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 then 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)P_2] into two second messengers: inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol (DG). While InsP_3 difuses 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 PtdIns4P and PtdIns(4,5)P_2 are not just 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. S260–S265.

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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 InsP_3 receptor (6). An exception is *Chlamydomonas*, a unicellular green algae with two flagella, where an InsP_3 receptor has been identified. Other ciliated organisms, such as *Paramecium*, also contain an InsP_3 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 mammalians. 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 PtdInsP_2. Labeling experiments using $^{32}P_i$ or ^{3}H-Inositol, but also recent PtdInsP_2 biosensor (GFPPH$_{PLC\delta1}$) studies, have shown that plant cells contain extremely low amounts of PtdInsP_2 (8–10). In contrast, PtdIns4P levels seem to be normal, i.e., similar to those observed in animals. Typical ^{32}P-ratios between PtdIns$P$ and PtdInsP_2, measured in many different plant cells and tissues, reveal 30- to 100-fold lower PtdInsP_2 levels than PtdIns$P$. In animals and *Chlamydomonas*, the PtdIns$P$:PtdInsP_2 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; InsP_3, 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; PtdInsP_2, phosphatidylinositol4,5-bisphosphate; ROS, reactive oxygen species.

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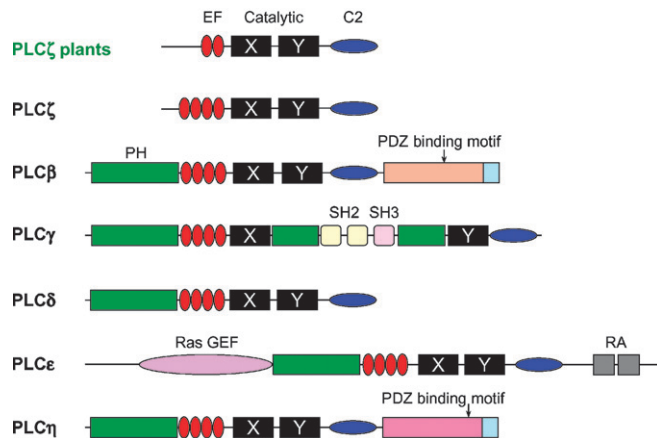   e-mail: t.munnik@uva.nl (T.M.); c.s.testerink@uva.nl (C.T.)
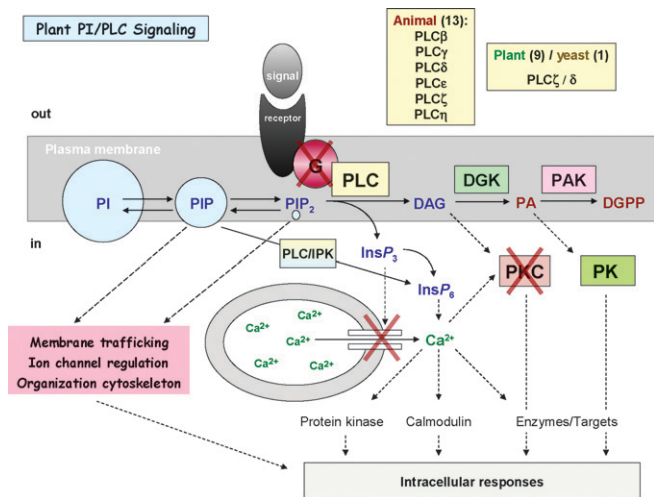
Fig. 1. Plant PI/PLC signaling: Differences and similarities to the mammalian paradigm. Higher plants lack both InsP_3 receptor, a ligand-gated Ca^{2+} channel, and PKC; hence, these are striked-out (X). Instead, plants seem to use their phosphorylated products, InsP_6 and PA, as signaling molecules. PA can also be generated by PLD and is attenuated by PA kinase (PAK), a novel lipid kinase that is absent from mammalian cells. PAK generates diacylglycerol-pyrophosphate (DGPP), which might function as a signaling molecule itself (19). Plant PLCs belong to the PLCζ subfamily. It is not known how they are regulated but not through heterotrimeric G-proteins (G), which is therefore striked out. Plant PtdIns(4,5)P_2 (PIP$_2$) quantities are extremely low, and plant PLCs lack the PH domain (Fig. 2). Instead, PtdIns4P (PIP) is a better candidate to be the in vivo PLC substrate. The resulting InsP_2 can be phosphoryated to InsP_6 via two dual-specificity inositolpolyphosphate kinases (IPK), while DG is phosphorylated to PA via DGK. Evidence is also emerging that PtdIns4P and PtdIns(4,5)P_2 themselves function as signaling molecules, involving membrane trafficking, organization of the cytoskeleton, and regulation of ion channels. In such a scenario, PLC would function as an attenuator of PIP and PIP$_2$ signaling. Solid arrows indicate metabolic conversions. Dashed arrows represent mechanisms of regulation.

Fig. 2. Domain structure and organization of PI/PLC isozymes. Plant PLCs belong to the most simple group, the PLCζs. PLCη undergoes alternative splicing, generating variable C termini with a PDZ binding motif being only present in the longer forms. Adapted from (11).

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 PLCδ 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 PLCβs to be regulated by trimeric G-proteins, PLCγs by tyrosine kinases, and PLCεs by trimeric G-proteins and Ras. It is still not clear how PLCδ, -η, and -ζ isoforms are regulated, but this may involve calcium, especially for PLCη (Fig. 2) (11).

Plant PLC activity indeed requires calcium. At low micromolar Ca^{2+} concentrations, both PtdIns4P and PtdIns(4,5)P_2 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)P_2. However, plants have no, or very little, PtdInsP_2 in their membranes, and an InsP_3 receptor is lacking, so wouldn't it make more sense to propose PtdIns4P as the in vivo substrate? In vitro, PtdIns4P is equally well hydrolyzed as PtdIns(4,5)P_2, and in vivo, PtdIns4P turnover and quantities are much more in agreement with the PA responses resulting from DG phosphorylation than PtdIns(4,5)P_2. Moreover, plant PLCs lack the PH domain and are thus unlikely to find the few molecules of PtdIns(4,5)P_2. Recent studies using a PtdIns4P biosensor indicate that there is plenty of PtdIns4P in the plasma membrane, with at least one additional pool occurring at the Golgi (8).

InsP_6 rather than InsP_3. When microinjected, or released via photoactivation of a caged variant, InsP_3 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 InsP_6 (14). InsP_6 was shown to release Ca^{2+} at a 10-fold lower concentration than InsP_3, and when InsP_3 was microinjected, it was rapidly converted into InsP_6. Also, the hormonal stimulation via abscisic acid (ABA) (to which it was linked) was shown to generate an InsP_6 response rather than InsP_3 (14).

In yeast, InsP_6 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 InsP_3 to InsP_6 (15). Could this reflect the pathway that is operational in plants too? PLCζ could hydrolyze PtdIns4P to produce Ins(1,4)P_2, which would then be sequentially phosphorylated by similar inositol dual-specificity polyphosphate multikinases (IPK). Two such *Arabidopsis*

IPK genes, *AtIPKβ1* and *AtIPKβ2*, 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, PtdIns$P$ 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 ^{32}P$_1$-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, wounding, 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 PLDζ 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, *PLDα1-3, β1-2, γ1-3, δ*, and *ε*, and two PX-PH-PLDs, *PLDζ1-2*.

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 (Table 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*

Class	*Arabidopsis* Gene	Enzymatic Activity (in Vitro)	Phenotype	Refs.
PLA	*AtPLAI*	Acylhydrolase (*sn*-1 and *sn*-2) preferring galactolipids over phospholipids	Less resistant to necrotrophic fungus *Botrytis cinerea*	(35)
	AtDAD1	*sn*1-acylhydrolase in JA pathway	Anther dehiscence, pollen maturation, and flower opening	(41)
PLC	*AtPLC*	PI/PLC	Lateral root growth	$-^a$
PLD	*AtPLDα1*	Phospholipase D	Reduced ABA responses; enhanced seed quality	(55, 56)
	AtPLDα3	Phospholipase D	Reduced salt tolerance	(57)
	AtPLDδ	Phospholipase D	Increased sensitivity to oxidative stress; reduced freezing tolerance	(58, 59)
	AtPLDα1/PLDδ	Phospholipase D	Reduced salt and osmotic stress tolerance	(60)
	AtPLDζ2	Phospholipase D	Reduced auxin sensitivity; increased sensitivity to Pi starvation	(61, 62)
	AtPLDζ1/2	Phospholipase D	Increased sensitivity to Pi starvation	(63)
PI3K	*AtVPS34*	PI 3-kinase	Lethal; antisense plants are severely affected in growth and development	(43–45)
PI4K	*AtPI4Kβ1*	PI 4-kinase	*AtPI4Kβ1/β2* double mutants display distorted root hair development	(24)
	AtPI4Kβ2	PI 4-kinase		
PIP5K	*AtPIP5K3*	PI4P 5 kinase	Shorter root hairs	(22, 23)
3PTase	*AtPTEN1*	Dual specificity 3-PTase and Tyr phosphatase	Pollen development (RNA interference) (pollen cell death after mitogenesis)	(54)
4PTase	*RHD4, AtSAC7*	PtdIns4*P* 4-phosphatase	Bulging root hairs	(25)
5PTase	*FRA7 AtSAC1*	PtdIns(3,5)P_2 5-phosphatase	Alterations in actin cytoskeleton organization and reduced cell wall thickening	(53)
	AtSAC9	Ins(1,4,5)P_3- and PtdIns(4,5)P_2 5-phosphatase	Reduced growth, hyponastic, purple stress leaves	(64)
	At5Ptase1 and *2*	Type I inositol polyphosphate 5-phosphatase toward Ins(1,4,5)P_3	Germination and seedling development in double mutant	(65)
	FRA3	Type II inositol poly-phosphate 5-phosphatase; PtdIns(4,5)P_2 > Ins(1,4,5)P_3	Reduction in secondary wall thickness and stem strength, alterated actin deposition in fiber cells	(66)
	At5Ptase13	Type I inositol polyphosphate 5-phosphatase toward Ins(1,4,5)P_3	Altered auxin levels, blue light signaling	(67, 68)
	MRH3 (At5Ptase5)	Inositol polyphosphate 5-phosphatase	Root hair initiation	(69)
	CVP2 (At5Ptase6)	Inositol polyphosphate 5-phosphatase	Vascular patterning cotyledon	(70)
IPK	*AtIPK1/AtIPK2β*	Inositolpolyphospate kinase	InsP_6 levels down, P_i sensing, and root hair growth	(16)

[a] Unpublished observations.

PDK1, 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 lyso-phospholipids and free fatty acids, either at the *sn*-1 (PLA$_1$) or 2-position (PLA$_2$) 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 PLA$_1$s. For most, it is not clear what their substrate is or which position they hydrolyze; some exhibit acyltransferase or acylhydrolyse 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 PLA$_1$ (41).

Lysophopholipids 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 PtdIns*P* pool in plants is PtdIns3*P*, with the majority (~80%) being PtdIns4*P* and containing a few percent of PtdIns5*P* (46). PtdIns3*P* has been imaged in living cells by expressing the PtdIns3*P* biosensor YFP-2xFYVE, revealing predominant labeling of late endosomes, multivesicular bodies, and prevacuolar membranes (9). Only ~20% of the early endosomes were labeled. The YFP-2xFYVE labeling pattern is sensitive to wortmannin, as all labeled structures disappeared within 20 min of treatment, with all fluorescence reappearing in the nucleus. HPLC-head-group analyses revealed that the FYVE-overexpressing cells

contained double amounts of PtdIns3*P*. Apparently, cells sense free PtdIns3*P* levels, and since overexpression of FYVE leads to preoccupation of PtdIns3*P*, 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 promotor 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 PtdIns3*P* 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)P_2 but lack PtdInsP_3. Earlier, the presence of PtdIns(3,4)P_2 had been reported, but this was before the discovery of PtdIns(3,5)P_2 and has not been reproduced so far (50). Like yeast, plants make PtdIns(3,5)P_2 in response to osmotic stress (50). Yeast PtdIns(3,5)P_2 is involved in the retrograde trafficking between organelles and the endocytic/lysosomal system and made by a PtdIns3*P* 5-kinase called Fab1p (51). *FAB1* mutants (for *formation of aploid and binucleate 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)P_2 include the PROPPIN family of seven-bladed β-propellers (51) of which *Arabidopsis* homologs are present.

Degradation of PtdIns(3,5)P_2 occurs through 3- or 5-phosphatases. In vitro, At5PTase11 and AtSAC1/FRA7 have been shown to dephosphorylate PtdIns(3,5)P_2 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)P_2 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 phosphotyrosine and PtdInsP_3. It is exclusively found in pollen grains and expressed during the later stages of development. Knock-out 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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