown to have affinity for several phospholipids, including PIP_2 and PA, using *in vitro* lipid-binding assays (Zegzouti *et al.*, 2006*b*; C. Testerink, unpublished data). Interestingly, also RCN1, one of the PP2A regulatory subunits that is required for dephosphorylation and proper targeting of PIN2 (Michniewicz *et al.*, 2007) was identified in a screen for PA-binding proteins (Testerink *et al.*, 2004). Thus, several lines of genetic and biochemical

evidence implicate a role for PA in polar auxin transport and the direction of root growth. Still, the actual presence of a protein kinase pathway, linking PA formation to PIN polarity and auxin transport, remains to be established *in vivo*.

Balancing growth with nutrient availability

Phosphate and nitrogen are essential macronutrients for plant growth. Under limiting conditions, plants improve uptake and utilization of these nutrients by adapting their metabolism, root architecture, and growth (Amtmann and Armengaud, 2009; Gojon *et al.*, 2009). PLDε, which belongs to the C2-domain-containing PLDs that are mainly involved in stress signalling, also appears to play a role in responses to nutrient availability. It specifically seems to be required for plants to sense and/or deal with low N availability, and to balance nutrient status with growth and biomass production (Hong *et al.*, 2009). *npc3* and *npc4* KO mutants showed mild phenotypes in root system architecture on low P_i medium, depending on brassinolide concentration (Wimalasekera *et al.*, 2010).

PA targets that have been implicated in nutrient sensing and growth have been identified and include PDK1 in plants (Anthony *et al.*, 2004) and TOR in mammals (Fang *et al.*, 2001). Gene expression of Arabidopsis *TOR* in response to salt or drought was found to be lower in *pldα3* KO plants compared to wild type (Hong *et al.*, 2008*a*). It will be interesting to establish whether not only expression but also TOR or PDK1-AGC kinase signalling would be dependent on PLDζ, ε, or α3 function and the production of PA *in vivo*.

Pollen tube growth, the cytoskeleton, and PA

PLD and PA have been identified as important regulators in the membrane–cytoskeleton interface. Both actin and microtubules have been implicated to interact with PLDs (Gardiner *et al.*, 2001, 2003; Munnik and Musgrave, 2001; Dhonukshe *et al.*, 2003; Kusner *et al.*, 2003; Pleskot *et al.*, 2010; Potocky *et al.*, 2003) and both structures are sensitive to primary alcohols, which affect PLD activity and the production of PA (Munnik *et al.*, 1995). Most recent knowledge comes from the Zarsky lab, which showed the involvement of NtPLDβ1 in regulating the actin cytoskeleton of tobacco pollen tubes (Pleskot *et al.*, 2010). Transient knock-down studies using antisense constructs revealed a moderate but significant impairment of pollen tube growth, which could be reversed by addition of exogenous PA. Interestingly, PA has been shown to induce actin polymerization in soybean cells (Lee *et al.*, 2003), while AtCP (actin capping protein), the ArfGAP AGD7, and also tubulins have been identified as potential PA targets (Testerink *et al.*, 2004; Huang *et al.*, 2006; Min *et al.*, 2007).

Plant (lipid) metabolism

Besides being produced by PLC/DGK and PLD pathways, PA is also formed *de novo* by acylation of lyso-PA at the ER, as a precursor of all phospholipids (Fig 1; Athenstaedt and Daum, 1999). Generally, this was considered as a separate PA pool, not participating in signalling. However, it is becoming apparent that lipid synthesis pathways also respond to stress and nutrient starvation, and that the presence of this PA pool is perceived by specific protein targets, leading to cellular responses. Similarly, PLD, which is considered a signalling enzyme, in some cases seems to function in membrane lipid degradation, rather than signalling (Bargmann *et al.*, 2009*a*). Thus, the distinction between stress signalling and lipid synthesis is becoming more and more vague. Signalling aspects of PA produced in lipid synthesis pathways will be discussed here.

De novo lipid synthesis at the ER

In yeast, PA was shown to act as part of a lipid-sensor complex on the ER to sequester the transcriptional repressor Opi1 (Loewen *et al.*, 2004). When sufficient inositol is present in the yeast growth medium, lipid synthesis turnover causes PA depletion from the ER, thus releasing Opi1, resulting in its translocation to the nucleus, where it coordinately represses the expression of genes involved in inositol biosynthesis. Recently, the PA–Opi1 interaction and its downstream responses were found to be dependent on intracellular pH, with lower pH resulting in decreased PA binding of Opi1 *in vitro* and its dissociation from the ER *in vivo* (Young *et al.*, 2010).

Also in plants, a central regulatory role of PA produced in primary metabolism, either as an intermediate in *de novo* lipid synthesis, or produced by NPC/DGK or PLDs, is emerging (Fig. 2). The recently identified phosphohydrolases PAH1 and PAH2 (also called lipins) negatively regulate synthesis of plant phospholipids at the ER (Eastmond *et al.*, 2010). In analogy to the yeast system, PA has been proposed to have a regulatory role in PC synthesis, as *pah1/2* double KO mutants not only had elevated PA levels, but also an increased rate of PC synthesis. This is in accordance with increased expression of several genes encoding enzymes involved in phospholipid synthesis in these mutants (Eastmond *et al.*, 2010). Interestingly, a wheat homologue of one of these, the PEAMT that catalyses the first committed step of choline synthesis, is itself regulated by PA binding (Jost *et al.*, 2009). Activity of two wheat PEAMT isoforms were shown to be inhibited by PA, which was suggested to be part of a feedback loop, limiting choline production under conditions of rapid phospholipid turnover or high PA levels induced by abiotic stress (Jost *et al.*, 2009).

Remodelling of lipid metabolism in response to P_i starvation

Galactolipids are essential building blocks of the chloroplast membranes, and include the non-phosphor-containing mono- and di-galactosyldiacylglycerols. A significant proportion of the synthesis of these lipids is derived from DAG synthesized at the ER (Moellering and Benning, 2011). The

PAH1 and 2 enzymes were postulated to be required for the synthesis of plastidial galactolipids via this pathway, since *pah1pah2* mutant plants contained lower amounts of galactolipids (Nakamura *et al.*, 2009). However, the observed reduction might rather be relative, and in fact, caused by the massive increase in phospholipid synthesis (Eastmond *et al.*, 2010).

Another pathway that has been suggested to play a role in the remodelling of galactolipids involves the recently identified NPCs. Gene expression of two homologues, *AtNPC4* and *5*, was shown to be upregulated by P_i starvation in leaves (Nakamura *et al.*, 2005; Gaude *et al.*, 2008). NPC5 specifically was shown to be involved in redirecting phospholipid metabolism to increased galactolipid production under these conditions.

Based on *pldζ1/ζ2* double mutants, also the PLDζs have been proposed to play a role in membrane remodelling under low-phosphate conditions. They were suggested to promote primary root elongation and to inhibit lateral root formation when starved of P_i. Loss of PLDζ2 not only resulted in decreased PA levels but also in an overall decrease in galactolipids in roots (Cruz-Ramirez *et al.*, 2006; Li *et al.*, 2006). On the other hand, analysis of *npc5/pldζ2* double mutants indicated that contribution of pldζ2 to galactolipid synthesis was negligible (Gaude *et al.*, 2008).

Several proteins involved in regulating galactolipid synthesis, including the TGD chloroplast import machinery and the MGD1 enzyme, have been found to bind PA (Fig. 2). The TGD2 protein binds ER-derived PA, thus allowing its import into the chloroplast (Awai *et al.*, 2006; Lu and Benning, 2009), where it is dephosphorylated to DAG, to serve as substrate for the synthesis of galactolipids. The MGD1 enzyme, which catalyses the formation of MGDG, is dependent on PA as a co-activator (Dubots *et al.*, 2010).

In summary, the role of PA in lipid metabolism might be broader than its function as a precursor for phospholipids. It also seems to play a role in regulating the net rate of lipid synthesis, as well as the balance between phospholipids and galactolipids, which becomes especially apparent under phosphate starvation. Whether the observed effects on tolerance to phosphate starvation and remodelling of chloroplast lipids are the direct result of a metabolic or signalling role of PA remains to be established. In the case of the *pldζ* mutants, the phenotypes could even reflect a general defect in membrane trafficking. While localization and molecular properties of mammalian and yeast PLDs have been well described, there is an urgent need for further characterization of the PLDζs in plants.

PA and phosphoenolpyruvate carboxylase function in photosynthesis and stress

Another metabolic enzyme whose activity is affected by PA is phosphoenolpyruvate carboxylase (PEPC). In C_4 plants, the PEPC enzyme is involved in carbon fixation, while in C_3 plants it has no such function. C_3 PEPC isoforms have been implicated in fine-tuning primary metabolism in response to P_i deprivation and osmotic stress (Gregory *et al.*, 2009; Chen *et al.*, 2010). The C_4 PEPC form was found to be inhibited by direct binding of anionic phospholipids and was shown to partially localize to non-soluble fractions of *Sorghum* leaf extracts (Monreal *et al.*, 2010*b*). Also upstream regulation of the C_4 PEPC by phosphorylation was shown to be regulated by PA (Monreal *et al.*, 2010*a*). C_3 PEPC was not inhibited by anionic phospholipids, but did bind PA directly and this binding was modulated by osmotic stress treatment (Testerink *et al.*, 2004). Thus, accumulation of PA, or other anionic phospholipids, could be a factor influencing PEPC activity, in both C_3 and C_4 plants.

How does PA signal?

Although the molecular and cellular mechanism by which PA exerts its effects is still largely unclear, data from plants, mammals, and yeast indicate that the formation of PA functions as a membrane-localized signal, affecting downstream responses by binding specific protein targets (Fig. 3; Testerink and Munnik, 2005; Raghu *et al.*, 2009). Targets include protein kinases, phosphatases, and various proteins involved in vesicular trafficking (Testerink and Munnik, 2005; Arisz *et al.*, 2009; Raghu *et al.*, 2009). PA binding regulates their activity, in some cases simply by recruitment, or alternatively by inducing direct conformational changes (Fig. 3b; reviewed in Testerink and Munnik, 2005).

A local increase in PA can also have a profound effect on membrane curvature and surface charge (Kooijman *et al.*, 2003), allowing it to positively modulate membrane fission and fusion (Fig. 3; Roth, 2008). So, even without binding of target proteins, the formation of the negatively charged, cone-shaped, PA alone is predicted to affect vesicle formation. In the case of mammalian BARS' promotion of

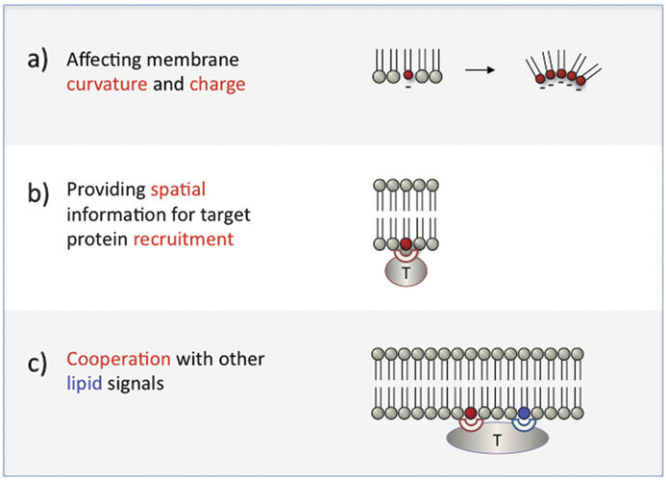

Fig. 3. Why are lipids so useful as signals? Schematic representation of the molecular mechanisms of PA's action as a lipid second messenger. Lipids with red head groups represent PA, T represents target protein.

COPI vesicle fission (Yang *et al.*, 2008) and yeast Spo20 function in sporulation (Nakanishi *et al.*, 2006), protein target binding as well as PA's direct effect on membrane curvature have been shown to play a role. Likely, the effects on membrane architecture, combined with the binding of specific protein targets, will be central to many cellular responses to PA formation (Kooijman *et al.*, 2003; Nakanishi *et al.*, 2006; Zeniou-Meyer *et al.*, 2007; Kooijman *et al.*, 2007; Roth, 2008; Yang *et al.*, 2008).

Molecular basis of PA–target binding

A model, called the 'electrostatic/hydrogen-bond switch' has been put forward to describe what actually makes PA different from other anionic phospholipids, and how protein domains can selectively bind PA (Kooijman and Burger, 2009; Kooijman and Testerink, 2010). In short, the phosphate headgroup of PA will likely carry a charge of $-1e$ at neutral pH. Upon hydrogen bonding with a positively charged residue, typically involving several lysine and/or arginine residues within a PA-binding domain, the headgroup will be further deprotonated to $-2e$. This increase in negative charge enhances the electrostatic interaction, and subsequent hydrogen bond formation results in docking of the PA-binding protein on di-anionic PA molecules (Kooijman and Testerink, 2010; Kooijman *et al.*, 2007). The model predicts that protein binding depends on local interfacial pH, since a decrease in cellular pH is predicted to increase the protonation of PA, thus weakening the PA–protein interactions. This was recently verified by Loewen and co-workers who showed that Opi1 binding to PA is indeed strongly pH dependent (Young *et al.*, 2010). Further validation of this model still awaits elucidation of the first crystal structure of a PA-binding domain in complex with the lipid.

PA cooperating with other cellular signals

A major unresolved question is how PA manages the multitasking required to perform all its different functions. Part of the answer lies in the close cooperation with other cellular signals, including other signalling lipids. For example, several PA-binding proteins have also been shown to bind PPIs (Fig. 3c). This can occur through the same domain, as in e.g. the AtPDK1 PH domain (Deak *et al.*, 1999) and the $p47^{PHOX}$ PX domain (Karathanassis *et al.*, 2002) or through another domain, as is the case for the C1 and C2 domains of mammalian PKCε (Lopez-Andreo *et al.*, 2003).

Besides the interaction with other signalling lipids, PA responses are integrated with many other cellular signals, including Ca^{2+}, ROS, nitric oxide (NO), cellular pH, and the cytoskeleton. These interactions are complex and at the moment their physiological impact is far from clear. For example, Ca^{2+} has been shown to be required for activity of certain PLDs (Li *et al.*, 2009), while also some target proteins require it for PA binding (Baillie *et al.*, 2002; Dominguez-Gonzalez *et al.*, 2007). ROS production has been shown to be a critical factor in responses to PA, too, by acting both upstream and downstream of PA formation (Sang *et al.*, 2001; Zhang *et al.*, 2003, 2009) and there appears to be a close relationship between PA and NO in plant defence, auxin and ABA signalling (Distefano *et al.*, 2008; Lanteri *et al.*, 2008; Raho *et al.*, 2011).

Conclusions and perspective

Since our previous review on PA's function in plant stress responses (Testerink and Munnik, 2005), many PI-PLC, NPC, DGK, and PLD enzymes have been further characterized, and specific physiological functions have been assigned to individual isoforms. Unexpectedly, besides their role in stress signalling, PLD enzymes also appear to have a function in general metabolism and plant development. Conversely, enzymes that were primarily thought to be involved in lipid metabolism, such as PA hydrolases and NPCs, might also have signalling roles. Whether these enzymes are also involved in the fast PA responses measured in response to stress, or whether the reported phenotypes rather reflect an overall change in physiology, is still an open question.

Another important factor will be to find out where exactly in the cell PA is being produced. While localization data are available for an increasing number of PA-generating enzymes, the plant field is still in urgent need of a *bona fide* PA biosensor to establish cellular location of PA formation, similar to those developed to image the phosphoinositides PI3P, PI4P, and PIP_2 (Vermeer *et al.*, 2006, 2009; van Leeuwen *et al.*, 2007).

Finally, significant progress has been made in identifiying and characterizing several of PA's protein targets. A model has been proposed to explain PA's unique properties and interaction with its targets. However, even 15 years after the identification of the first PA targets from mammals (Jenkins *et al.*, 1994; Ghosh *et al.*, 1996), and the identification of >30 PA targets from several eukaryotes, no consensus PA-binding motif has become apparent yet. Therefore, one of the current challenges is to solve the crystal structure of a PA-binding site in the presence of the lipid.

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