Plant phospholipid signaling: “in a nutshell”

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Abstract Since the discovery of the phosphoinositide/phospholipase C (PI/PLC) system in animal systems, we know that phospholipids are much more than just structural components of biological membranes. In the beginning, this idea was fairly straightforward. Receptor stimulation activates PLC, which hydrolyses phosphatidylinositol4,5-bisphosphate [PtdIns(4,5)P2] into two second messengers: inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DG). While InsP3 diffuses into the cytosol and triggers the release of calcium from an internal store via ligand-gated calcium channels, DG remains in the membrane where it recruits and activates members of the PKC family. The increase in calcium, together with the change in phosphorylation status, (in)activates a variety of protein targets, leading to a massive reprogramming, allowing the cell to appropriately respond to the extracellular stimulus. Later, it became obvious that not just PLC, but a variety of other phospholipid-metabolizing enzymes were activated, including phospholipase A, phospholipase D, and PI 3-kinase. More recently, it has become apparent that PLC, which hydrolyses phosphatidylinositol4,5-bisphosphate, is not just a signal precursors but can also function as signaling molecules themselves. While plants contain most of the components described above, and evidence for their role in cell signaling is progressively increasing, major differences between plants and the mammalian paradigms exist. Below, these are described “in a nutshell.”—Munnik, T., and C. Testerink. Plant phospholipid signaling: “in a nutshell.” J. Lipid Res. 2009. 50: S260–S265.

Supplementary key words phosphatidic acid • phosphoinositide • phospholipase

PLC SIGNALING

If we did not know about the existence of the PI/PLC system from animals and only had the data from plants today (1–5), then we would have never come up with a signaling system as depicted in Fig. 1. The reasons for this are briefly summarized below.

No inositol 1,4,5-trisphosphate receptor nor PKC. Several plant genomes have been sequenced nowadays, including Arabidopsis, rice, and poplar, and many expressed sequence tag libraries of various higher plant species are available, but none of them seem to encode an InsP3 receptor (6). An exception is Chlamydomonas, a unicellular green algae with two flagella, where an InsP3 receptor has been identified. Other ciliated organisms, such as Paramecium, also contain an InsP3 receptor. Apparently, higher plants have lost this in evolution (6). Similarly, the most important diacylglycerol (DG) target, PKC, is lacking from plant genomes, including lower plants. There are numerous articles describing an effect of “PKC-specific” inhibitors, but these most likely reflect protein kinases that are not present in mammals. These include calcium/calmodulin-dependent protein kinase (CDPK), calcineurin B-like proteins that interact specifically with a group of CBL-interacting protein kinases (CIPK), and AGC kinases (with similarities to PKA, PKC, and PKG) (7). CDPKs and CIPKs respond to calcium, while some members of the CDPK and AGC family may be regulated by phosphoinositides and/or phosphatidic acid (PA; see below). Although we were hopeful before the genomic era (3), we have to accept now that plants lack PKC.

Low on PtdInsP2. Labeling experiments using 32P, or 3H-Inositol, but also recent PtdInsP2 biosensor (GFPPHPLC2) studies, have shown that plant cells contain extremely low amounts of PtdInsP2 (8–10). In contrast, PtdIns4P levels seem to be normal, i.e., similar to those observed in animals. Typical 32P-ratios between PtdInsP and PtdInsP2, measured in many different plant cells and tissues, reveal 30– to 100-fold lower PtdInsP2 levels than PtdInsP. In animals and Chlamydomonas, the PtdInsP:PtdInsP2 ratio is usually close to 1 (1–3).

Abbreviations: ABA, abscisic acid; CDPK, calcium/calmodulin-dependent protein kinase; DG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerolpyrophosphate; InsP3, inositol 1,4,5-trisphosphate; IPK, inositol dual-specificity polyphosphate multikinase; JA, jasmonic acid; PA, phosphatidic acid; PAK, phosphatidic acid kinase; PH, Pleckstrin homology domain; PI/PLC, phosphoinositide/phospholipase C; PLA, phospholipase A; PLD, phospholipase D; PtdInsP2, phosphatidylinositol4,5-bisphosphate; ROS, reactive oxygen species.

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Plant PLCs belong to the PLCζ class. Eukaryotic PI/PLCs have been classified into β, γ, δ, ε, η, and ζ subfamilies (Fig. 2). While mammalian cells contain all six isoforms (13 in total), plants exhibit only one class of PLCs. Originally, these were classified as PLCα isoforms (4); however, after the recent discovery of the sperm-specific mammalian PLCζ, which lacks the typical Pleckstrin homology (PH) domain, it became clear that plant PLCs belong to the PLCζ class (Fig. 2) (12).

The ζ-isoform represents the most simple PI/PLC, consisting of the minimal core structure: the catalytic X- and Y-domain, an EF-hand domain, and a C2 lipid binding domain (Fig. 2). Additional subfamilies contain the PH domain and various other conserved sequence regions, allowing PLCζs to be regulated by trimeric G-proteins, PLCβs by tyrosine kinases, and PLCεs by trimeric G-proteins and Ras. It is still not clear how PLCζ, -η, and -ζ isoforms are regulated, but this may involve calcium, especially for PLCζ (Fig. 2) (11).

Plant PLC activity indeed requires calcium. At low micromolar Ca^{2+} concentrations, both PtdIns4P and PtdIns(4,5)P2 are hydrolyzed and at millimolar concentrations, PLC also uses PtdIns as substrate (3). In vivo, PLC has always been assumed to hydrolyze PtdIns(4,5)P2. However, plants have no, or very little, PtdInsP2 in their membranes, and an InsP3 receptor is lacking, so wouldn’t it make more sense to propose PtdInsP3 as the in vivo substrate? In vitro, PtdInsP4 is equally well hydrolyzed as PtdIns(4,5)P2, and in vivo, PtdInsP4 turnover and quantities are much more in agreement with the PA responses resulting from DG phosphorylation than PtdIns(4,5)P2. Moreover, plant PLCs lack the PH domain and are thus unlikely to find the few molecules of PtdIns(4,5)P2. Recent studies using a PtdInsP4 biosensor indicate that there is plenty of PtdInsP4 in the plasma membrane, with at least one additional pool occurring at the Golgi (8).

InsP2 rather than InsP3. When microinjected, or released via photoactivation of a caged variant, InsP2 was shown to release Ca^{2+} from an intracellular store in the early 90s. Obviously, this fitted the paradigm, so the plant PI system was a fact, even though Robin Irvine was still skeptical (13). It now seems he was right! Recent work from Brearley’s lab indicates that the Ca^{2+} release is actually caused by InsP6 (14). InsP6 was shown to release Ca^{2+} at a 10-fold lower concentration than InsP3, and when InsP3 was microinjected, it was rapidly converted into InsP6. Also, the hormonal stimulation via abscisic acid (ABA) (to which it was linked) was shown to generate an InsP6 response rather than InsP3 (14).

In yeast, InsP6 is not related to Ca^{2+} signaling but directly regulates gene transcription and mRNA export from the nucleus. This pathway involves a PLC and two inositol polyphosphate multikinases, which can stepwise phosphorylate InsP3 to InsP6 (15). Could this reflect the pathway that is operational in plants too? PLCζ could hydrolyze PtdInsP4 to produce Ins(1,4)P2, which would then be sequentially phosphorylated by similar inositol dual-specificity polyphosphate multikinases (IPK). Two such Arabidopsis...
IPK genes, *AtIPK*1 and *AtIPK*2, have recently been identified (Table 1) (16).

Plants are well known for their phytate (InsP$_6$) content in seeds, which mainly reflects the mechanism to store huge amounts of phosphate and inositol required for germination. Nonetheless, InsP$_6$ may play a completely different role during plant development and in response to agonists, similar to the one that is emerging in mammalian and yeast fields (15). An exciting example of such may be the unexpected discovery of InsP$_6$ in the crystal structure of the auxin (a plant hormone) receptor, TIR1 where it is structurally required for auxin binding and receptor function (17). The major question now is, does this reflect InsP$_6$ signaling and does it require a PLC-mediated pathway?

*PA signaling rather than DG.* A convincing role for DG as a plant signaling molecule has never really been reported. Though it cannot be excluded, the lack of evidence over the last 20 years, together with the absence of its primary target PKC, leaves very little ground to put it into the plant PI/PLC model today (Fig. 1). As a precursor for glycolipids, storage lipids, and the major structural phospholipids, together accounting for ~90% of all plant lipids, DG does not seem to be the most favorable molecule as a membrane-localized-second messenger either. Instead, we see that the PLC-generated DG is rapidly phosphorylated to PA by DG kinase (DGK) and that PA has typically emerged as the plant’s second messenger (5, 18, 28). Over the years, a number of biotic (pathogens) and abiotic (e.g., temperature, osmotic) stress signals have been shown to activate this pathway; meanwhile, a number of plant PA targets have been identified (5). *Arabidopsis* contains seven DGK genes, which are differentially expressed throughout the plant and in response to stress, but knockout mutants have not resulted in a phenotype yet (unpublished observations), indicating a high degree of redundancy. PA can also be generated via the PLD pathway. More about PLD, PA signaling, and downstream targets can be found below.

*PAK and DGPP.* What is also different from mammalian systems is that PA can be phosphorylated into DGPP by a PA kinase (PAK) (Fig. 1). The gene encoding this novel lipid kinase is still unknown, but the enzyme seems to be present in every tissue and is enriched in plasma membrane fractions. The idea is that PAK attenuates PA signaling, but DGPP could also be a signaling molecule itself (19).

*Inositol lipids and phosphates.* Apart from being signaling precursors, it is evident that PtdIns4P and PtdIns(4,5)P$_2$ can also function as signaling molecules themselves. Both lipids are involved in polar growth of pollen tubes and root hairs and during cell plate formation, as shown by lipid biosensors, but also judged from the recent phenotypes of PI 4-kinase, PtdInsP$_5$ 5-kinase and PtdIns4P phosphatase mutants (Table 1) (8, 20–25). Similarly, several cell wall assembly mutants have been identified that may reflect defects in vesicular trafficking (fragile fibers; FRA mutants) and vascular patterning (Table 1). These include several 5-phosphatase mutants, of which it is not always clear whether this affects the lipid or the inositolphosphate, nonetheless emphasizing their importance.

Transgenic plants constitutively overexpressing a (human) InsP$_3$ 5-phosphatase have been reported to have various phenotypes. While these have been interpreted as “attenuation of InsP$_3$ signaling” (26), they might very well reflect defects in InsP$_6$, or raffinose family oligosaccharide metabolism. The latter requires inositol as a precursor and functions to protect cellular structures during desiccation and as carbon reserves for early germination (27).

**PHOSPHOLIPASE D**

As mentioned above, PA is emerging as an important plant lipid second messenger. PA is rapidly and transiently generated in response to a variety of biotic and abiotic stresses, either via the PLC/DGK pathway as discussed above, or directly via PLD.

This enzyme catalyzes the hydrolysis of structural lipids, like PC and PE, to produce PA and the respective headgroup. Using differential 32P$_3$-labeling techniques and PLD-specific transphosphatidylation assays, it is possible to distinguish between both PA-generating pathways (28). In this way, a variety of environmental cues have been shown to activate the PLD pathway, including plant defense elicitors, cold,ounding, heat, oxidative stress, and osmotic stress (5, 29, 30).

Plants are true PLD champions. While humans only contain 2 PLD genes, and yeast 1 (*SPO14*), *Arabidopsis* contains 12 PLD genes and rice even 17 (29–31). Plant PLDs can be classified into 2 groups based on their lipid binding domains. Those with a combined PX and PH domain belong to the PLD$_{12}$ class and are homologous to the mammalian and yeast PLDs. The others, representing the majority of plant PLDs, belong to the C2 class, containing a C2 (calcium and lipid binding) domain. *Arabidopsis* has 10 C2-PLDs, *PLD*$_{1-3}$, *β1-2*, *γ1-3*, *δ*, and *ε*, and two PX-PH-PLDs, *PLD*$_{1-2}$.

Using T-DNA insertion knockout mutants, individual PLDs have been linked to specific plant responses, including ABA signaling, osmotic stress, reactive oxygen species (ROS), freezing, auxin, Pi starvation, and root and root hair development (1).

*PA properties and its targets.* PA formation has a profound effect on membrane curvature and surface charge. Its small anionic phosphomonoester headgroup resides very close to the hydrophobic interior of the lipid bilayer, which 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 (32). The combined effects are likely to be crucial for specific PA responses (33). Major progress has been made in identifying molecular PA targets. Like in mammalian cells, these include protein kinases, phosphatases, and proteins involved in membrane trafficking and the organization of the cytoskeleton. Both activation of positive regulators and inhibition of negative regulators have been reported (5, 18). Examples include...
TABLE 1. Phospholipid signaling KO mutants in *Arabidopsis*

<table>
<thead>
<tr>
<th>Class</th>
<th><em>Arabidopsis</em> Gene</th>
<th>Enzymatic Activity (in Vitro)</th>
<th>Phenotype</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td><em>AtPLAI</em></td>
<td>Acylhydrolase (s1 and s2) preferring galactolipids over phospholipids</td>
<td>Less resistant to necrotrophic fungus</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td><em>AtDAD1</em></td>
<td>sn1-acylhydroxase in JA pathway</td>
<td>Anther dehiscence, pollen maturation, and flower opening</td>
<td>(41)</td>
</tr>
<tr>
<td>PLC</td>
<td><em>AtPLC</em></td>
<td>PI/PLC</td>
<td>Lateral root growth</td>
<td>…</td>
</tr>
<tr>
<td>PLD</td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Reduced ABA responses; enhanced seed quality</td>
<td>(55, 56)</td>
</tr>
<tr>
<td></td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Reduced salt tolerance</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Increased sensitivity to oxidative stress; reduced freezing tolerance</td>
<td>(58, 59)</td>
</tr>
<tr>
<td></td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Reduced salt and osmotic stress tolerance</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Reduced auxin sensitivity; increased sensitivity to Pi starvation</td>
<td>(61, 62)</td>
</tr>
<tr>
<td>PI3K</td>
<td><em>AtVPS34</em></td>
<td>PI 3-kinase</td>
<td>Increased sensitivity to Pi starvation</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td><em>AtPLD</em></td>
<td>Phospholipase D</td>
<td>Increased stress tolerance</td>
<td>(43–45)</td>
</tr>
<tr>
<td></td>
<td><em>AtPTEN1</em></td>
<td>Dual specificity 3-PTase and Tyr phosphatase</td>
<td>Shorter root hairs</td>
<td>(22, 23)</td>
</tr>
<tr>
<td></td>
<td><em>AtVPS43</em></td>
<td>PI 4-kinase</td>
<td>Pollen development (RNA interference)</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td><em>AtPTEN1</em></td>
<td>Dual specificity 3-PTase and Tyr phosphatase</td>
<td>Bulging root hairs</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td><em>AtCT1</em></td>
<td>PtdIns4P 4-phosphatase</td>
<td>Altered cell wall thickening</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td><em>AtSAC</em></td>
<td>PtdIns(3,4,5)P3 5-phosphatase</td>
<td>Reduced growth, hypostatic, purple stress leaves</td>
<td>(64)</td>
</tr>
<tr>
<td></td>
<td><em>AtSAC9</em></td>
<td>Ins(1,4,5)P3 and PtdIns(4,5)P2 5-phosphatase</td>
<td>Germination and seedling development in double mutant</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td><em>At5Pase1 and 2</em></td>
<td>Type I inositol polyphosphate 5-phosphatase</td>
<td>Reduction in secondary wall thickness and stem strength, altered actin deposition in fiber cells</td>
<td>(66)</td>
</tr>
<tr>
<td></td>
<td><em>AtSAC2</em></td>
<td>Ins(1,4,5)P3 and PtdIns(4,5)P2 5-phosphatase</td>
<td>Altered auxin levels, blue light signaling</td>
<td>(67, 68)</td>
</tr>
<tr>
<td></td>
<td><em>At5Pase2</em></td>
<td>Type II inositol polyphosphate 5-phosphatase</td>
<td>Root hair initiation</td>
<td>(69)</td>
</tr>
<tr>
<td></td>
<td><em>MRH3</em></td>
<td>Ins(1,4,5)P3 and PtdIns(4,5)P2 5-phosphatase</td>
<td>Vascular patterning cotyledon</td>
<td>(70)</td>
</tr>
<tr>
<td></td>
<td><em>CIP</em></td>
<td>Ins(1,4,5)P3 and PtdIns(4,5)P2 5-phosphatase</td>
<td>InsP3 levels down, P3 sensing, and root hair growth</td>
<td>(16)</td>
</tr>
</tbody>
</table>

* Unpublished observations.

PLA, mediating responses to ROS in root hair development and pathogens, CTR1, a crucial protein kinase in ethylene signaling, ABI1, a protein phosphatase in ABA signaling, AtCP, an actin capping protein, and AGD7, an ArfGAP (32). Though several PA binding motifs have been recognized (5, 34), a general PA binding domain still remains obscure.

**PHOSPHOLIPASE A**

PLA catalyzes the hydrolysis of phospholipids into lysophospholipids and free fatty acids, either at the sn-1 (PLA1) or 2-position (PLA2) of the glycerol backbone, or both (PLB). Plants contain numerous PLAs. In *Arabidopsis*, three different families can be distinguished: four small secretory sPLAs, 10 patatin-like pPLAs, and 14 lipase-like PLA1s. For most, it is not clear what their substrate is or which position they hydrolyze; some exhibit acyltransferase or acylhydrolyase activity, sometimes even toward nonphospholipids, such as galactolipids (35). As such, it is not always clear whether effects reflect general lipid metabolism or signaling. Nonetheless, evidence is increasing for PLA’s involvement in disease resistance, auxin, and light (Table 1; 35–39).

Similar to the eicosanoid (C20) pathway in animals, plants exhibit an octadecanoid (C18) pathway, playing an important role in, for example, the plant’s defense against pathogens and herbivores, in particular, jasmonic acid (JA) and its volatile, methyl JA (40). JA is also important in flower development. The latter involves DAD (for *defective in anther dehiscence*), a gene encoding a PLA1 (41).

Lysophospholipids have also been also implicated in cell signaling (3). In particular, lysophosphatidylcholine, which is proposed to activate a vacuolar H^+ /Na^+ antiporter to regulate the cytosolic pH in response to a pathogenic elicitor (36, 42).

**PI3 KINASE**

*Arabidopsis* only contains one PI3K, which is Vps34p-like (type III). Silencing *AtVPS43* causes severe defects in development, while knockout mutants are lethal, indicating an important function (43–45). About 5–15% of the PtdInsP pool in plants is PtdInsP3, with the majority (~80%) being PtdInsP4 and containing a few percent of PtdInsP5 (46). PtdInsP3 has been imaged in living cells by expressing the PtdInsP3 biosensor YFP-2xYFVE, revealing predominant labeling of late endosomes, multivesicular bodies, and prevacuolar membranes (9). Only ~20% of the early endosomes were labeled. The YFP-2xYFVE labeling pattern is sensitive to wortmannin, as all labeled structures disappeared within 20 min of treatment, with all fluorescence reappearing in the nucleus. HPLC-headgroup analyses revealed that the FYVE-overexpressing cells...
contains double amounts of PtdIns3P. Apparently, cells sense free PtdIns3P levels, and since overexpression of FYVE leads to preoccupation of PtdIns3P, competing with endogenous targets, cells simply make more. This probably also explains why there is no apparent phenotype in cell suspensions or Arabidopsis seedlings that constitutively express the sensor (9), although overexpression behind a root-hair-specific promoter did have a dose-dependent effect on root hair elongation (47). Wortmannin and LY294002 inhibit root hair growth. The same inhibitors have been used to imply the involvement of PtdIns3P in the production of ROS and actin dynamics (43, 47, 48).

In Arabidopsis, 11 proteins with a PX and 16 with a FYVE domain have been predicted (49).

Other D3-PPI. Plants contain small amounts of PtdIns(3,5)P2 but lack PtdInsP3. Earlier, the presence of PtdIns(3,4)P2 had been reported, but this was before the discovery of PtdIns(3,5)P2 and has not been reproduced so far (50). Like yeast, plants make PtdIns(3,5)P2 in response to osmotic stress (50). Yeast PtdIns(3,5)P2 is involved in the retrograde trafficking between organelles and the endocytic/lysosomal system and made by a PtdIns3P 5-kinase called Fab1p (51). EABI mutants (for formation of atophid and binucelate cells) have enlarged vacuoles that do not acidify correctly and have nuclear segregation defects. Arabidopsis contains 4 putative FAB genes (1). Proposed effectors for PtdIns(3,5)P2 include the PROPPIN family of seven-bladed β-propellers (51) of which Arabidopsis homologs are present.

Degradation of PtdIns(3,5)P2 occurs through 3- or 5-phosphatases. In vitro, At5PTase11 and AtSAC1/FRA7 have been shown to dephosphorylate PtdIns(3,5)P2 at the 5-position (52). AtSAC1/FRA7 mutants have defects in the organization of the actin cytoskeleton and exhibit reduced cell wall thickening (53). Whether PtdIns(3,5)P2 is the (only) substrate in vivo remains to be shown. In total, Arabidopsis contains 15 At5PTase and 9 SAC genes (80–89). The SAC (for suppressor of actin) family of phosphatases contains both 4- and 5-specific phosphatases (Table 1).

3-Phosphatases. Arabidopsis contains three homologs of PTEN (54) and two potential MTM (myotubularin-type phosphatase) genes that are predicted to be catalytically active. AtPTEN1 is a dual-specificity phosphatase which, in vitro, has phosphatase activity toward phosphatidylinositol and PtdInsP5. It is exclusively found in pollen grains and expressed during the later stages of development. Knockout mutants are lethal, and RNA interference suppression results in cell death after mitosis, indicating that this gene is essential for pollen tube development (54).

The authors apologize to those whose original work could not be cited due to heavy restrictions in the number of references.

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