Novel roles for phospholipase C in plant stress signalling and development

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Citation for published version (APA):
Chapter 1

General Introduction

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1. PLC signaling in plants

In nature, plants live in an open environment and face various challenges, including heat- and cold stress, drought, salinity, wounding by herbivores, or infection by pathogens. Since plants cannot run away, they must quickly recognize and respond to outside signals and adjust themselves to the challenging surroundings. This process, called 'signal transduction', starts at the cellular level. Signal perception is through proteins or other molecules located in the plasma membrane, which is a selective barrier between the interior cell and the external environment (Fig. 1a). The plasma membrane is comprised of a lipid bilayer with associated integral and peripheral membrane proteins. The lipid part consists of phospholipids (~40-60%), sphingolipids (~10-20%) and sterols (~40-50%) (Furt et al., 2011). The bilayered structure results from the biophysical properties of the phospholipids, which are composed of a hydrophilic, phosphate-containing head group and, in general, two hydrophobic glycerol-fatty acyl tails (Fig. 1b). The fatty acids tend to face each other inwards while the hydrophilic head groups interact with the aqueous surroundings on the outside (Fig. 1a). The most abundant phospholipids in the plasma membrane are phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which together account for ~70-80%, followed by phosphatidylycerol (PG) and phosphatidylinositol (PI) (~5-10% each). Together, they are called structural phospholipids, because they make-up the mass- and structure of membranes, which is not only the plasma membrane, but includes all intracellular organelles, i.e endoplasmic reticulum (ER), Golgi, vacuole, mitochondria, peroxisomes and plastids. In each membrane, phospholipids play a key role in the dynamics and maintenance of the fluid, bilayer structure, with the desaturation (double bonds) and saturation (no double bonds) of the fatty acids determining a great deal of the fluidity of membranes (Van Hooren and Munnik, 2017).

In addition to the structural phospholipoids, there are also some minor phospholipids, which are present in relatively low quantities and have signaling rather that structural role. These include, phosphatidic acid (PA), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2), which play important roles in vesicular trafficking (endo- and exocytosis) and signal transduction. Although the study of phospholipid signaling in plants is relatively young, their importance in plant stress and development is emerging and holds great potential for future research (Munnik & Nielsen, 2011; Testerink & Munnik, 2011; Gillaspy, 2013; Gujas & Rodriguez-Villalon, 2016; Heilmann, 2016).

Phospholipid signaling systems would not function without various phospholipases, i.e.: phospholipase A1 (PLA1), PLA2, phospholipase C (PLC) and phospholipase D (PLD), which attack different positions of a phospholipid (Fig. 1b; Hong et al., 2016). In this thesis, research is focused on PLC that is involved in signaling. It specifically hydrolyzes phosphoinositides and is referred to PI-PLC. Plants also contain non-specific PLCs, called NPCs, which act on structural lipids like PC and PE, and mainly function in membrane dynamics.
Figure 1. Schematic representation of the plasma membrane consisting of phospholipids and embedded proteins. (a) The plasma membrane consists of two phospholipid layers with their hydrophobic part facing each other and the hydrophilic heads facing outward. (b) Phospholipid has a three-carbon glycerol backbone containing two fatty acid chains and a phosphate-containing head group. The phospholipid X group can vary, as listed in the blue box. The cleavage sites of phospholipases are indicated by arrows.
PLC cleaves PIP2 into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which, in mammalian systems, are crucial second messengers, regulating various cellular processes. IP3 is water soluble and diffuses into cytosol to bind the IP3 receptor, which is a ligand-gated Ca\(^{2+}\) channel at the ER that releases Ca\(^{2+}\) into the cytosol, leading to a transient increase in cytosolic Ca\(^{2+}\) that triggers a cascade of downstream changes. The lipid moiety of PIP2, i.e. DAG, remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family that phosphorylate various downstream protein targets, altering numerous biochemical pathways. Alternatively, DAG can activate so-called TRP (Transient receptor potential) channels, which triggers Ca\(^{2+}\) influx across the plasma membrane.

**Figure 2.** Comparison between mammalian and plant PLC signaling pathways. In mammalian cell, PLC is regulated by a G protein after receiving the signal. PLC cleaves PIP2 into IP3 and DAG. IP3 releases Ca\(^{2+}\) via Ca\(^{2+}\) channel and DAG activates PKC, leading to activation of downstream signaling cascades. In higher plants, G protein, targets for IP3 and DAG are missing, indicated by the red crosses. Instead, plant seems to use IP6 and PA as second messengers. The picture is modified from Munnik et al. (2009)

While the above is typical for the mammalian PLC signaling pathway, a number of differences have been found for the plant PLC system in terms of second messengers, regulation and targets (Fig. 2). Perhaps the most striking difference is that plants are lacking homologs of the IP3 receptor, PKC or TRP-channel (Munnik, 2014). Earlier, PLC and IP3 had been coupled to Ca\(^{2+}\) signaling in ABA-induced stomatal closure (Blatt et al., 1990; Gilroy et al., 1990; Allen and Sanders, 1994; Lee et al., 1996; Staxen et al., 1999), but later this was shown to result from its phosphorylation into IP6, with the latter being responsible for the release of the intracellular Ca\(^{2+}\) (Lemtiri-Chlieh et al., 2000, 2003; Munnik and Vermeer, 2010). To produce IP6 from IP3, two inositolpolyphosphate kinases, IPK2 and IPK1, are required (Munnik and Vermeer, 2010; Gillaspy, 2013). Apart from releasing Ca\(^{2+}\), IP6 has also been implicated in auxin signaling where it binds the auxin receptor, TIR1 (Tan et al., 2007), mRNA export (Lee et al., 2015), phosphate homeostasis (Kuo et al., 2014; Puga et al., 2014; Wild et al., 2016) and disease resistance (Murphy et al., 2008). Meanwhile, inositolpolyphosphates...
(IPPs) other than IP$_6$ have been implicated in signaling. For example, IP$_4$ has been shown to regulate a chloride channel (Zonia et al., 2002), while IP$_5$ was discovered in the crystal structure of COI1, which is the receptor for jasmonate signaling, (Sheard et al., 2010). Recently, another IPK, i.e. VIH2, has been identified, which is responsible for the production of pyrophosphorylated IPPs, i.e. IP$_7$ and IP$_8$. The latter are emerging as important signaling molecules in plants, as well as in animals and fungi (York, 2006; Michell, 2008; Burton et al., 2009; Shears, 2009; Desai et al., 2014; Laha et al., 2015, 2016; Thota and Bhandari, 2015). Importantly, cellular roles for IP$_6$ should not be confused with its role in plants as phosphate-storage molecule in seeds (phytate), like Ca$^{2+}$ signaling should not be confused with its abundant presence in bones.

Likewise, not DAG but its phosphorylated product, phosphatidic acid (PA) is emerging as the plant lipid second messenger (Munnik, 2001, 2014; Testerink & Munnik, 2005; Wang et al., 2006; Arisz et al., 2009; Pokotylo et al., 2014). The latter requires the help of DAG kinase (DGK). The metabolism of PA is complex though, as it is also an intermediate in glycerolipid biosynthesis and is directly produced via PLD hydrolysis of PC, PE and PG (Arisz et al., 2009; Arisz and Munnik, 2013).

2. PLC enzymes

2.1 PLC family

Eukaryotic cells contain multiple PLC isoforms and they fall into six subfamilies ($\beta$, $\gamma$, $\delta$, $\epsilon$, $\eta$ and $\zeta$) based on sequence similarity and domain structure (Fig. 3; Munnik and Testerink, 2009).

Mammalian cells contain all six subfamilies, resulting in 13 PLC isoforms. Most subfamilies share the conserved structure of an N-terminal pleckstrin homology (PH) domain, Ca$^{2+}$ binding EF-hands, catalytic X-and Y domains, and a lipid binding-C2 domain in the C-terminal, except for PLC$\zeta$, which lacks the PH domain that is known to bind phosphoinositides and certain proteins, which differs per PH domain (Rebecchi and Pentyala, 2000; Bunney and Katan, 2011). The PLC family has also been studied in various plant species, including Arabidopsis (Hunt et al., 2004; Tasma et al., 2008; Xia et al., 2017),
tomato (Vossen et al., 2010), rice (Singh et al., 2013; Li et al., 2017), soybean (Wang et al., 2015) and maize (Wang et al., 2008). The plant PLC protein structure is similar to the mammalian PLCδ- and PLCζ subfamilies, lacking a PH domain and one of the conserved EF-hand lobes (Munnik, 2014; Pokotylo et al., 2014; Hong et al., 2016) and containing a plant-specific N-terminal region. Arabidopsis contains 9 PLC genes, which can be divided into 4 different subfamilies, based on phylogenetic analysis of the encoded proteins (Fig. 4; Hunt et al., 2004). Among them, PLC8 and PLC9 are the most divergent as they contain mutations in the Y domain that would render them inactive as enzymes (Mueller-Roeber and Pical, 2002; Hunt et al., 2004; Tasma et al., 2008). Nonetheless, PLC9 has been implicated in heat-stress tolerance (Zheng et al., 2012).

![Figure 4](image)

**Figure 4.** Phylogenetic tree of the Arabidopsis PLC protein family together with mammalian δ and ζ isozymes. Bootstrap values for nodes that had > 50% support in a bootstrap analysis of 1000 replicates are shown. Data show is from Hunt et al. (2004).

### 2.2 Enzymatic activity

PLC catalyzes the hydrolysis of phosphoinositides by attacking the phosphodiester bond at the glycerol side to generate a phosphorylated-inositol headgroup and DAG. *In vitro*, the enzyme activity requires Ca$^{2+}$ and the substrate preference depends on the Ca$^{2+}$ concentration. For example, at low, physiological Ca$^{2+}$ concentrations (100 nM-10 μM), PLC hydrolyses PI4P and PI(4,5)P$_2$ equally well. At higher, non-physiological Ca$^{2+}$ concentrations (mM level), PLC also hydrolyses PI. However, PLCs cannot hydrolyze phosphoinositides that are phosphorylated at the D3-position of the inositol ring (i.e. PI3P, PI(3,4)P$_2$, and PI(3,5)P$_2$) nor other phospholipids (Munnik et al., 1998). Based on crude protein extracts from different species and tissues, both membrane-associated and soluble PLCs have been described (Munnik et al., 1998). Membrane-associated PLCs preferred PI4P and PI(4,5)P$_2$ as substrates requiring
low µM Ca\(^{2+}\), whereas soluble PLCs preferred PI at higher (mM) Ca\(^{2+}\). The pH optimum for both PLC forms was between 6 and 7 (Munnik et al., 1998).

Mammalian PLCs are known to mainly use PI(4,5)P\(_2\) as in vivo substrate, which is facilitated by the fact that mammalian cells have relatively high concentrations of PI(4,5)P\(_2\) in their plasma membranes, and because the PLCs are equipped with PIP\(_2\)-binding domains, which is either the PH domain in PLC\(\beta\), -\(\gamma\), -\(\delta\), -\(\epsilon\), -\(\eta\) or the XY linker in PLC\(\zeta\) (Swann and Lai, 2016). For plants, the in vivo substrate remains unclear. The PI(4,5)P\(_2\) levels in plants are 30-100 times lower and the PLCs lack a PH domain or the XY-linker sequence from PLC\(\zeta\). Since PI4P can be hydrolyzed equally well in vitro and plants contain relatively high amounts of it in the plasma membrane in vivo (Munnik et al., 1998; Vermeer et al., 2009), the latter could be the common substrate for plant PLCs.

2.3 Gene expression and (sub)cellular localization

PLC genes from different plant species (Arabidopsis, rice, maize, tomato, potato, pea, mung bean, etc.) are expressed in various tissues (leaf, stem, flower, root) during development, and levels are influenced by biotic- and abiotic stresses (Hirayama et al., 1995; Hunt et al., 2004; Kim et al., 2004; Lin et al., 2004; Das et al., 2005; Vergnolle et al., 2005; Liu, Liu, et al., 2006; Tasma et al., 2008; Song et al., 2008; Sui et al., 2008; Vossen et al., 2010; Zheng et al., 2012; Singh et al., 2013; Wang et al., 2015; Li et al., 2017). Several putative regulatory elements have been identified in the promoter regions of the Arabidopsis PLCs, confirming their potential involvement in developmental and stress responses (Tasma et al., 2008; Hsieh et al., 2013; Singh et al., 2013).

Based on subcellular-fractionation and activity assays, but also by imaging of fluorescent-protein fusions, PLCs have been localized to the plasma membrane in various species, including Arabidopsis PLC2 (Otterhag et al., 2001) and PLC9 (Zheng et al., 2012), Petunia PLC1 (Dowd et al., 2006), tobacco PLC3 (Helling et al., 2006) and mung bean PLC3 (Kim et al., 2004). PLCs have also been found in the cytoplasm (Shi et al., 1995; Rupwate and Rajasekharan, 2012; Singh et al., 2013), but it remains unknown by which genes these are encoded.
3. PLC in plant stress and development

Although PLC signaling in plants is still obscure, various roles in plant development and stress have been proposed (Munnik, 2014; Pokotylo et al., 2014).

3.1 PLC in plant-stress responses

Over the years, several papers on the involvement of PLC in stress signaling have appeared, bringing new insights of its role in plants (Munnik and Vermeer, 2010; Munnik, 2014; Pokotylo et al., 2014).

3.1.1 Osmotic-, heat- and cold stress

Upon abiotic stress, such as salinity, drought, heat and cold, several PLC genes are induced and increased PLC activity has been reported in different plant systems (Hirayama et al., 1995; Ruelland et al., 2002; Hunt et al., 2004; Das et al., 2005; Vergnolle et al., 2005; Zhai et al., 2005; Skinner et al., 2005; Liu et al., 2006; Sui et al., 2008; Tasma et al., 2008; Munnik and Vermeer, 2010; Wang et al., 2015; Li et al., 2017). Accumulation of IP$_3$ and increases of cytosolic Ca$^{2+}$ have been correlated and claimed to reflect PLC’s activity (DeWald et al., 2001; Takahashi et al., 2001; Ruelland et al., 2002; Im et al., 2007; König et al., 2007; Zheng et al., 2012; Gao et al., 2014; Li et al., 2017). However, these IP$_3$ measurements were mainly based on so-called $^3$H-IP$_3$-displacement assays, which in plants are quite inaccurate because they contain very low IP$_3$ levels, while IPPs that can affect the displacement assay, are very abundant (see Munnik and Vermeer, 2010). Hence, whether "measured IP$_3$" originates from PLC hydrolysis or results from changes in other IPPs is still debatable. Yet increases of IP$_3$ upon heat or salt stress are typically accompanied by increases of PIP$_2$ (Pical et al., 1999; DeWald et al., 2001; Zonia and Munnik, 2004; Liu et al., 2006; van Leeuwen et al., 2007; Darwish et al., 2009; Mishkind et al., 2009; Horvath et al., 2012; Simon et al., 2014). The latter is likely due to the activation of PIPK rather than inhibition of PLC or PIP$_2$ phosphatases (Mishkind et al., 2009; Zarza, 2017). Theses PIP$_2$ responses are relatively slow, however, accumulating after 15-30 min while Ca$^{2+}$ responses already occur within minutes, so these do not correlate at all. A decrease of PIP has also been reported in some cases (Cho et al., 1993; Pical et al., 1999; DeWald et al., 2001; Zonia and Munnik, 2004; van Leeuwen et al., 2007; Darwish et al., 2009; Mishkind et al., 2009; Vermeer et al., 2009; Horvath et al., 2012; Munnik, 2014; Zarza et al., unpublished). Whether this reflects activation of PIPK or hydrolysis by PLC remains unknown too. During salt stress, PIP$_2$ has also been implicated in the formation of clathrin-coated vesicles (CCV) (König et al., 2008). Whether PIP$_2$ is used for IP$_3$ or functions as a signaling molecule itself remains to be addressed.

3.1.2 Abscisic acid (ABA)

PLC signaling in response to ABA has been studied for many years, especially in relation to the induction of stomatal closure. Initially, most studies tried to link IP$_3$/Ca$^{2+}$-signaling with this event (Gilroy et al., 1990; Blatt et al., 1990; Lee et al., 1996; Staxen et al., 1999; Blatt, 2000; Schroeder et al.,
However, the finding that (i) microinjected IP$_3$ was converted into IP$_6$ within seconds, (ii) that IP$_6$ was 10-100 times more efficient than IP$_3$ in releasing intracellular Ca$^{2+}$, and (iii) that ABA triggered IP$_6$ formation in vivo within minutes of ABA treatment (Flores and Smart, 2000; Lemtiri-Chlieh et al., 2000, 2003), raised new ideas for PLC signaling in response to ABA (Zonia and Munni, 2006; Munnik, 2014) However, IP$_6$-activated Ca$^{2+}$ channel (an "IP$_6$ receptor") has still not been identified.

Apart from guard cells, ABA-induced IP$_3$ responses have been reported for Arabidopsis seedlings (Burnette et al., 2003; Perera et al., 2006). However, these studies only focused on IP$_3$ and no other IPPs were measured.

Whether PIP$_2$ levels change upon ABA treatment remains unclear (Munnik and Vermeer, 2010). Nevertheless, PIP$_2$ has been shown to inhibit anion channels (Lee et al., 2007) and K$^+$ efflux channels (Ma et al., 2009), which would facilitate stomatal opening. The importance of PLC in ABA signaling is illustrated by several reports. For example, silencing of a PLC in tobacco made the plant less sensitive to ABA-induced stomatal closure, leading to a wilting phenotype (Hunt et al., 2003). Similarly, silencing of Arabidopsis PLC1 was shown to be important for secondary ABA responses (Sanchez and Chua, 2001). Several PLC genes are also induced by ABA treatment (Hirayama et al., 1995; Lin et al., 2004; Tasma et al., 2008; Pokotylo et al., 2014).

3.1.3 Biotic stress
A role for PLC in plant defense has also been emerging (Laxalt and Munnik, 2002; Vossen et al., 2010; Canonne et al., 2011; Abd-El-Haliem et al., 2012, 2016, Gonorazky et al., 2014, 2016; D’Ambrosio et al., 2017). Based on changes in PPI-labeling profiles, PLC signaling is thought to be induced by various pathogen-associated molecular patterns (PAMPs), also called elicitors. These include xylanase, flagellin and chitin (Luit et al., 2000; Den Hartog et al., 2003; Yamaguchi et al., 2005). It is also triggered by many other chemical and biological inducers, such as benzothiadiazole (BTH), salicylic acid (SA), jasmonic acid (JA) (Song and Goodman, 2002) and avirulence (Avr) proteins (De Jong et al., 2004; Andersson et al., 2006). Rapid PA production through PLC/DGK activity has been demonstrated to occur after pathogen attack (Luit et al., 2000; Laxalt and Munnik, 2002; Hartog et al., 2003; De Jong et al., 2004; Canonne et al., 2011) and some of the responses have been shown involve nitric oxide (NO) signaling (Laxalt et al., 2007; Lanteri et al., 2011; Raho et al., 2011). Interestingly, PA accumulation is accompanied by reactive oxygen species (ROS) production which are playing an important role in defense response (Yamaguchi et al., 2003; De Jong et al., 2004). In addition, it has been suggested that PA might activate plant defenses via mitogen-activated protein kinase (MAPK) signaling cascades (Laxalt and Munnik, 2002; Canonne et al., 2011). Through gene silencing, several tomato PLCs have been shown to be involved in disease resistance (Vossen et al., 2010; Gonorazky et al., 2014, 2016; Abd-El-Haliem et al., 2016; D’Ambrosio et al., 2017).

3.2 PLC in plant development
In addition to stress, PLC is also involved in various aspects of plant growth- and development. *Arabidopsis PLC2* has been found to regulate reproductive development (Li *et al.*, 2015; Di Fino *et al.*, 2017) and a similar function was observed for *PLC1* in *Torenia fournieri* (Song *et al.*, 2008). Petunia *PLC1* and tobacco *PLC3* regulate tip growth in pollen tubes (Dowd *et al.*, 2006; Helling *et al.*, 2006). During pollen tube elongation, PLC was found at the flanks of tip, whereas PIP2 mainly accumulated in the apex (Ischebeck *et al.*, 2010; Grierson *et al.*, 2014). It is well known that PIP2 is essential for polar tip growth, and this distribution of PLC would keep the PIP2 gradient directed to the tip, which is important for polarized growth (Ischebeck *et al.*, 2010; Heilmann and Ischebeck, 2016). In *Physcomitrella patens*, loss of *PLC1* resulted in insensitivity to cytokinin and exhibited a paler phenotype, caused by reduced chlorophyll. The response to gravitropism was also reduced in mutant filaments (Repp *et al.*, 2004). IP3 has been suggested to play an important role in gravitropism (Perera *et al.*, 2001). However, this was based on the same inaccurate (section 3.1.1) IP3 kit and before the realization that plants lack IP3 receptor. Auxin plays an important role in gravitropism too and as such IP3/TIR1 could play a potential role here as well. In *Brassica napus*, overexpression of PLC2 enhanced photosynthesis, changed hormone distributions, caused an earlier shift to reproductive phase and decreased maturation time (Georges *et al.*, 2009).

4 Scope of this thesis: dissection of the role of *Arabidopsis PLC3*, *PLC5* and *PLC7* in plant signaling

To be able to study the role of PLC signaling in plants more precisely, we needed additional molecular tools. While the model system *Arabidopsis* contains 9 PLCs (section 2.1), very little was known about their individual function/contribution in plant stress and development. Hence, we used a reversed-genetics approach, using Arabidopsis T-DNA insertion lines from different collections. As such, we found that *plc3* mutants exhibited a lateral-root phenotype (Chapter 2). Since the phenotype was quite mild and *PLC3*-promotor GUS lines revealed specific expression in the vascular tissue, with a particular segmentation pattern that indicated a role for this PLC in lateral root formation, we decided to search for redundant PLCs in this process. As such we found *PLC5* (Chapter 3) and *PLC7* (Chapter 4), with each revealing novel features.

4.1 Characteristics of the *Arabidopsis PLC3*, *PLC5* and *PLC7* genes

*PLC3* (At4g38530), *PLC5* (At5g58690) and *PLC7* (At3g55940) belong to different subfamilies (Fig. 4). Figure 5 shows the multiple sequence alignment of the amino acid sequences, with the four conserved domains underlined in colour. No obvious differences between the PLCs were noted except for a stretch of negatively-charged aspartic acids (Asp, D) in the linker-region between domain X and -Y of PLC7. Predicted proteins were for ~45-53% identical (Fig. 5b). Expression analyses based on Genevestigator data indicate that all 3 PLCs are expressed throughout development, with *PLC7* being relatively lowest but quite abundant in siliques, i.e. during seed development (Fig.6).
Figure 5. Sequence alignment of Arabidopsis PLC3, PLC5 and PLC7.
(a) Multiple sequence alignment of the amino acid sequences. Amino acids are color coded, with Red indicating hydrophobic; Green, polar; Blue, negatively charged; Pink, positively charged amino acids. The four conserved domains are underlined with colors too. Blue, EF-hand; Red, X domain; Pink, Y domain; Green, C2 domain. ‘*’ represents identical residues in all sequences; ‘:’ means conserved substitutions between similar residues have occurred; ‘.’ indicates the semi-conserved substitutions between similar residues. Multiple sequence alignment by MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle/). (b) Amino acid identity of PLC3, PLC5 and PLC7.
Figure 6. Gene expression of PLC3, PLC5 and PLC7 based on Genevestigator.
(a) Ten developmental stages from data selection AT_AFF_ATH1-2 showing 3 measures or 3 PLC genes. (b) Similar to (a) but showing the relative expression levels of each gene per developmental stage.

4.2 Techniques to study PLC signaling in plant

To study the role of PLC in plant cell signaling, biochemical and genetic approaches have been used in this study. Here, these two techniques will be briefly described.

PLC activity can be determined *in vivo* by the measuring the turnover of its substrate, i.e. PIP and PIP₂, and the appearance of products, i.e. IPPs and DAG. The latter, can be witnessed from its conversion into PA via DGK. Since the concentration of the PPIs is very low, a radioactive ³²P⁻(³²PO₄⁻₁⁻) labeling technique is required to visualize the PPI and PA (DAG is no labeled). When taken up by cells, the ³²P first labels the ATP-pool and is then quickly incorporated into those phospholipids that are produced via lipid kinases using this ATP, such as PI- and PIP kinase. The ³²P-label is also incorporated into structural phospholipids but this pathway is much slower (Arisz and Munnik, 2013; Arisz *et al.*, 2013). In fact, relatively short- and long-labeling times, can be used to distinguish between PA formation from either PLC/DGK (short labeling, requiring DAG kinase activity and ATP) or *de novo*- and PLD pathways (require long labeling) (Arisz and Munnik, 2013; Arisz *et al.*, 2013). Phospholipids can be separated by thin layer chromatography (TLC) and phosphoinositides can be
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further analyzed by High Performance Liquid Chromatography (HPLC) but then the fatty acids are removed first, and the resulting glycerophosphoinositides separated by a strong anion-exchanger (Munnik, 2013; Munnik and Zarza, 2013). For IPP measurements, \(^3\text{H-Myo-Inositol}\) labeling is used in combination with HPLC anion-exchange analyses (Lemtriri-Chliel et al., 2000; Stevenson-Paulik et al., 2005; Perera et al., 2008; Laha et al. 2015). These studies showed that IP\(_1\), IP\(_2\) and IP\(_6\) are the most predominant IPPs, whereas IP\(_3\) levels are extremely low.

PLC function can also be studied by genetically modulating its gene expression, preferably in the popular model plant system *Arabidopsis thaliana* (thale cress). With a fully mapped genome sequence and short life cycle, many genetic tools have been developed, including *Agrobacterium tumefaciens*-mediated gene transformation (Krysan et al., 1999), in which T-DNA insertion knock-out (KO), knock down (KD) and overexpression (OE) mutants, as well as GUS/GFP reporter lines can be obtained.

**References**


DeWald DB, Torabinejad J, Jones C, Shope JC, Cangelosi R, Thompson JE, Prestwich GD, and Hama H (2001) Rapid...


Zarza X (2017) Polyamine metabolism and activation of lipid signaling pathways in Arabidopsis thaliana.


