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REVIEW

Phosphoglycerolipids are master players in plant hormone signal transduction

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Abstract Phosphoglycerolipids are essential structural constituents of membranes and some also have important cell signalling roles. In this review, we focus on phosphoglycerolipids that are mediators in hormone signal transduction in plants. We first describe the structures of the main signalling phosphoglycerolipids and the metabolic pathways that generate them, namely the phospholipase and lipid kinase pathways. In silico analysis of Arabidopsis transcriptome data provides evidence that the genes encoding the enzymes of these pathways are transcriptionally regulated in responses to hormones, suggesting some link with hormone signal transduction. The involvement of phosphoglycerolipid signalling in the early responses to abscisic acid, salicylic acid and auxins is then detailed. One of the most important signalling lipids in plants is phosphatidic acid. It can activate or inactivate protein kinases and/or protein phosphatases involved in hormone signalling. It can also activate NADPH oxidase leading to the production of reactive oxygen species. We will interrogate the mechanisms that allow the activation/deactivation of the lipid pathways, in particular the roles of G proteins and calcium. Mediating lipids thus appear as *master players* of cell signalling, modulating, if not controlling, major transducing steps of hormone signals.

Keywords Phospholipase · Phosphoglycerolipids · Lipid kinase · Abscisic acid · Salicylic acid · Auxin · Signalling · Phosphatidic acid

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Introduction

Importance of hormones for plant physiology and development

Hormones are molecules produced by plant cells in response to environmental stresses and during plant development. The chemical natures of plant hormones are diverse. Some are related to adenine (cytokinins), others are terpenoids (gibberellins; abscisic acid, ABA) or polyhydroxysteroids (brassinosteroids) or are related to phenol derivatives (salicylic acid, SA) or derived from oxidised fatty acid (jasmonate). Some peptide hormones have also been described. Some plant hormones are volatile such as ethylene and some jasmonates. All hormones have in common that they act at extremely low concentrations. The first step of their action initiates when they are recognised by a receptor, thus triggering intracellular transduction and

amplification of the signal. This will ultimately lead to cellular and organ responses.

The importance of hormone in plant physiology can be briefly illustrated by a few examples. Pathogen attacks induce an increase in SA that then triggers a response leading to systemic acquired resistance. ABA is synthesised during drought stress and promotes stomatal closure and inhibits stomatal opening, thereby reducing transpiration and water loss. ABA therefore has a major role in protection against dehydration. In development, auxins, for instance, are well known to stimulate cell elongation, stimulate differentiation of phloem and xylem, mediate the tropic response of bending in response to gravity and light, to stimulate lateral root development and delay leaf senescence (Taiz and Zeiger 2010).

These few examples clearly illustrate that hormones intervene at many levels during the plant's lifetime. As a consequence, studying hormone signal transduction has always been considered primordial in plant biology. Hormone production, signalling and responses can affect vegetative growth, plant health and development of flowers, fruits and seeds, all aspects that are of high importance for agriculture.

For all hormone signalling pathways, major advances were achieved using genetics, mainly but not exclusively with Arabidopsis, where the focus is on the regulation of gene expression, the protein/protein interactions, the protein post-translational modifications and, more recently, the role of noncoding RNAs. For instance, now classical studies of promoter analysis allowed the identification of ABA-responsive element motifs (ABRE; Marcotte et al. 1989); the first step is to identify ABRE binding factors and their regulators. The isolation of ABA-insensitive mutants made it possible to show the importance of protein phosphorylation status in ABA responses (Leung et al. 1994). Those major findings and the ease of molecular biology tools using protein encoding genes eclipsed the fact that lipids were found early on to be signal mediators in hormone transduction (Scherer and André 1989). Recent studies have nevertheless put the production and involvement of lipids in hormone signalling back in the spotlight. Nevertheless, knowledge on lipids needs to be better integrated into some of the genetically defined hormone pathways.

We attempt this here by reviewing the current knowledge of the role of phosphoglycerolipids in hormone signalling. For each hormone signalling cascade known to require a phosphoglycerolipid pathway, we will describe which specific lipid signalling pathways are activated or inhibited. Ideas on how these pathways, and the molecules they generate, act and interact will be discussed. In doing so, lipid mediators, and in particular phosphatidic acid, will be shown to be involved in controlling well-established hormone signal transducers such as protein phosphatases or NADPH oxidases. Finally, we will discuss how the pathways can be activated or inhibited downstream of hormone perception. This review will thus propose mediating lipids to be *master players* of cell signalling, modulating, if not controlling, major transducing steps of hormone signals.

Overview of phosphoglycerolipid metabolic pathways

Phosphoglycerolipids are built from a glycerol backbone that is acylated by fatty acids on hydroxyls at positions *sn*-1 and *sn*-2. The hydroxyl group in the *sn*-3 position is esterified by phosphoric acid which is further esterified by another alcohol such as ethanolamine, serine, choline or *myo*-inositol (Fig. 1a). Signalling phosphoglycerolipids are generated through the action of phospholipases or lipid kinases (Fig. 1b). Structural phosphoglycerolipids such as phosphatidylcholine (PC), phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) can be hydrolysed by phospholipase D (PLD, EC 3.1.4.4) into phosphatidic acid (PA). PA can also be formed in the reaction catalysed by diacylglycerol kinase (DGK, EC 2.7.1.107). The diacylglycerol (DAG) substrates of DGK can be generated by the action of phospholipase C enzymes (PLCs) of which there are two kinds. Phosphoinositide-specific PLC (PI-PLC, EC 3.1.4.11) hydrolyses phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) into DAG and inositol-1,4,5-triphosphate (IP3). IP3 can be phosphorylated into higher phosphorylated forms of soluble inositol by inositol kinases (IPK). Phosphatidylinositol-4-phosphate (PI4P) may be an in vivo substrate of PI-PLC in plants as it is known to be an in vitro substrate and PI4P is 10- to 100-fold more abundant than PI-4,5-P_2 in plants (Delage et al. 2013). The so-called non-specific PLC (NPC, EC 3.1.4.3) hydrolyses structural phosphoglycerolipids into the corresponding phosphoalcohol and DAG (Pokotylo et al. 2013). Phospholipase A (PLA) catalyses the hydrolysis of ester bonds at the *sn*-1 and/or *sn*-2 positions of phosphoglycerolipids, thus liberating a free fatty acid (FFA) and forming a lysophosphoglycerolipid. PLAs may act specifically at the *sn*-1 or the *sn*-2 positions, and are named PLA1 (EC 3.1.1.32) or PLA2 (EC 3.1.1.4) accordingly (Ryu 2004; Scherer et al. 2010). PI4P and PI-4,5-P_2 result from the sequential phosphorylations of phosphatidylinositol (PI) catalysed by PI-4-kinases (PI4K, EC 2.7.1.67) and PI4P-5-kinases (PI4P5K, EC 2.7.1.68). PI can also be phosphorylated into phosphatidylinositol 3-phosphate (PI3P) by a PI-3-kinase (PI3K, EC 2.7.1.137). PI3P can then be phosphorylated into PI(3,5)P_2 (Delage et al. 2013). Finally, PA can be phosphorylated into diacylglycerol pyrophosphate (DGPP) by PA kinase (PAK) or dephosphorylated by phosphatidate phosphatase (PAP, EC. 3.1.3.4) or lipid phosphate

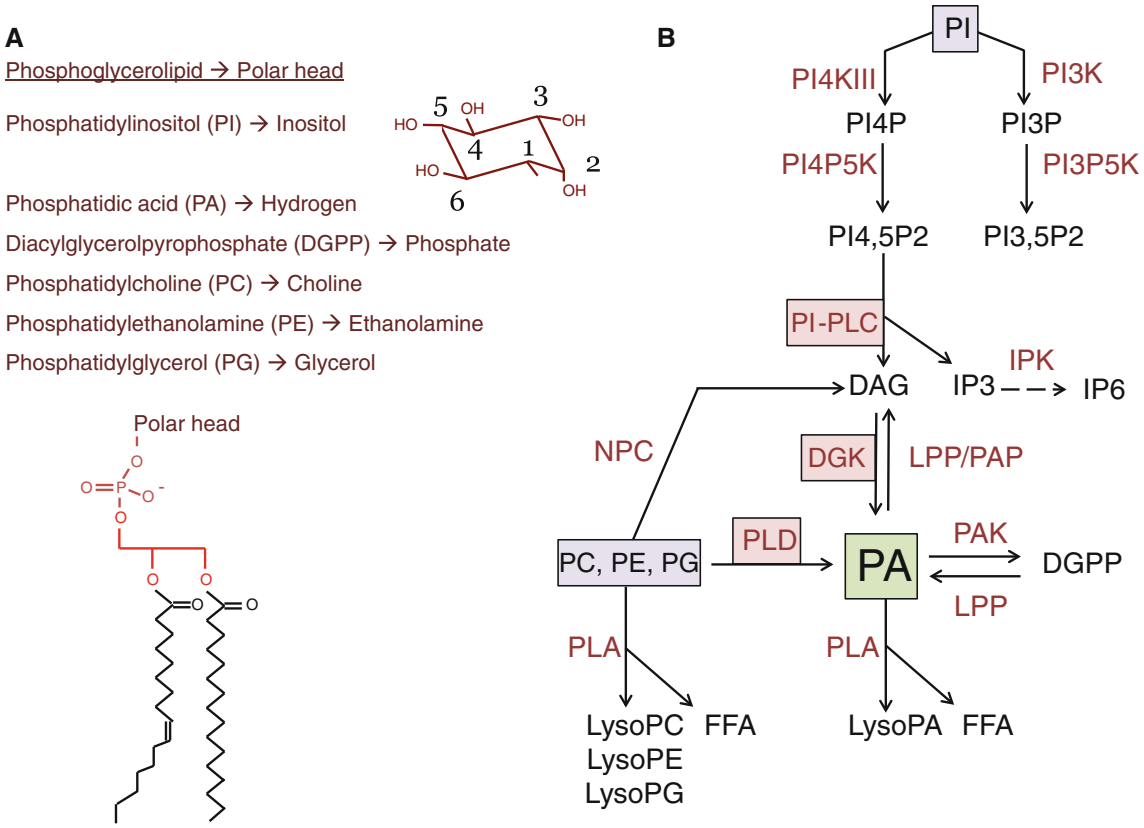

Fig. 1 Structure of glycerophospholipids (**a**) and metabolic pathways generating phosphoglycerolipid signal mediators (**b**). Each phosphoglycerolipid class differs according to the nature of the polar head. The structure of inositol is indicated; its hydroxyl at position 1 is the one engaged in forming the phosphodiester bond; the other hydroxyls can be phosphorylated leading to different phosphoinositides. *PI* phosphatidylinositol, *PI4P* phosphatidylinositol-4-phosphate, *PI3P* phosphatidylinositol-3-phosphate, *PI4,5P2* phosphatidylinositol-4,5-bisphosphate, *DAG* diacylglycerol, *IP3* inositol triphosphate, *IP6* inositol hexakisphosphate, *PA* phosphatidic acid, *DGPP* diacylglycerolpyrophosphate, *FFA* free fatty acid, *PI4KIII* type-III phosphatidylinositol-4-kinase, *PI3K* phosphatidylinositol-3-kinase, *PI3P5K* phosphatidylinositol-3-phosphate-5-kinase, *PI4P5K* phosphatidylinositol-4-phosphate-5-kinase, *PI-PLC* phosphoinositide-specific phospholipase C, *NPC* non-specific phospholipase C, *DGK* diacylglycerol kinase, *IPK* inositol phosphate kinase, *LPP* lipid phosphate phosphatase, *PAP* PA-phosphatase, *PAK* PA kinase, *PLA* phospholipase A, *PLD* phospholipase D

phosphatases (LPP, EC 3.1.3.76). DGPP can also be a substrate of LPPs (Pleskot et al. 2012). Phosphates in the inositol group of either soluble polyphosphate-inositol or in lipid phosphoinositides can be hydrolysed by *myo*-inositol polyphosphate phosphatases (EC 3.1.3.57) and PI phosphatases (EC 3.1.3.64/66) that are more or less specific for the soluble or lipid inositol forms (Williams et al. 2005).

Gene expression in response to hormones

A great deal of data implicating phosphoglycerolipids in hormone signal transduction is coming from transcriptome analysis (Lin et al. 2004). If a gene encoding an enzyme of the phosphoglycerolipid signalling pathways is upregulated in response to a hormone, it could mean that the encoded protein has a role in transducing the hormone signal. We analysed the relative levels of expression of genes encoding enzymes involved in phosphoglycerolipid pathways in *Arabidopsis thaliana* using Genevestigator (Hruz et al. 2008). We chose experiments in which RNA was sampled no later than 4 h after hormone treatment (Fig. 2). The transcript level of most genes encoding enzymes involved in the phosphoglycerolipid pathway changed in response to hormone. Within individual families of enzymes, some isoforms are induced and some repressed by the same hormone treatment, which suggests that different isoforms have different roles. For instance, *PLDβ*s, *γ1*, *γ3*, *ζ1* and *α1* are repressed by ABA. They are also repressed by the cytokinin zeatin, but induced by SA. The fact that ABA and zeatin have the same effect, which is opposite to that of SA, cannot be generalised though. *PLDδ* and *ζ2* are induced by ABA but are repressed by zeatin. It is striking that transcript levels of some PLA1 and plastid LPP genes are very much controlled by hormones, while their role in hormone transduction in plants is as yet poorly documented (Fig. 2).

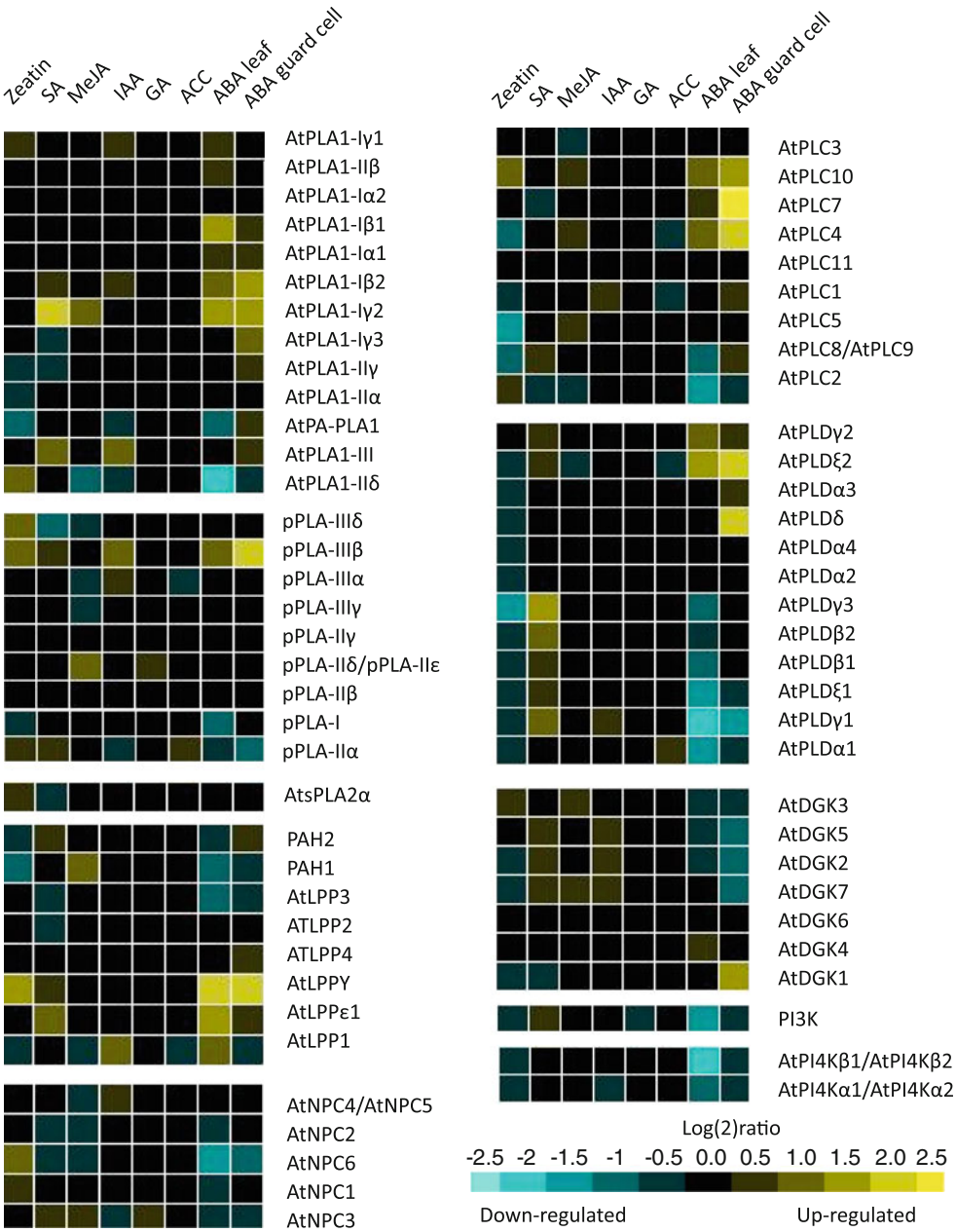

Fig. 2 Relative expression of genes encoding enzymes involved in phosphoglycerolipid signalling. The hormone, treatment duration and Gene Expression Omnibus code for each set of experimental data used in this analysis are as follows: zeatin, 3 h (AT-00239); SA, 4 h (AT-0039); methyl jasmonate (MeJA), 3 h (AT-00110); auxin indole-3-acetic acid (IAA), 1 h (AT-00407); gibberellic acid (GA), 3 h (AT-00110); ethylene precursor 1-aminocyclopropane-1-carboxylic-acid (ACC), 3 h (AT-00110); ABA, 3 h on leaf (AT-00433); and ABA, 3 h on guard cell (AT-00433). The AGI references for gene names are in Supplementary Table 1. Genes of each enzyme class were then sorted out after a Pearson's correlation clustering analysis in Genevestigator to evidence genes with close expression pattern in response to hormone treatments

Involvement of phosphoglycerolipids in hormone signalling

While transcriptome studies can indicate possible involvement, it is important to show that enzymes of these pathways are actually active and/or activated or inhibited in response to hormones. Mostly in vivo lipid labelling has been used for which suspension cells are the most amenable and relevant models. The availability of mutant plants is helpful to further narrow down the exact role of the in vivo activities measured.

Abscisic acid

The most documented description of phosphoglycerolipids in hormone signal transduction is for ABA. In *Arabidopsis thaliana* suspension cells, ABA plasmalemma perception results in a transient stimulation of phospholipase D (PLD) activity that is necessary for the activation of anion channels in the pathway leading to *RAB18* expression (Hallouin et al. 2002). This PLD activity leads to a transient increase in PA that is followed by an increase in DGPP, showing that a PAK is active. While exogenous PA activates

plasmalemma anion currents, it does not trigger the expression of *RAB18* or *RD29A*. On the contrary, exogenous DGPP mimics ABA in stimulating similar changes in both anion currents and gene expression. This suggests that DGPP could be the lipid mediator resulting from ABA activation of the PLD pathway in Arabidopsis suspension cells (Zalejski et al. 2005, 2006).

Guard cells generate reactive oxygen species (ROS) in response to ABA, which triggers stomatal closure. Inhibiting the production of PI3P or PI4P, or sequestrating them by the expression of PI3P-binding or PI4P-binding domains, inhibits ABA-induced stomatal closure (Park et al. 2003). The role of PI4P could be linked to its role as a precursor of substrates for PI-PLC. Indeed, endogenous IP3 levels were found to increase within 2 min of ABA application to stomata (Lee et al. 1996). The induction of some ABA-responsive genes was reduced in transgenic lines expressing an antisense *AtPLC1* (Sanchez and Chua 2001). The signalling molecules generated by the PI-PLC pathway activated in stomata in response to ABA are probably soluble phosphorylated inositols (polyphosphoinositols) as microinjection of IP3 into stomata initiates their closure (Gilroy et al. 1990). Reducing the level of polyphosphoinositols by overexpressing inositol polyphosphate phosphatases decreases the induction of some ABA-responsive genes (Sanchez and Chua 2001). By contrast, Arabidopsis *fiery1* (*fry1*), in which an inositol polyphosphate 1-phosphatase is mutated, accumulates more IP3 than the wild type upon ABA treatment with stronger induction of ABA-responsive genes (Xiong et al. 2001).

PLDs are also involved in ABA signal transduction in guard cells. PLDs can use primary alcohols as substrates to form a phosphatidylalcohol (in the so-called transphosphatidylation reaction) to the detriment of PA production. Secondary or tertiary alcohols are not substrates of the PLD-catalysed reaction. *n*-Butanol (*n*-ButOH) inhibits ABA-induced stomatal closure, pointing to a role for PLD-produced PA in this process. Similarly, in an Arabidopsis *pldα1 pldδ* double mutant, ABA-induced stomatal closure was suppressed. Accumulation of ROS and nitric oxide (NO), alkalisation of the cytosol, and inhibition of inward-rectifying K^+ channel currents that occur in wild-type ABA-treated guard cells are all inhibited in this double mutant. However, the calcium response to ABA is not altered in double-mutant guard cells (Uraji et al. 2012). While the above results suggest PLDs are upstream of the NO response, PLDδ could also be downstream of NO as *pldδ* mutants fail to close their stomata in response to NO (Distéfano et al. 2012). The mode of action of the PA produced by PLD*α1* in response to ABA is detailed in "Phosphatidic acid targets".

PA and DGPP levels increase when seeds are treated with ABA. In Arabidopsis, LPP-coding genes *AtLPP2* and *AtLPP3* are expressed during seed germination. *AtLPP2* insertion mutants are hypersensitive to ABA in terms of germination inhibition. This is correlated with greater accumulation of PA during mutant germination compared to WT, showing that PA relays the ABA effect on germination. Similarly, in rice, the expression of α-amylase, a key step in germination, is inhibited when seeds are treated with PA. Double-mutant analysis in Arabidopsis showed that ABA-insensitive 4 (ABI4) is epistatic to AtLPP2. PA thus appears to be upstream of the activity of ABI4, an AP2-type transcription factor. The origin of PA involved in ABA inhibition of germination might be due to PLD activities. In *Oryza sativa*, mutation of PLDβ1 inhibits the repression of germination by ABA and leads to a reduction in the repression of α-amylase by ABA (Li et al. 2007). PI-PLC could also participate in ABA inhibition of germination. Germination of seeds overexpressing the AtPLC1 gene is not inhibited by ABA (Sanchez and Chua 2001). Here again the active molecule generated by PI-PLC in seeds might be polyphosphoinositol. Seeds with a mutated inositol polyphosphate phosphatase gene, which have increased levels of IP3, have an enhanced sensitivity to ABA (Gunesekera et al. 2007).

Salicylic acid

In Arabidopsis suspension cells, we showed that SA stimulation led to the rapid activation of PLD. In the presence of primary alcohols that quench PA formation by PLDs, transcriptomic changes due to the hormone were strongly affected (Krinke et al. 2009). Interestingly, in the same model, SA also activated a type-III PI4K, leading to the formation of PI4P and PI-4,5-P_2. There was considerable overlap between PLD-controlled genes and PI4K-controlled genes. Amongst the genes controlled similarly by both pathways were *WRKY38* and *PR1*. This led us to hypothesise that SA activates PI4K, increasing PI4P and PI-4,5-P_2, with the latter lipid activating some PLD isoforms. However, *NPR1* induction by SA was abolished by including *n*-ButOH but not by inhibiting type-III PI4K, suggesting that in response to SA, both PI-4,5-P_2-dependent PLDs and PI-4,5-P_2-independent PLDs are brought into play (Krinke et al. 2007a, 2009). Identifying the PLDs activated in response to SA will help in validating this hypothesis. In Arabidopsis, the PI-4,5-P_2-dependent PLDs, *PLDβ2* and *PLDγs*, are induced by SA (see "Gene expression in response to hormones"), which might point to them having a role in SA transduction.

Do the pathways involved in the suspension cell SA response have the same role in plants? Possibly. The addition of primary alcohols blocks *PR1* expression in response to SA in Arabidopsis plantlets (Janda et al., unpublished). PI-PLC could also have a role in SA signal

transduction in plants. *PR1* is overexpressed in Arabidopsis plants expressing a mammalian inositol polyphosphate 5-phosphatase in control conditions (Perera et al. 2008).

It is also important to understand how SA activates PI4K. In in vitro enzymatic assays of microsomes extracted from *Capsicum chinense* Jacq. suspension cells 30 min after SA treatment, it was found that SA provoked an increase in lipid kinase activities leading to PI4P and PI-4,5-P_2 and a decrease in PI-PLC activity (Altuzar-Molina et al. 2011). This fits well with what we observed in vivo in Arabidopsis suspension cells. The very short period in which these in vitro changes occur after SA treatment most likely implies that PI4K and/or PI-PLC are subject to post-translational modifications, which will be confirmed by further investigation.

Auxins

As early as 1989, Scherer and André showed that when auxins were added to soybean suspension cells prelabelled with radioactive ethanolamine or choline, an increase in radioactive lysoPE or lysoPC occurred within 5 min. This was a strong indication that a PLA was activated. Interestingly, some PLA2 inhibitors were found to inhibit the growth of etiolated Arabidopsis hypocotyls, an auxin-driven mechanism (Holk et al. 2002), as well as the auxin-induced activation of the promoter of the *DR5* gene (Scherer et al. 2007). Plant PLA2 enzymes are encoded either by patatin-related PLA (pPLA) genes or by secreted PLA2 (sPLA2) genes. All single Arabidopsis pPLA mutants show delays in the up-regulation of early auxin-induced genes compared to WT. Thus, *ppla* mutants are considered to be auxin-signalling mutants (Labusch et al. 2013). Overexpression of *AtsPLA2β* promotes cell elongation, whereas silencing of *AtsPLA2β* expression retards it. *AtsPLA2β* is also rapidly induced by auxin treatments. To explain the elongation phenotypes, it is proposed that AtsPLA2β has a role in transducing auxin (Lee et al. 2003). PLAs are not the only phospholipases that have been shown to be involved in passing on the auxin signal. In cucumber, a PLD was activated, and PI4P and PI-4,5-P_2 also accumulated within 1 min of auxin treatment. Interestingly, the auxin-induced accumulation of these signalling lipids is dependent on auxin-induced NO production (Lanteri et al. 2008).

When considering auxin signalling, it is important to bear in mind that auxin has an asymmetrical distribution. This asymmetry is achieved through polar transport of auxin by influx or efflux auxin carriers. PINs are the best-characterised proteins controlling auxin efflux. Auxin distribution is altered in an inositol polyphosphate 5-phosphatase 13 mutant (Wang et al. 2009), whereas PIN distribution is affected in AtPLA_2α and AtPIP5K2 mutants (Lee et al. 2010). It has also been suggested that an At-PLDζ2 mutant was affected in PIN cycling (Li and Xue 2007). Finally, auxin synthesis might be controlled through PI-PLC. *Myo*-inositol and indole-3-acetonitrile, a precursor of the auxin indole-3-acetic acid, were up-regulated in the *rotunda1-1* (*ron1-1*) mutant. RON1 gene is identical to FRY1, mentioned in "Abscisic acid", which encodes an enzyme with inositol polyphosphate 1-phosphatase activity (Robles et al. 2010).

Which molecules act as mediators

Phosphoglycerolipid pathways generate molecules that can act as mediators by binding and thus altering the activity of effectors, or targets. Altering may imply activating or inhibiting the target and also sequestering it in a membrane, thus preventing the effector from reaching its site of action. Identifying these targets is a field of research in full swing. Although some targets have been identified, their roles in hormone signal transduction may not necessarily have been described. We will summarise current knowledge of the targets of molecules generated by phosphoglycerolipid pathways, always bearing in mind how these pathways may act in hormone signal transduction and how new actors may be identified.

Phosphatidic acid targets

PA is the common product of PLC/DGK and PLD pathways. Besides its involvement in hormone signalling as reviewed above, it has been shown to be involved in response to various abiotic and biotic stresses. Some PA binding proteins have been found in plants (Dubots et al. 2012; Wang et al. 2006). Table 1 lists a selection of plant proteins that bind PA and also have a role in signalling. The list is not exhaustive as protein kinases, protein phosphatases, lipid kinases and lipid phosphatases also bind PA in a way that modifies their activity.

The role of some of these proteins has been well described in studies of ABA responses. PLDα1 produces PA that binds to the ABI1 protein phosphatase 2C, a negative regulator of ABA action. This interaction inhibits the function of ABI1 by inhibiting its phosphatase activity and tethering it to the plasma membrane. PA formation by PLDα1 thus alleviates the inhibiting effect of ABI1 and allows gene expression through ATHB6. PA produced by PLDα1 has also been shown to interact with and stimulate NADPH oxidases. Interestingly, the interaction between PA and ABI1 is not required for the ABA-induced production of ROS and NO but is important for mediating the ROS effect on stomatal closure (Zhang et al. 2009). Finally another PA binding protein related to ABA signal

Table 1 Proteins that bind PA and have a role in signalling

Protein	Function	PA effect	References
Protein kinase			
ZmCPK11	CDPK	Stimulation	Klimecka et al. (2011)
SnRK2.4 SnRK2.10	Class 1 SnRK2 kinases	ND	McLoughlin et al. (2012)
PDK1	Protein kinase	Stimulation	Anthony et al. (2004)
MPK6	MAP-kinase	Stimulation	Yu et al. (2010)
CTR1	MAPKKK	Inhibition	Testerink et al. (2007)
Protein phosphatase			
ABI1	PP2C	Inhibition	Zhang et al. (2004)
PP1	Type 1 protein phosphatase	Inhibition	Takemiya and Shimazaki (2010)
Lipid kinase			
SPHK1 SPHK2	Sphingosine kinases	Stimulation	Guo et al. (2011)
PI4Kbeta	PI4 K	ND	Lou et al. (2006)
Lipid phosphatase			
PTEN2a	P3P phosphatase[a]	ND	Pribat et al. (2012)
Other mechanisms			
RbohD RbohF	NADPH oxidase	Stimulation	Zhang et al. (2009)
14-3-3	14-3-3 proteins	Inhibition[b]	Camoni et al. (2012)
AtCP	Capping protein	Inhibition	Huang et al. (2006)

ND not determined

[a] Dephosphorylates 3′ phosphate group of PI3P, PI-3,4-P_2 and PI-3,5-P_2

[b] 14-3-3 Proteins are regulatory proteins that bind client proteins in a phosphorylation-dependent manner, but PA binding significantly hampers the interaction with the client protein tested

transduction is sphingosine kinase (SPHK). The interplay between PLD and SPHK pathways could be complex. PA is involved in the activation of SPHK, but activation of PLDα1 requires SPHK activity, suggesting that SPHK and PLDα1 are co-dependent in amplification of the ABA response-mediating stomatal closure in Arabidopsis (Guo et al. 2012).

It would be interesting to know whether the same protein targets play the same roles in other hormone signalling pathways involving the production of PA. Kalachova et al. (2013) showed that Arabidopsis NADPH oxidase AtRbohD is also downstream of PA production in guard cells challenged with SA.

The other proteins listed in Table 1 have not been directly linked to hormone signal transduction, but they are very good candidates as mediators of hormone signals that lead to PA accumulation. Many of these proteins belong to multigene families, so other members of the families may also be involved in hormone signalling.

Diacylglycerol

Very few proteins that bind DAG have been identified in plants (Dong et al. 2012). In mammals, DAG binds protein kinase C (PKC) isoforms through their cysteine-rich C1 domain. Higher plants lack genes encoding PKCs. However, some kinases having the enzymatic properties of PKC have been detected, and some of them are activated by DAG, such as a *Brassica campestris* kinase (Nanmori et al. 1994). Plant genomes also have genes encoding proteins with C1 domains. For instance, the *Arabidopsis thaliana* genome encodes 30 confirmed or putative C1 domain proteins (Supplemental figure S1), including 2 DGKs. It is possible that, for some of these proteins, the C1 domain is an actual DAG binding domain. More data are necessary to decipher the exact role of DAG in hormone signal transduction.

Phosphorylated inositols

IP3 is produced through the action of PI-PLC on PIP2. In mammals, IP3 is well documented to bind to some calcium channels, thus allowing the release of calcium from internal stores into the cytosol. No IP3-binding channels have been found in land plants (Krinke et al. 2007b), although they do occur in Chlamydomonas (Wheeler and Brownlee 2008). It has been suggested that the more highly phosphorylated forms of inositol could be mediators in higher plants. For instance, inositol hexakisphosphate (IP6, also named phytic acid) has been shown to act as a more potent endomembrane calcium-release agent than IP3 in guard cells of *Vicia faba* (Lemtiri-Chlieh et al. 2003).

The auxin receptor transport inhibitor response 1 (TIR1) was unexpectedly found to have crystallised with IP6 (Tan et al. 2007), as was the jasmonate receptor COI1 (Sheard et al. 2010). Does this mean that they are polyphosphoinositide receptors and that PI-PLC signalling controls auxin and jasmonate perception? If so, this would be very interesting. However, we need to keep in mind that the PI-PLC pathway is not the only source of phosphorylated inositols. Glucose-6-P can be converted into IP3 by *myo*-inositol monophosphate synthase and IP3 can then be sequentially phosphorylated (Raboy 2009).

Phosphoinositides

Several phosphoinositide-binding domains are known, such as the pleckstrin homology (PH), FYVE, HEAT, C2 and Phox homology (PX) domains (Catimel et al. 2008). These domains are more or less specific for phosphoinositides as

some can also bind to other anionic phospholipids or proteins. The *Arabidopsis thaliana* genome encodes ca. 168 proteins with at least one of these domains (Supplemental figure S2). Many of these proteins are involved in signal transduction, in cell fate determination or intracellular trafficking. For most of these proteins, however, it has yet to be established whether they act in hormonal signal transduction.

Some phosphoinositides control cellular processes. In animal cells, a wide range of channels are controlled by PI-4,5-P_2. In *Nicotiana tabacum* protoplasts isolated from cultured cells with genetically altered plasma membrane levels of PIP2, the net steady-state outward K^+ current was inversely related to the plasma membrane PIP2 level. Increasing PIP2 levels in controls or in ABA-treated cells with high PI levels, using the PI-PLC inhibitor U73122, decreased the outward-rectifying potassium channel (NtORK) activity. These results would be consistent with the negatively charged PI-4,5-P_2 in the inner plasma membrane leaflet inhibiting NtORK (Ma et al. 2009).

Tubby and Tubby-like proteins (TLPs) were first discovered in mammals where they are involved in the development and function of neuronal cells. The binding of the carboxyl-terminal Tubby domain to PI-4,5-P_2 sequestrates these proteins to the plasma membrane. When PI-PLC is activated, PIP2 is hydrolysed and thus TLPs are released from the plasma membrane. TLPs are conserved across eukaryotic kingdoms. Hydrogen peroxide stimulates the release of AtTLP3 from the plasma membrane in a mechanism involving PI-PLC (Reitz et al. 2012). Therefore, Tubby proteins could be a link between PI-PLC and oxidative stress, and hence to hormone signalling. However, in Arabidopsis, no phytohormone treatment (with methyljasmonate, ABA, SA, auxin, brassinosteroid, or GA) leads to relocalisation of AtTLP3. Therefore, the possible role of other Arabidopsis TLPs in hormone transduction in conjunction with PI-PLC is uncertain.

Lysophosphoglycerolipids and free fatty acids

Lysophosphoglycerolipids and FFA are released by the action of PLA. Both types of molecules have mediating roles. For instance, oxidised fatty acids in potato suspension cells in vivo and saturated fatty acids in vitro stimulate protein kinase activity. Unsaturated fatty acids inhibit MP2C, a protein phosphatase, while polyunsaturated fatty acids can modulate K^+ channels. Oleic acid activates PLDδ. The lysophosphoglycerolipid lysoPE inhibits a PLD in fruit senescence. It has been postulated that lysoPC could activate an H^+/Na^+ exchange vacuolar transporter, thus mobilising the vacuolar proton pool for pH signalling (references in Scherer et al. 2010). Clearly, more work is necessary to identify other targets of FFA and lysophosphoglycerolipids.

Conclusion

Open questions

Clearly lipid signalling is of major consequence in plant hormone signal transduction. Yet many questions remain open that will define the immediate future of this fascinating research field.

Lipid signalling in the signal transduction of other hormones

Phosphoglycerolipid signalling is now well documented for ABA, SA and auxins. Does it also play a role in the transduction of other hormone signals? Very promising data suggest so. Methyljasmonate has been shown to activate PLD in *Brassica napus*, *Taxus cuspidata* and *Silybum marianum* cells (Profotová et al. 2006; Yang et al. 2008; Madrid and Corchete 2010; Sánchez-Sampedro et al. 2009). In Arabidopsis plantlets, mechanical wounding known to induce jasmonate led to a 4- to 5-fold increase in IP3 with concomitant depletion in phosphoinositides. No such IP3 increase was observed in *dde2-2* mutant plants that are deficient in JA biosynthesis (Mosblech et al. 2008). Cytokinins activate PLD as a step in the control of gene expression in Arabidopsis (Romanov et al. 2002) and *Catharanthus roseus* suspension cells (Amini et al. 2008). In Amaranthus seedlings, a PI-4,5-P_2-dependent PLD controls the accumulation of amaranthin (a pigment) that follows cytokinin application (Kravets et al. 2010). Ethylene activates PLD in carrot suspension cells (Lee et al. 1998). PLDα-deficient transgenic plants have slower rates of senescence induced by ethylene than WT (Fan et al. 1997). Gibberellic acid (GA) leads to a rapid and transient IP3 accumulation in barley aleurone (Villasuso et al. 2003). Interestingly, in rice seeds, ABA prevents the gibberellin-induced formation of IP3, although ABA alone does not alter the IP3 level (Kashem et al. 2000). Brassinosteroid treatment of Arabidopsis seedlings leads to increased expression of *NPC3* and *NPC4* monitored by GUS activity in leaves (Wimalasekera et al. 2010). LysoPE, a product of PLA2 activity, collaborates with brassinosteroids during stimulation of Arabidopsis root growth and development (Jeong et al. 2012).

How are the lipid signalling pathways activated or inhibited in response to hormones?

The upstream events leading to the regulation of phospholipid signalling pathways are far less well understood than the downstream processes.

Calcium has an important role in controlling the activity of enzymes involved in lipid signalling. PI-PLCs are

strictly dependent on calcium for their activity, except AtPLC4 that retains 20 % of its activity with no calcium. Most PLDs need calcium for their activity and only PLDζ isoforms have shown to be calcium independent. PLDα enzymes need higher concentrations of calcium than other calcium-dependent PLDs, which may indicate that this is an important regulatory mechanism. Some PLAs, both pPLA2 and sPLA2 isoforms, have shown to be calcium dependent (Chen et al. 2011).

Some enzymes of the phosphoglycerolipid pathway may be post-translationally modified in a way that modulates their activities. In *Brassica oleracea*, a PIP2-dependent PLD is phosphorylated and this phosphorylation might positively regulate its activity (Novotná et al. 2003). Arabidopsis PLDα1 also seems to be regulated by this mechanism (Novotná, unpublished results). Arabidopsis patatin-related PLA2 pPLA-IIδ and pPLA-IIε are phosphorylated by calcium-dependent protein kinases in vitro (Rietz et al. 2010). Regulation by phosphorylation is probably a major component of the activation/inhibition of lipid pathways. The PhosPhAt database curates the sequences of experimentally detected phosphorylated peptides obtained in Arabidopsis phosphoproteomics experiments (http://phosphat.mpimp-golm.mpg.de/download.html; Durek et al. 2010), and includes many that originate from enzymes of the phosphoglycerolipid pathway (Supplemental Table 2). However, the significance of these phosphorylations in relation to hormone signalling has not been investigated.

An important mechanism in phosphoglycerolipid signalling seems to be the coupling with heterotrimeric G protein (Tuteja and Sopory 2008). Potential G-protein activators and inhibitors, such as toxins, nonhydrolyzable guanine nucleotide analogues, and alcohols, affect plant PLD (Munnik et al. 1995; Ritchie and Gilroy 2000), and PLD can also control G proteins. The direct interaction between AtPLDα1 and the α-subunit of heterotrimeric G proteins (GPA1) has been characterised using protein co-precipitation. PLDα1 binds to GPA1 and activates the intrinsic GTPase activity that converts active Gα-GTP to inactive Gα-GDP. In turn, Gα-GDP binds to PLDα1 and attenuates its activity. Thus, the PLDα1–GPA1 interaction mutually regulates both partners (Mishra et al. 2006). An intriguing possibility that follows on from this observation is that PLD might be regulated by G protein-coupled receptors. G proteins also regulate PI-PLC. In tobacco cells overexpressing either Arabidopsis G protein-coupled receptor (GCR1) or the Gα-subunit of the heterotrimeric G protein, PI-PLC is active and IP3 levels are elevated (Apone 2003). The G-protein activator Mas7 is able to trigger α-amylase secretion in barley aleurone just as strongly as GA does. U73122 inhibits this Mas7 effect, linking the Mas7 effect with PI-PLC activity (Villasuso et al. 2003). *Pisum sativum* Gα interacts with the C-terminal C2 domain of pea PLCδ (Misra et al. 2007). It is still not known whether all PI-PLC and PLD isoforms are dependent on G protein activity.

An intriguing aspect of lipid signalling regulation may be the role played by the interaction of phospholipases and signalling phospholipids with components of the cytoskeleton. In tobacco, monomeric G-actin inhibits and polymeric F-actin enhances the PIP2-dependent activity of NtPLDβ1, while PIP2-independent PLDα activity and oleate-activated PLDδ activity are unaffected by either form of actin (Pleskot et al. 2010). PA has also been shown to modulate actin dynamics both in vitro and in vivo by binding to capping protein (Li et al. 2012; Pleskot et al. 2012).

Finally, it should be noted that the different phosphoglycerolipid pathways are likely to be intertwined. For example, plant PLDs need PI-4,5-P_2 as cofactors, the exceptions being the α-type PLDs. Therefore, PI-PLC activity might indirectly modulate PLD activity, by modulating the level of phosphoinositide cofactors.

What is the link with known hormone receptors?

The first step in signalling is when a receptor perceives the hormone. However, when phosphoglycerolipid signalling is known to be involved in a specific hormone signalling pathway, the link to the hormone receptor(s) has not been made. It has recently been proved that the PYR/PYL/RCAR family of soluble proteins in Arabidopsis are ABA-binding receptor-like proteins. Upon ABA binding, these PYR/PYL/RCAR receptors bind to and inhibit PP2C allowing the phosphorylation and activation of SnRK2 proteins. It is not certain how PA fits into this scenario given that PA interacts with ABI1. Is PLDα1 activated upon ABA binding to PYR/PYL/RCAR soluble receptors? Or does PLDα1 activation involve a yet-to-be-discovered receptor? We note that a membrane impermeant ABA–bovine serum albumin conjugate, which cannot enter the cell, is able to activate PLD in Arabidopsis suspension cells (Hallouin et al. 2002). Different proteins are known to bind SA, such as catalase, ascorbate peroxidase, chloroplast carbonic anhydrase, methyl salicylate esterase, some α-ketoglutarate dehydrogenases and some glutathione S-transferases (Tian et al. 2012). NPR1 (Wu et al. 2012) and its paralogues NPR3 and NPR4 (Fu et al. 2012) are proteins that have also been shown to bind SA. Thus, it needs to be elucidated whether or not lipid signalling is connected to these "receptors", and if so how (whether directly or via other proteins such as G proteins). Auxin is perceived either by ABP1 or TIR1/auxin-signalling F box proteins. It has been suggested that the receptor responsible for pPLA activation by auxin is ABP1, but more

experimental data are necessary to establish this (Scherer et al. 2012).

How lipid mediators act

More work is needed to discern the modes of action of the lipid mediators. There is now quite a long list of PA binding proteins, but it is unlikely to be exhaustive. No DGPP binding proteins have been identified so far either in plants or in other biological kingdoms. Nevertheless, the specific role of DGPP as a hormone signal transduction player seems beyond doubt since DGPP has effect that PA has not (Zalejski et al. 2006). Finding DGPP binding proteins is therefore a challenge ahead.

Most of the molecules generated by these pathways are lipids and stay in membranes. This gives rise to two questions. First, how do these lipids affect the physical state of the membrane—its fluidity, flexibility, or capacity to curve. PA, for instance, is considered to be a cone-shaped lipid. Lysophospholipids have inverted cone shapes. FFAs make membranes more fluid. Do such changes in the physical state of membranes have a role in hormone signal transduction as part of lipid signalling? It is believed that vesicle budding is favoured by PLA2 activity, because its action on a single leaflet of the bilayer asymmetrically increases the number of inverted cone-shaped lysophospholipids, thus enhancing membrane curvature towards the said leaflet side (Brown et al. 2003). PLD products of PA could have similar effects. PA is a fusogenic lipid (Jenkins and Frohman 2005). Vesicle formation, mobility and fusion are necessary for hormone signalling as exemplified by the trafficking of PIN proteins in auxin responses. Discovering the importance of lipid mediators not through binding proteins but through membrane effects will be a fascinating field of research.

In conclusion, phosphoglycerolipids appear to be major players in hormone transduction. The data obtained so far suggest they are involved in the signalling of most, if not all, plant hormones. Much more data will be necessary to understand how these pathways are regulated and how they act to transduce hormone signals. The same phosphoglycerolipid players are involved in the responses to different hormones and this could explain in part the crosstalk between hormones. However, the exact timing, duration and amplitude of the phosphoglycerolipid responses, and their interacting partners, probably differ going some way to explain the specificity of hormone responses.

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