Novel roles for phospholipase C in plant stress signalling and development

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Novel roles for phospholipase C
in plant stress signalling and development

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

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ten overstaan van een door het College voor Promoties ingestelde commissie,
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Promotor: Prof. dr. M.A. Haring, Universiteit van Amsterdam

Copromotor: Dr. T. Munnik, Universiteit van Amsterdam

Overige leden: Prof. dr. C.S. Testerink, Universiteit van Amsterdam
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Faculteit der Natuurwetenschappen, Wiskunde en Informatica

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Chapter 1

General Introduction

Qianqian Zhang,¹,² Michel A. Haring¹ & Teun Munnik¹,²

¹Section Plant Physiology, ²Section Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, Amsterdam, 1098 XH, The Netherlands
Chapter 1

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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 phosphatidylglycerol (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 than 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 PIP$_2$ into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG), which, in mammalian systems, are crucial second messengers, regulating various cellular processes. IP$_3$ is water soluble and diffuses into cytosol to bind the IP$_3$ 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 PIP$_2$, 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 PIP$_2$ into IP$_3$ and DAG. IP$_3$ 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 IP$_3$ and DAG are missing, indicated by the red crosses. Instead, plant seems to use IP$_6$ 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 IP$_3$ receptor, PKC or TRP-channel (Munnik, 2014). Earlier, PLC and IP$_3$ 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 IP$_6$, 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 IP$_6$ from IP$_3$, two inositolpolyphosphate kinases, IPK2 and IPK1, are required (Munnik and Vermeer, 2010; Gillaspy, 2013). Apart from releasing Ca$^{2+}$, IP$_6$ 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 IP₆ have been implicated in signaling. For example, IP₄ has been shown to regulate a chloride channel (Zonia et al., 2002), while IP₅ 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. IP₇ and IP₈. 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 IP₆ should not be confused with its role in plants as phosphate-storage molecule in seeds (phytate), like Ca²⁺ 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 (β, γ, δ, ε, η and ζ) 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, Ca²⁺ binding EF-hands, catalytic X-and Y domains, and a lipid binding-C2 domain in the C-terminal, except for PLCζ, 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. 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 Ca²⁺, whereas soluble PLCs preferred PI at higher (mM) Ca²⁺. The pH optimum for both PLC forms was between 6 and 7 (Munnik et al., 1998).

Mammalian PLCs are known to mainly use PIP(4,5)P₂ as in vivo substrate, which is facilitated by the fact that mammalian cells have relatively high concentrations of PIP(4,5)P₂ in their plasma membranes, and because the PLCs are equipped with PIP₂-binding domains, which is either the PH domain in PLCβ, -γ, -δ, -ε, -η or the XY linker in PLCζ (Swann and Lai, 2016). For plants, the in vivo substrate remains unclear. The PIP(4,5)P₂ levels in plants are 30-100 times lower and the PLCs lack a PH domain or the XY-linker sequence from PLCζ. 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 (leave, 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, Huang, 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.,
2001). 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.

<table>
<thead>
<tr>
<th>Identity</th>
<th>AtPLC3</th>
<th>AtPLC5</th>
<th>AtPLC7</th>
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<td>AP1LC3</td>
<td>45.2</td>
<td>52.4</td>
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<tr>
<td>AP1LC5</td>
<td>53.1</td>
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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 PIP$_2$, 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 $^{32}$P$_i$-($^{32}$PO$_4^{3-}$) labeling technique is required to visualize the PPI and PA (DAG is no labeled). When taken up by cells, the $^{32}$P 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 $^{32}$P-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
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, \(^{3}\)H-Myo-Inositol labeling is used in combination with HPLC anion-exchange analyses (Lemtiri-Chlieh 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 (the 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


General introduction

2893.


van Leeuwen W, Vermeer JEM, Gadella TWJ, and Munnik T (2007) Visualization of phosphatidylinositol 4,5-bisphosphate in


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


Chapter 2

A role for Arabidopsis phospholipase C3 (PLC3) in seed germination, lateral root formation and stomatal closure

Qianqian Zhang,1,2 Ringo van Wijk,1,2 Muhammad Shahbaz,1# Wendy Roels,1 Bas van Schooten,1 Joop E.M. Vermeer,1,3 Aisha Guardia,4 Denise Scuffi,4 Carlos García-Mata,4 Deabrating Laha,5 Phoebe Williams,6 Leo A.J. Willems,7 Wilco Ligterink,7 Susanne Hoffmann-Benning,8 Glenda Gillaspy,6 Gabriel Schaaf,5 Michel A. Haring,1 Ana M. Laxalt,4 & Teun Munnik1,2

1Section Plant Physiology or 2Section Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, Amsterdam, 1098 XH, The Netherlands, 3Dept. of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland, 4Instituto de Investigaciones Biológicas (IIB-CONICET-UNMdP), Universidad Nacional de Mar del Plata, Mar del Plata, Argentina, 5Center for Plant Molecular Biology, University of Tübingen, Tübingen, Germany, 6Dept. of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, 7Lab. of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands, and 8Dept. of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, USA, #Current address: Dept. of Botany, University of Agriculture, Faisalabad, Pakistan.
ABSTRACT

Phospholipase C (PLC) is best known for its role in generating second messengers by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) in mammalian cells. In plants however, PLC’s role is less clear as plants lack the prime targets for both inositol 1,4,5-trisphosphate (i.e. a ligand-gated Ca\textsuperscript{2+} channel) and diacylglycerol (i.e. protein kinase C and TRP-type ion channels). The genome of Arabidopsis thaliana encodes for 9 PLC genes. Here, we analyzed the role of PLC3. Promoter-GUS analyses revealed that PLC3 is specifically expressed in the vascular tissue (most likely phloem) of roots, leaves and flowers, but also in guard cells and at the base of trichomes. Knock-out mutants of PLC3 were found to be affected in seed germination, root development and stomatal closure. Using in vivo \textsuperscript{32}P\textsubscript{i}-lipid labeling analyses, we found that ABA stimulated the formation of PIP\textsubscript{2} in wild type germinating seeds, seedlings and guard cell-enriched leaf peels, but not in plc3 mutants. The latter displayed decreased sensitivity to ABA during seed-germination inhibition and ABA induced-stomatal closure. Overexpression of PLC3 enhanced drought tolerance and decreased stomatal aperture. Together, our results uncovered novel roles for PLC3 in ABA signaling and plant development.

Key words: PLC; seed germination; lateral root formation; stomatal closure; drought tolerance.
INTRODUCTION

Phospholipase C (PLC) is well known for its role in phospholipid signaling in animals. In this classical paradigm, extracellular receptor occupation leads to the activation of intracellular PLC, which hydrolyzes the minor lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce two second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ diffuses into the cytosol where it triggers the release of Ca$^{2+}$ from an intracellular store via an IP$_3$ receptor that is a ligand-gated Ca$^{2+}$ channel, whereas DAG remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family or stimulates TRP- (transient receptor potential-) ion channels. Subsequent changes in Ca$^{2+}$ and phosphorylation status affect multiple protein targets and hence, downstream cellular processes (Irvine, 2006; Michell, 2008; Balla, 2013).

Less is clear about the PLC-signaling paradigm in plants (Munnik, 2014). Most importantly, all higher plant genomes sequenced so far, lack homologs of an IP$_3$ receptor, PKC or TRP channel, which are supposed to be the primary targets of this signaling system (Wheeler and Brownlee, 2008; Munnik and Testerink, 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Munnik, 2014; Heilmann, 2016). Initially, microinjected IP$_3$ had been shown to release Ca$^{2+}$ from an intracellular store (Gilroy et al., 1990; Blatt et al., 1990; Allen and Sanders, 1994) indicating that plants cells exhibited a genuine IP$_3$ receptor (Hunt and Gray, 2001) but Brearley's lab later provided evidence that this IP$_3$ is phosphorylated into IP$_6$ within seconds, and that the latter compound is likely to be responsible for the store-operated Ca$^{2+}$ release (Lemtiri-Chlieh et al., 2000, 2003; Munnik and Vermeer, 2010). Similarly, not DAG but its phosphorylated product, phosphatidic acid (PA) is emerging as the plant lipid-second messenger (Munnik, 2001; Testerink and Munnik, 2005; Arisz et al., 2009; Pokotylo et al., 2014; Munnik, 2014; Vermeer et al., 2017).

Evidence that PLC is important for plants has come from various studies. Silencing of PLC1 in Arabidopsis and tobacco has indicated a role for a PLC in ABA signaling and stomatal closure (Sanchez and Chua, 2001a; Hunt et al., 2003). ABA also induces the expression of some PLC genes (Hirayama et al., 1995; Lin et al., 2004; Tasma et al., 2008; Pokotylo et al., 2014). A link between ABA and polyphosphoinositide (PPI) turnover has been reported, but the data is quite controversial (Munnik and Vermeer, 2010). Nonetheless, ABA has been shown to trigger IP$_6$ responses within minutes in potato guard cell protoplasts and duck weed turions (Flores and Smart, 2000; Lemtiri-Chlieh et al., 2000, 2003), and to elevate intracellular Ca$^{2+}$ levels in a variety of plant (Lee et al., 1996; Staxen et al., 1999; Blatt, 2000; Schroeder et al., 2001; Munemasa et al., 2015; Assmann and Jegla, 2016) Whether the formation of IP$_6$ is PLC-dependent and related to ABA-mediated signaling is still unknown.

Apart from ABA, PLC signaling has been linked to several other abiotic stresses, including salt, drought (mimicked by sorbitol, mannitol or PEG) and heat stress. Interestingly, these stresses are also known to trigger an increase in the level of PIP$_2$ (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; Horvath et al.,
In some cases, IP$_3$ responses were reported, but none addressed IP$_6$ levels or other inositolpolyphosphates (IPPs) that are emerging as signalling molecules, i.e. IP$_5$, IP$_7$ and IP$_8$ (Takahashi et al., 2001; Huang, et al., 2006; Liu, et al., 2006; Zheng et al., 2012; Gilaspy 2013; Laha et al., 2015, 2016). Decreases of PIP have also been reported (Cho et al., 1993; Pical et al., 1999; DeWald et al., 2001; Vermeer et al., 2009; Zarza et al., unpublished) and theoretically, PLC could use PIP as a substrate as well. In vitro, both PPIs are hydrolyzed equally well and in vivo, there is enough PIP in the plasma membrane of plants, where PIP$_2$ is typically missing, and in general only present at very low concentrations (20-100x less than mammalian cells; Munnik et al., 1994; 1998a,b Munnik, 2014; Zarza et al., unpublished). Interestingly, overexpression of PLC in maize, canola and tobacco has been shown to increase the plant's tolerance to salinity-, drought- and/or cold stress (Wang et al., 2008; Georges et al., 2009, Tripathy et al., 2011; Nokhrina et al., 2014), although it is not yet clear how the plant achieves this (Das et al., 2005; Georges et al., 2009).

PLC has also been implicated in plant-microbe interactions (Laxalt and Munnik, 2002) both symbiotic and pathogenic (Luit et al., 2000; Hartog et al., 2003; De Jong et al., 2004; Gonorazky et al., 2014, 2016). Some of the pathogenic responses have been shown comprise nitric oxide (NO) signalling (Lanteri et al., 2011; Raho et al., 2011). Recently, Vossen et al. (2010) presented the first genetic evidence for PLC's contribution in tomato disease resistance (Vossen et al., 2010).

Apart from stress, PLC signalling has also been connected to various growth- and developmental responses. For example, Arabidopsis PLC2 and Torenia fournieri PLC1 are involved in auxin modulated-reproductive development (Song et al., 2008; Li et al., 2015; Di Fino et al., 2017), while petunia PLC1 and tobacco PLC3 regulate tip growth in pollen tubes (Dowd et al., 2006; Helling et al., 2006). In Physcomitrella patens, PLC1 has been shown to play a role in the cytokinin- and gravity response (Repp et al., 2004).

The Arabidopsis genome encodes 9 PLC genes (Mueller-roeber and Pical, 2002). So far, no developmental disorders other than the reproduction mentioned above have been reported for Arabidopsis KO mutants, presumably due to genetic redundancy. Here, we show that PLC3 plays various, yet subtle roles in plant development and ABA signaling, and that overexpression increases drought tolerance.
RESULTS

Loss of PLC3 affects root development

To investigate PLC3 function, we isolated two homozygous T-DNA insertion mutants of PLC3, plc3-2 (SALK_037453) and plc3-3 (SALK_054406) with T-DNA inserts in exon 3 and intron 3 located in the X-domain, respectively (Fig. 1a). Reduction of PLC3 expression was verified by both reverse transcription (RT)-PCR (Fig. 1b) and Q-PCR (Fig. 1c).

Growing seedlings on ½MS plates, a subtle difference in root system architecture between wild type and plc3 mutants was found. Both PLC3 deficient lines exhibited slightly shorter primary roots (~5%) and developed less (~15%) lateral roots than wild type. The mutants also showed low lateral root density (~10%) (Figs. 1d, 1e).

Figure 1. Effect of PLC3 knockout on seedling root development
(a) Representation of PLC3 gene and T-DNA insertion positions of plc3-2 and plc3-3. Filled boxed and lines represent exons and introns, respectively. Open, grey boxes and triangle represent untranslated region, X- and Y- domains and T-DNA insertions, respectively. (b) Confirmation of reduction of PLC3 expression in plc3 lines by cDNA amplification. PLC3-specific primers were used to detect PLC3 mRNA by RT-PCR. TUBULINα4 (TUB) was used as loading control. (c) PLC3 expression level in wild-type, plc3-2 and plc3-3 lines measured by Q-PCR. Relative expression is based on comparison to expression of the SAND gene. Values are means ± SD (n = 3) for one representative experiment. (d) Seedling morphology of wild-type and plc3 mutants. Seeds were germinated on ½ MS with 0.5% sucrose for 4 days, then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (e) Primary root (PR) length, lateral root (LR) number and lateral root (LR) density at 12 DAG. Values are means ± SE of three independent experiments (n=20). Asterisk (*) indicate significance at P<0.05 compared to wt based on Student’s t test.
Expression of *PLC3* during plant development

Changes in transcript level of *PLC3* in various organs and upon induction by hormones or abiotic stress have been reported using quantitative RT-PCR (Hunt *et al.*, 2004; Tasma *et al.*, 2008). To investigate this further, we generated β-glucuronidase- (GUS-) and YFP- reporter lines, driven by a 2.4 kb *PLC3* promoter fragment (*PLC3*<sub>pro</sub>:GUS-YFP). As shown in Figure 2, GUS activity was mainly found in the vasculature, throughout all stages of development, including seedling, cotyledons, leaves, hypocotyl, flower, incl. stamen and style, and during seed development. Interestingly, the base of the trichomes revealed GUS expression (Fig. 2j-k), which again appears to be linked to the vascular system (Fig. 2k). The expression in the main root was not homogenous. At the distal side of the root maturation zone, the GUS expression tended to be 'segmented', while in the apical maturation zone it was continuous, and expression stopped near the transition zone (Fig. 2c-i). Interestingly, lateral roots always emerged from a segment, but not every segment led to a lateral root (Fig. 2c, d). To search for a potential correlation, we analysed GUS expression in seedlings grown on a plate that was positioned in a 45º angle, which forces lateral roots to emerge at the curved sites. Under these conditions, less segments were found but all lateral roots did emerge from a segment (Supplemental Fig. S1). A similar segmented pattern was observed in tertiary root formation (Fig. 2e). Together these results confirm that PLC3 is expressed throughout the plant, but the expression is mainly restricted to the vasculature (Hunt *et al.*, 2004; Tasma *et al.*, 2008).

To obtain more detailed information about the *PLC3* expression within the vasculature, optical cross- and longitudinal sections were made by confocal microscopy (Supplemental Fig. S2). From this data, YFP expression appeared to be localized to the phloem and this correlated with data from the eFP browser, where *PLC3* seems to be predominantly expressed in the companion cells (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).
**Figure 2.** \(PLC3_{pro}^{GUS-YFP}\) expression analyses in Arabidopsis seedlings and mature tissues.

GUS activity was present in the vasculature of 2-d-old (a) and 10-d-old seedlings including shoot and root (b-i). GUS staining was also observed in vascular tissue of mature plants (j), trichome base (indicated by arrows) (j, k), hydathodes (l), silique (l), developing seed chalaza (m) and different parts of the flower (n), including style, filament, receptacle and pedicel (indicated by arrows).
Analysis of PPI-, PA- and IP₆ levels in Arabidopsis seedlings

To determine whether loss-of-PLC3 caused changes in the level of PLC substrates (i.e. PIP and PIP₂) or products (i.e IP₆ or other IPPs), various isotope labelling studies were performed. Since PLC-generated DAG can be rapidly converted into PA (Munnik et al., 1998; Ruelland et al., 2002; Arisz et al., 2009, 2013), we also measured PA levels. Lipids were analyzed by labelling five-day-old seedlings O/N with ³²P. As shown in Figures 3a and 3b, wt and plc3 seedlings were found to contain similar amounts of PIP₂, PIP and PA. For the IPP analyses, ³H-Inositol labelling of seedlings and HPLC analyses were performed, but also here, no significant changes in the level of IP₆ or lower IPPs were found. On closer inspection of the extremely low levels of the pyrophosphate-IPPs (PP-IPPs), i.e IP₇ and IP₈, we did observe some differences, however. Both plc3 mutants were found to contain ~30% less IP₈ than wt (Fig. 3d). While the latter analyses were performed on seedlings of 18-days old (11d + 7d of labelling), we also tested younger seedlings with shorter labelling times (4d old + 4d labelling). Again no differences in IPPs were found but in this case, plc3 mutants were found to contain ~30-40% less IP₇ (see Supplemental Fig. S4).

Figure 3. PPI-, PA- and IPP levels in wild type- and plc3-mutant seedlings.

For lipid analyses, five-days old seedlings were labelled with ³²PO₄³⁻ overnight and the next day their lipids were extracted and separated by TLC. (a) Autoradiograph of a typical experiment. Each lane represents the extract of 3 seedlings. (b) Quantification of ³²P-labeled PIP₂-, PIP- and PA levels in wild-type and plc3 mutants. Values are calculated as the percentage of total ³²P-labeled phospholipids and represented as means ± SD (n=3). This experiment was repeated twice with similar results (c) Inositol polyphosphates (IPP) levels in wild type and plc3-mutant seedlings. (d) IP₇ in wild type and plc3-mutant seedlings. Eleven-day old seedlings were labelled with ³H-myoinositol for 7 days, after which the IPPs were extracted and resolved by HPLC-SAX chromatography. Fractions were collected every minute, and the radioactivity was determined by liquid scintillation counting. The quantities are expressed as percentage of total. Data shown are the means ± SD (n=10) of one representative experiment. Similar results were obtained in an independent experiment.
Loss of *PLC3* affects seed germination

We noticed another subtle phenotype: *plc3* mutants always germinated slower on agar plates. Normally, we imbibed seeds on ½MS plates in the dark at 4 °C for 48 h, after which they are transferred to the light. After 24 h in the light, *plc3-2* and *plc3-3* mutants had germinated 54% and 60% less than wt, respectively, and after 28 h this was 17% and 34% (Fig. 4a). These results also primed us to check the *pPLC3::GUS* expression during seed germination. As shown in Fig. 4b, GUS activity was found during testa rupture and radical emergence in the embryo cotyledons and shoot apical meristem, confirming a role for *PLC3* in seed germination.

To investigate whether this was due to changes in sugar quantity or composition, soluble carbohydrates in *plc3* mutants and wt seeds were measured. As shown in Fig. 4C, small changes in trehalose and stachyose were observed, but these differences were not found to be significant (*P*<0.05). Data from three different seed batches was analysed.

![Figure 4](image-url)

*Figure 4. PLC3 is expressed in germinating seeds and plc3 mutants exhibit a delayed germination rate and soluble carbohydrates content in seeds of wild-type and plc3 mutants*

(a) Seed-germination rate was determined by radical emergence and scored in wild-type and *plc3* mutants. Seeds were stratified on ½MS with 0.5% sucrose plates at 4°C for 2 days and allowed to germinate at 22°C. Data shown are the means ± SD for one representative experiment (n=55 seeds for each genotype). Asterisks (*) mark that *plc3* values are significantly different from wild-type based on Student’s *t*-test (*P*<0.05). (b) GUS activity was determined in embryo cotyledons during seed germination from testa rupture until radical emergence (20-28hrs after transfer from 4°C to 22°C). These experiment were repeated twice with similar outcome. (c) Soluble carbohydrates were extracted from dry seeds and analyzed by Dionex HPLC. Sugar quantities were corrected by means of an internal standard and transformed to µg of sugar per mg of dry material. Values are the means of triplicates ± SE of three independent seed batches.
Decreased sensitivity to ABA in plc3 mutants

Since germination could involve ABA sensitivity, this was analysed in more detail. Wild type and plc3 mutants were germinated on ½MS plates containing different concentrations of ABA (0, 1 and 2 µM). In the absence of ABA, plc3 mutants germinated slower than wt as described above (Fig. 4a). However, in the presence of ABA, plc3 mutants were found to germinate faster than wt (Fig. 5a). For example, after 40 h at 1 µM ABA, 12.5% of plc3-2 and 10.5% of plc3-3 seeds had germinated whereas only 2.5% of the wt seeds. In time, these differences remained or even increased (Fig. 5a, left panel). At higher ABA concentrations (2 µM), seed germination was inhibited more strongly, but again, both plc3 mutants had higher germination rates than wt (Fig. 5a, right panel). These results indicated that plc3 mutants are less sensitive to ABA during germination.

Since ABA is also involved in guard-cell closure (Munemasa et al., 2015), and since antisense-PLC expression in Nicotiana tabacum had been shown to reduce the stomatal-closure response upon ABA treatment (Hunt et al., 2003), we decided to investigate this further for PLC3. As shown in Fig. 5b, GUS activity of the pPLC3::GUS line indicated that PLC3 was indeed active in guard cells. To investigate its putative involvement in ABA-induced stomatal closure, epidermal peels of wt and plc3 mutants were treated with different concentrations of ABA (i.e. 0, 0.1, 1 and 10 µM). In the absence of ABA, no significant differences in the stomatal aperture between wild type and plc3 mutants were found (Fig. 5c). However, with increasing concentrations of ABA, both plc3-2 and plc3-3 showed reduced closure responses compared to wt. In summary, these results indicate that loss-of-PLC3 leads to decreased sensitivity to ABA, in both germinating seeds and guard cells.

**Figure 5.** Decreased ABA sensitivity of plc3 mutants in seed germination and stomatal movement. (a) Seeds germination rate of wild-type and plc3 mutants in the presence of 1 or 2 µM ABA. Seeds were germinated on ½MS with 0.5% sucrose plates with different concentration of ABA at 22 °C after 2 days of stratification at 4 °C. Germination is defined by radical emergence and was scored at the indicated times. Data shown are the means ± SD from one representative experiment of at least 3 experiments (n=55 seeds for each genotype). Asterisks (*) mark that plc3 value are significantly different from wild-type based on Student’s t-test (P< 0.05). This experiment was repeated 3 times with similar results. (b) PLC3pro::GUS-YFP expression in guard cells, using epidermal leaf peels of 3 weeks-old Arabidopsis plants. (c) Effect of ABA on stomatal aperture in wild-type and plc3-2 (left) or plc3-3 (right). Epidermal strips were incubated in opening buffer with light for 3 h until stomata were fully open. Strips were then treated with different concentration of ABA for 90 mins, after which stomata were digitized and the aperture width measured. Data was analyzed by 2-way ANOVA. Statistically significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method). Values are means ± SE of at least three independent experiments (n ≥ 100).
ABA triggers PIP2 formation in germinating seeds, seedlings and guard cells

Since loss-of-PLC3 increased the ABA insensitivity during seed germination and stomatal closure, we decided to analyse the phospholipid levels in more detail in these tissues and to analyse the effect of ABA. First the effect in germinating seeds was analysed (Fig. 6). In the absence of ABA, no major differences in the structural phospholipids (not shown) or PIP and PA (Fig. 6a, b) were found between wild type and plc3 mutants. However, PIP2 levels in plc3 mutants were significantly higher than in wild type seeds (i.e. 23% and 22% for plc3-2 and plc3-3, respectively). Upon ABA treatment, no major changes in PIP or PA were found (Fig. 6a, b). However, while a significant increase in PIP2(27%) was found in wild-type seedlings upon ABA treatment, both plc3 mutants lacked this response (Fig. 6b).

Figure 6. PPI- and PA levels in germinating seeds and effect of ABA in wild type and plc3 mutants.

Seeds of wild-type and plc3 mutants were pre-germinated on ½ MS with 0.5% sucrose plates until testa ruptured, then labelled with $^{32}$PO$_4^-$ for 24 h, after which they were treated for 2 h with buffer ± 100 µM ABA. (a) Autoradiograph of a typical experiment is shown, each lane representing the extract of ± 200 seeds. (b) Quantification of the $^{32}$P-levels of PIP2, PIP and PA. Three independent experiments were performed; data shown are means ± SD (n=3) from one representative experiment. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).

Next, we analysed the response in guard cells (Fig. 7). For this we used epidermal-leaf peels that are enriched in guard cells (Munnik & Laxalt, 2013) from wt and mutant leaves, and labelled these with $^{32}$P, for 3 hrs. Longer labelling times were found to have a negative effect on the viability of the guard cells (not shown). Similar to what we found for germinating seeds, plc3 mutants contained slightly higher PIP2 levels (Fig. 7), while PIP and PA and the major structural phospholipids levels remained unchanged (Fig. 7; not shown). With ABA (15 min treatment), again a significant increase of PIP2 was observed for wild type, but not in the plc3 mutants (Fig. 7). A small decrease in PIP and increase in PA was found, but these changes were not statistically significant (P<0.05). No changes in the structural phospholipids were found. We also tested the effect of ABA after 2, 5, 30 and 60 min, but no clear differences were found before or after treatment in both wild type and plc3 mutants.

Similarly, the effect of ABA on seedlings was analysed. Time-course analyses in wt seedlings revealed an increase in PIP2, which was found to be significant after ~30-60 min of treatment (Supplemental Fig. S6). PIP, PA and structural-phospholipids levels remained the same during that period (Supplemental Fig. S6 b-d). Testing plc3 mutants after 1 h of ABA treatment showed in this case
an accumulation of PIP$_2$, which was not significantly different from wild type (Supplemental Fig. S6f).

PIP, PA and structural-phospholipid levels remained similar.

**Figure 7.** PPI and PA levels in leaf peels and effect of ABA in wild-type and plc3 mutants.

Three-week-old rosette leaf peels from wild-type and plc3 mutants were $^{32}$P$_i$-labeled for 3h and then treated in buffer ± 100 µM ABA for 15 min. Lipids were then extracted and separated by TLC. Radioactivity levels in PIP$_2$, PIP and PA were determined as percentage of total phospholipids. Three independent experiments were performed. Data shown are the means ± SD (n=3) from one representative experiment. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).

**Over expression of PLC3 enhances drought tolerance**

Overexpression of PLC has been shown to promote drought tolerance in maize, canola and tobacco (Wang *et al.*, 2008; Georges *et al.*, 2009; Tripathy *et al.*, 2011). It is unknown whether specific PLCs were chosen for this or whether any PLC can achieve this. For Arabidopsis, this is unknown either. Hence, we generated transgenic plants overexpressing PLC3 under the control of the UBQ10 promoter. Transgenic plants were selected from T0 to T3 and independent homozygous lines were obtained.

Two homozygous lines, PLC3-OE9 and PLC3-OE16, were selected for further studies, overexpressing PLC3 48-fold and 20-fold, respectively (Fig. 8a). No obvious phenotypes were observed comparing wild type and the PLC-OE lines on either agar plates or soil. In soil, four weeks old plants from PLC3-OE lines were found to be more drought tolerant than wt (Fig. 8b) and to show significantly higher survival rates (Fig. 8b, c).

During drought stress, the shoot FW of wild type decreased by ~21%, which was less in the PLC3-OE9 and PLC3-OE16 lines (17% and 12%, respectively)(Fig. 8d). Their DW, however, was higher with or without drought (Fig. 8e). PLC3-overexpression lines also lost less water when water loss of detached 4-week-old rosettes were compared (Fig. 8f).

ABA synthesis is stimulated by dehydration stress and known to induce stomatal closure to reduce water loss (Sean *et al.*, 2010). In the absence of ABA, stomatal aperture of PLC3-OEs was found to be strongly reduced by ~30%. Upon ABA treatment (0.1 µM), stomata closed rapidly for all
genotypes, but the aperture of the \textit{PLC3-OEs} was still significantly smaller than wild type. Above 1 µM, this difference was not observed (Fig. 8g).

\textbf{Figure 8.} Overexpression of \textit{PLC3} enhances drought tolerance.
(a) \textit{PLC3} expression levels in wild-type and two homozygous \textit{PLC3} overexpression lines, \textit{PLC3-OE9} and \textit{PLC3-OE16} as measured by Q-PCR and based on the expression of the \textit{SAND} reference gene. Values are means ± SD (n = 3) for one experiment. (b) Phenotype of wild type- and \textit{PLC3-OE} plants. Four-week old soil-grown plants were exposed to drought stress by water withholding for 2 weeks. (c) Survival rates were determined by counting the visible, green plants after re-watering. (d, e) Fresh- and dry weights were determined from shoots under control and drought (1 week water withholding) conditions. (f) Water loss of detached rosette. Water loss was measured at indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment (n=36). (g) ABA-induced stomatal closure in wild-type, \textit{PLC3 OE9} (left), \textit{PLC3 OE16} (right) plants. Epidermal peels from 3-weeks old plants incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentration of ABA for 90 mins, after which stomata were digitized and the aperture width measured. Values are means ± SE of at least three independents (n >100). Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).
Higher accumulation of PIP$_2$ in PLC3 overexpressing plants under osmotic stress

To determine whether overexpression of PLC3 caused any changes in PPI- and/or PA levels, $^{32}$P-labelling experiments were performed on seedlings, and the effect of 600 mM sorbitol was tested to mimic water stress. As shown in Fig. 9, no major differences between wild type and PLC3-OE lines were found under control conditions. However, upon sorbitol treatment, a much stronger PIP$_2$ response was observed in the OE lines. In wt, PIP$_2$ levels increased by about 300%, while in the OE lines a ~600% increase was witnessed. The PA response appeared slightly higher (200% vs 300%) but this was not statistically significant. The osmotic stress-induced decrease in PIP was similar to wild type (Fig. 9b). These results suggest that PLC3-OE lines are capable of enhancing the PIP$_2$ response under osmotic stress.

![Figure 9](image)

**Figure 9.** Osmotic stress triggers PIP$_2$ and PA responses in wild type- and PLC3-OE seedlings. Six-day-old seedlings were $^{32}$P-labeled for 3h and then treated with buffer ± 600 mM sorbitol for another 30 min. Extracted lipids were analyzed by TLC and quantified through phosphoimaging. (a) Typical TLC profile with each lane representing the extract of 3 seedlings. (b) $^{32}$P-levels in PIP$_2$, PIP and PA of wild-type and PLC3-OE lines #9 and #16 with and without sorbitol. Three independent experiments were performed. Data shown are the means ± SE (n=3) from three independent experiments. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05).
DISCUSSION

In this paper, new roles for PLC in plant stress and development are described. Using loss-of-function mutants in Arabidopsis, we found that AtPLC3 is involved in seed germination, root development, stomatal movement and ABA signaling, whereas overexpression of PLC3 enhanced the plant's tolerance to drought stress. While these findings underline the importance of PLC signaling in plant stress and development, we still know very little of how this is achieved at the molecular level. Theoretically, there are several possibilities. First of all, PLC can produce DAG and IP$_2$ or IP$_3$, (depending on whether PIP or PIP$_2$ is used as a PLC substrate), and while plants lack the classical targets of the mammalian paradigm (i.e. IP$_3$ receptor, PKC), it is likely that their phosphorylated products, i.e. PA and higher IPPs (incl. PP-IPPs) fulfill this second-messenger role in plants. Various biological processes have already been linked to these molecules, and several protein targets involved in signal transduction and metabolism have been identified too (see below). In guard cells, IP$_6$ has been show to release Ca$^{2+}$ (Lemtiri-Chlieh et al., 2000, 2003), so the PLC system in plants could potentially do that.

In non-stressed cells, it is more likely that PLC will hydrolyse PIP than PIP$_2$. The concentration of the latter in plasma membranes is extremely low in plants (30-100 fold lower than mammalians; Munnik et al., 1994; Meijer and Munnik, 2003), while PI4P concentrations appear comparable to those found in mammalian cells (Munnik, 2014). Moreover, in order to make IP$_6$ out of IP$_2$ or IP$_3$ involves the same two inositolpolyphosphate kinases (IPKs). IPK2 is an inositol multiphosphate kinase that can phosphorylate the 3-, 5-, and 6- position of the inositol ring to produce IP$_5$. IPK1 specifically phosphorylates IP$_5$ at the 2-position to produce IP$_6$. VIH2 is a recently discovered IPK that is responsible for the production of the pyrophosphorylated IPPs, i.e. IP$_8$. Like in animal- and yeast cells, these compounds are emerging as important signaling molecules in plants (York, 2006; Michell, 2008; Burton et al., 2009; Shears, 2009; Desai et al., 2014; Laha et al., 2015).

Another function of PLC could be to attenuate PIP$_2$ signalling. While the concentration of this lipid is extremely low under control conditions, PIP$_2$ is readily produced in response to certain hormones or stress signals, where it is suggested to fulfill a second messenger itself, regulating various aspects of plant growth, development, and stress signaling (Gillaspy, 2013; Rodriguez-Villalon et al., 2015; Heilmann, 2016; Zarza et al., unpublished). Potential targets include proteins involved in ion transport (e.g. K$^+$ channels), membrane trafficking (endo/exocytosis, e.g. clathrin and Exo70) and cytoskeletal organization (e.g. small G-protein, Rop) (Gillaspy, 2013; Munnik, 2014; Heilmann, 2016).

In vitro, plant PLCs hydrolyze PI4P and PI(4,5)P$_2$ equally well (Munnik, 2014), and since PI4P is emerging as a lipid second messenger too (Vermeer et al., 2009; Munnik and Nielsen, 2011; Heilmann, 2016), under certain conditions and in particular cells, PLC could also function as attenuator of PI4P signalling. As far as we know, PLCs are unable to use D3-phosphorylated inositol lipids as a substrate [i.e. PI3P and PI(3,5)P$_2$] or PI5P (Munnik, 2014). Whether the newly linked-PLC3 functions observed
here, reflect PLC's role as second messenger producer or -attenuator (or both), needs to be established. Below, a broader perspective of our results is given and some potential molecular mechanisms discussed.

**Role for PLC3 in seed germination**

Promoter-GUS expression in germinating seeds (Fig. 4b), together with the delayed germination phenotype of both plc3 mutants (Fig. 4a), indicates a role for PLC3 in seed germination. Since ABA is known to play an important role in this (Nambara et al., 2010; Nakashima & Yamaguchi-Shinozaki, 2013), we investigated whether the delayed germination of the plc3 seeds was caused by hypersensitivity to ABA. Surprisingly, plc3 mutants were found to be less sensitive to ABA (Fig. 5a). Such results are in agreement with Sanchez & Chua (2001), who found that the ABA sensitivity of seed germination and downstream-gene expression was lost when PLC1 was silenced in Arabidopsis. Guard cells of plc3 mutants were also found to be less sensitive to ABA, which could point to a more general role for PLC3 in ABA signaling (see below; Fig. 5). At least, the above results indicate that the basal, delayed germination rate in plc3 mutants is unlikely to be caused by ABA hypersensitivity.

Gibberellin (GA) is another important hormone involved in seed germination (Yamaguchi and Kamiya, 2001). In contrast to ABA, GA promotes seed germination, and there is data to suggest that this could involve PLC signaling too, i.e. induced PLC expression, changes in PPIs, and increased IP$_3$ levels (Murthy et al., 1989; Chen et al., 1997; Kashem et al., 2000; Villasuso et al., 2003; Fleet et al., 2009; Luo et al., 2012). We tested whether plc3 mutants were affected in GA responsiveness by comparing their germination to wt, with and without 1 mM GA (Supplemental Fig. S7). Although the initial germination rate of plc3-2 and plc3-3 mutants with GA was still slower than wild type (Supplemental Fig. S7a and b), after 24h, the fold-increase in plc3 mutants was around 20% more than in wild type. This was found in three independent experiments, despite the fact that the difference was not statistically significant (Supplemental Fig. S7c). These results point to a possible role for PLC3 in GA signaling, even though the hypersensitivity of the plc3 mutants to GA does not explain their slower germination phenotype. One hypothesis could be that PLC has a positive effect on GA levels and therefore lower GA levels in the mutant, which could delay germination. By adding external GA, some of this inhibition might then be released, which would become visible as hypersensitivity.

Results from our phospholipid measurements revealed that germinating plc3 seeds contained significantly higher levels of PIP$_2$ (Fig. 6), which would be consistent with a loss of PLC3 that would normally hydrolyze this lipid to produce IP$_3$. Unfortunately, the latter is very difficult to measure because seeds contain tiny amounts of IP$_3$ and huge amounts of IP$_6$, and are also extremely difficult to label with $^3$H-inositol (Stevenson-Paulik et al., 2005). Seeds hardly take-up this label, and this is probably also the reason why young seedlings require relatively long labelling times (i.e. 4-11 days vs hrs with $^{32}$P; see Methods). Seeds typically store high amounts of IP$_6$ during their development, where it is used as supply of phosphate (e.g. for DNA, ATP, membranes and sugars) and inositol (IPPs, PPIs,
precursor of cell wall sugars) (Munnik and Nielsen, 2011; Valluru and Van den Ende, 2011) when the seed germinates and the embryo develops into a seedling while growing in the dark. This so-called 'storage' IP₆ is easily confused with 'signalling' IP₆ (Munnik and Vermeer, 2010), but has totally different functions and is probably even differentially localized within cells or tissues. It is difficult, if not impossible, to distinguish between these two IP₆ sources at the moment (Munnik and Vermeer, 2010; Gillaspy, 2011; Munnik and Nielsen, 2011). During seed germination, IP₆ is rapidly broken down to IP₃ (Luo et al., 2012) and this could be an alternative explanation for what was assumed to be PLC-generated IP₃ (Murthy et al., 1989; Chen et al., 1997; Kashem et al., 2000; Villasuso et al., 2003; Fleet et al., 2009; Luo et al., 2012).

Another set of molecules related to inositol metabolism are Raffinose Family Oligosaccharides (RFOs), which serve as desiccation protectant in seeds, as transport sugar in the phloem and as storage sugar in various tissues (Sengupta et al., 2015). In Arabidopsis seeds, RFOs are required for the rapid germination in the dark (Gangl and Tenhaken, 2016). RFOs are sucrose derivatives to which a galactosyl unit is attached via galactinol (Gol). The latter is produced via UDP-galactose and myo-inositol by the enzyme, galactinol synthase (GolS). To make RFOs, free myo-inositol is required and this is predominantly formed through cyclization of glycolytic glucose 6-phosphate (G6P) into inositol-3-phosphate (Ins3P) by myo-inositol-3-phosphate synthase (MIPS) and subsequent dephosphorylation by inositol mono-phosphatase (InsPase). Theoretically, however, inositol could also be generated via dephosphorylation of PLC-generated IPPs (Munnik and Vermeer, 2010). We analysed the soluble carbohydrate composition in seeds and in the phloem sap but found no significant differences between wt and plc3 mutants. Of course, changes could be very local so it is possible these differences remain unobserved.

During seed development, PLC3 was expressed at the chalaza, the non-micropylar end of the seed, likely the chalaza endosperm and/or seed coat (Fig. 2m). Nutrients from the mother plant are transported via the vascular tissue through the chalaza into the nucellus. The vascular and chalaza expression of PLC3 might be necessary for nutrient transportation. Alternatively, PLC might be involved in the production of IP₆ for the storage of essential minerals. Developing seeds store these minerals in three locations, i.e. in the protein storage vacuoles of the embryo, and transiently in the endoplasmic reticulum (ER) and vacuolar compartments of the chalaza endosperm. X-ray analysis and enzyme treatments have suggested that these minerals are stored as IP₆-salts with distinct cation (Mg, Mn, Zn, K, and Ca) composition per compartment (Otegui et al., 2002). As such, loss of PLC3 may affect embryo development, germination and even plant development.

Role for PLC3 in lateral root formation and auxin signaling

Loss-of-function PLC3 mutants displayed shorter primary roots and fewer lateral roots (Fig. 1). The latter was due to less initiation sites, not development (data not shown). Promoter-GUS analyses indicated a very typical, segmented, PLC3-expression pattern at the lateral root-emerging site, whereby
lateral roots always emerged from a segment, but not every segment resulted in a lateral root. Normally, we grow our plates vertically in an angle of 70°. By tilting the agar plate more horizontally (45°), roots start to wiggle more and tend to grow a lateral root at every bend. Using the latter setup for GUS analyses, revealed that lateral roots only emerged from these segments, but that the number of segments was drastically reduced, which was almost 1:1 with the lateral roots whereas with the 70° setup, typically two or three segments were found near the lateral root. These results may indicate that PLC3 expression is required just before the lateral root is initiated, and that the primary root at the 70° setup is less determined as to where and when it will produce the lateral root compared to the 45° setup where this decision is forced at the bending sites (Ditengou et al., 2008). That the phenotype is quite mild may indicate that redundant PLCs are involved. Using the eFP browser data, we found that in addition to PLC3, expression of PLC2, PLC5 and PLC7 is also present in the phloem and/or companion cells.

Interestingly, the initiation of tertiary roots revealed a very similar GUS-expression pattern, showing segments in the lateral roots from which tertiary roots emerged. Root growth and branching are main events of root development. Root growth requires cell proliferation and division in the meristematic zone, and cell expansion in the elongation zone. Lateral root formation involves three major steps, which are initiation, primordial organogenesis, and emergence (Benková and Bielach, 2010). Auxin has been shown to be required in both primary root growth and lateral root formation (Péret, De Rybel, et al., 2009; Péret, Larrieu, et al., 2009; Benková and Bielach, 2010). The signaling pathway of auxin perception is well characterized. Auxin promotes the degradation of the transcriptional repressor Aux/IAA, resulting in massive auxin responsive-gene expression. The auxin receptor, TIR1 is a F-box protein and complex with SCF (ubiquitin protein ligase), which promotes ubiquitin-dependent proteolysis of Aux/IAAs (Kepinski and Leyser, 2005). Interestingly, IP₆ has recently been found in the crystal structure of TIR1 where it is thought to be required for auxin binding and TIR1 function (Tan et al., 2007). Where the IP₆ is coming from is unknown, but potentially this could be formed though PLC3-generated IP₂- or IP₃ formation at the above mentioned 'segments' and subsequent phosphorylation into IP₆. Less PLC-generated IP₆ in plc3 mutants would then lead to less auxin responsiveness during root development (Fig.10A). Redundant PLCs are likely to take over most of PLC3's function(s) though.

In contrast to the germinating seeds, no differences in PPI- or PA levels were found in ³²P₁-prelabeled seedlings (Fig. 3b). However, since PLC3 is expressed in a limited number of cells (especially in the phloem companion cells), analyses of whole seedlings might dilute any difference. We also did not find differences in IP₆ either. Tiny differences in the pyro-IPP levels were found, however, with lower levels of IP₇ and IP₈ in the plc3 mutants depending on their age (Fig. 3c; supplemental Fig. 4). Both IP₇ and IP₈ are implicated as novel signalling molecules (Laha et al., 2015, 2016) for which there is already lots of evidence in yeast and animals (York, 2006; Michell, 2008; Burton et al., 2009; Shears, 2009). That PLC3 could be involved in generating such signaling molecules is exciting, but requires further analysis. Similarly, on the role of IP₆. In guard cells, IP₆ may be
Role for PLC3 in Arabidopsis

role for the release of intracellular Ca\(^{2+}\) and since the latter is also important for auxin signaling (Zhang et al., 2011), this line of research is worth pursuing too. The main bottleneck, still, after the first discoveries over 25 years ago (Blatt et al., 1990; Gilroy et al., 1990; Allen and Sanders, 1994), is the identification of a genuine IP\(_{6}\) (or other IPP-) gated channel (Lemtiri-Chlieh et al., 2000, 2003).

As discussed above, PLC3 could also be involved in inositol-based RFO metabolism. Since RFOs are important for carbohydrate transport- and storage, potentially they could be involved in loading sucrose to sink organs, e.g in lateral root (Van den Ende, 2013; Sengupta et al., 2015; Gangl and Tenhaken, 2016). Analysing the sugar composition of the phloem sap revealed increased amounts of sucrose in the plc3 mutants and slightly decreased levels of myo-inositol levels, although the latter differences were not significant (Supplemental Fig. 3b). If sucrose is not properly transported to, or into, the lateral root via a PLC depended-RFO pathway, then sucrose levels could indeed be higher in the phloem sap and theoretically could affect root growth and lateral root formation.

Role for PLC3 in stomatal closure and ABA signaling

PLC has been linked to ABA signaling in several reports (Hirayama et al., 1995; Sanchez and Chua, 2001; Hunt et al., 2003; Sui et al., 2008). In Arabidopsis, a number of PLC genes are induced upon ABA treatment (Hunt et al., 2004; Lin et al., 2004; Tasma et al., 2008). We tested our plc3 mutants for their response to exogenous ABA with respect to inhibition of seed germination and ABA-mediated stomatal closure along with wild type. Results showed that down-regulation of PLC3 decreased the ABA sensitivity for both responses (Fig. 5). Similar results have been found for germinating seeds of PLC1-silenced Arabidopsis plants (Sanchez and Chua, 2001), and in guard cells of PLC-silenced tobacco plants(Hunt et al., 2003; Mills et al., 2004).

We also tested the effect of ABA on the turnover of phospholipids in germinating seeds and guard cell-enriched leaf peels and found an increase of PIP\(_2\) in both tissues after ABA stimulation, which was strongly reduced or even lost in the plc3 mutants (Figs. 6 and 7). We speculate that PLC3 is activated by ABA, thereby increasing the hydrolysis of PIP\(_2\) and the subsequent replenishment of the pool by PIPK. Increased turnover of PIP\(_2\) is ideally reflected by this type of \(^{32}\)P-labeling experiment (Munnik et al., 1994; Munnik and Zarza, 2013).

In Figure 10B, a model is presented of how PLC3 and PIP\(_2\) could be involved in regulating stomatal movement. The latter is controlled by changes in turgor of the surrounding guard cells. During stomatal opening and -closing, ion channels and cytosolic Ca\(^{2+}\) oscillations play key roles in this process, and these transporters need to be tightly regulated. Over the years, many genes and proteins have been implicated (Ward et al., 2009; Roelfsema et al., 2012; Munemasa et al., 2015; Assmann and Jegla, 2016). Here, we would like to draw the attention of how PPIs and IPPs could regulate stomatal movement. During light induced-stomatal opening, the H\(^+\)-ATPase pump is activated, which causes hyperpolarization of the plasma membrane and the opening of the voltage-gated K\(^+\) - influx channel, KAT1. The subsequent influx of K\(^+\) lowers the water potential and drives the net influx of water into
the guard cell (Dietrich et al., 2001; Schroeder et al., 2001; Ward et al., 2009; Roelfsema et al., 2012). Meanwhile, ABA-INSENSITIVE 1 (ABI1), a type 2C protein phosphatase (PP2C), inhibits SNF1-Related kinase (SnRK2, i.e. OST1) activity, which in its active form activates the slow anion channel 1 (SLAC1). PIP5K4 is essential for stomatal opening (Lee et al., 2007). This lipid kinase generates PIP2, which has been shown to inhibit SLAC1 (Lee et al., 2007) and the K\(^+\)-efflux channel (Ma et al., 2009) co-facilitating the low water potential, the subsequent influx of water, and the opening of stomata.

Upon ABA, the PYR/PYL-receptor dimer dissociates and forms PYR- or PYL-ABA complexes(Ma et al., 2009; Park et al., 2014) that bind PP2C (Melcher et al., 2009; Miyazono et al., 2009; Nishimura, 2009; Santiago et al., 2009; Yin et al., 2009), which can then no longer inhibit the protein kinase activity of SnRK2/OST1 (Hirayama and Umezawa, 2010). As a consequence, OST1 can now auto-phosphorylate itself (Soon et al., 2012) and activate SLAC1 (Kulik et al., 2011), which results in a decrease of intracellular Cl\(^-\). Activated PLC, hydrolyses PIP2, thereby releasing the inhibition of SLAC1 and the K\(^+\)-efflux channel, but also generates increased amounts of IP\(_3\) and IP\(_6\) through IPK1 and IPK2. The IP\(_6\) can release Ca\(^{2+}\) from internal stores (Lemtiri-Chlieh et al., 2000, 2003; Munnik, 2014), which inhibits the K\(^+\)-influx channel (Lemtiri-Chlieh et al., 2000) and co-activates SLAC1 (Siegel et al., 2009). Together these activities cause the net efflux of K\(^+\) and Cl\(^-\), which decreases the water potential and causes water to leave the guard cells and stomata to close. PLC3 seems to be one of the PLC genes involved in this process. Although plc3 mutants still respond to ABA by closing their stomata, the response is significantly reduced. How the PLCs involved are activated still remains elusive. Ca\(^{2+}\) is a potential factor since it stimulates PLC activity in vitro (Munnik et al., 1998), but this would first require an influx of Ca\(^{2+}\) into cytosol via another pathway. As such, PLC and PIP2 would act as facilitators in these biophysical events. The redundancy of 9 PLCs and 11 PIPKs may prevent more clear phenotypes.
Figure 10. Models for the role of PLC in (A) lateral root formation, and (B) regulating stomatal aperture.

Abbreviations: KAT1: voltage-gated K⁺ influx channel; AB11: ABA-INSENSITIVE 1; PP2C: type 2C protein phosphatase; SnRK2: SNF1-Related kinase; SLAC1: slow anion channel 1
Overexpression of PLC3 enhances drought tolerance

Plants cope with drought stress via many different strategies (Zhu, 2002, 2016; Osakabe et al., 2013; Mickelbart et al., 2015) and recently various lipid signalling pathways have been reported to be involved in (Munnik and Meijer, 2001; Zhu, 2002; Meijer and Munnik, 2003; Munnik and Vermeer, 2010; Hou et al., 2016). Moreover, overexpression of a PLC in maize, tobacco and canola have been shown to improve drought tolerance (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011). To investigate whether overexpression of Arabidopsis PLC3 could advance drought tolerance, homozygous pUBQ10::PLC3 overexpression lines were generated. Under control conditions, these plants appeared similar to wild type, but upon drought stress the PLC3-OE lines clearly performed better (Fig. 8b, 8c). They lose less water than wild type, which is likely due to increased number of closed stomata, as there was no difference in stomata number. The molecular mechanism behind this may well reflect what we discussed above and what is summarized in Figure 10.

In an attempt to mimic this in seedlings by using sorbitol, we found that PIP$_2$ and PA accumulated dramatically upon this water stress, and that the accumulation was much stronger in the PLC3-OE lines (Fig. 9). This may again reflect the increased turnover of PIP$_2$ and phosphorylation of DAG, which is readily picked-up by these $^{32}$P-labelling experiments. We also measured IPP levels with $^3$H-inositol labeling, but found no differences there between wt and PLC3-OE lines.

Besides guard-cell regulation, PA and PIP$_2$ may accumulate in various other cells and tissues, since the UBQ10 promoter is constitutively expressed, which is totally different from the endogenous PLC3 expression in the vasculature. Both lipids have been implicated as second messengers, playing roles in reorganization of the cytoskeleton, endo- and exocytosis, vesicular trafficking and ion channel regulation (Stevenson et al., 2000; Martin, 2001; van Leeuwen et al., 2007; Heilmann, 2016), which are all important cellular events. Therefore, PIP$_2$ and PA are very likely to play an important role in the plant's response to control water stress. Further unraveling of the molecular mechanisms involved here requires identification and characterization of some of the main targets of these lipid second messengers, but also for the IPPs, with IP$_6$ and the PP-IPPs in particular. How PLC is activated remains also an important issue to address.

Apart from osmotic stress, heat stress also triggers a PIP$_2$ and PA responses (Mishkind et al., 2009; Horvath et al., 2012). Recently AtPLC3 and AtPLC9 were claimed to be involved in heat stress. Their T-DNA insertion lines lacked the IP$_3$ response and exhibited decreased thermotolerance while overexpression lines showed more heat resistance (Zheng et al., 2012; Gao et al., 2014). Problem here is AtPLC9 is predicted to be “non-active” due to the lack of conserved amino acids in the X-Y domain that are required for the catalytic activity (Hunt et al., 2004). IP$_3$ was measured with the commercial displacement assay, this may reflect changes in the flux of other IPPs (Munnik, 2014). However, if the inactive AtPLC9 could bind PIP$_2$, then its competition with active PLCs might regulate PIP$_2$’s function as a second messenger.
For many years, PLC/IP₃/Ca²⁺ pathway has been claimed to involved in gravitropism (Perera et al., 1999, 2006; Stevenson et al., 2000; Boss et al., 2010). We tested our plc3 mutants response to gravitropism by changing the root growth direction (rotate the plate by 90°; Supplemental Fig. S8 a,b). The roots of both wild type and plc3 mutants bended around 90°due to gravitropism and no obvious difference in bending degree between them (Supplemental Fig. S8c). The reason could be PLCs redundancy. However, most evidence for PLC/IP₃/Ca²⁺ enrollment in gravitropism is based on IP₃ measurements using the commercial IP₃- displacement kit, which might reflect the flux of other IPPs as well (Munnik, 2014). This is equally interesting and deserves further investigation.

MATERIALS AND METHODS

Plant material

*Arabidopsis thaliana* (Columbia-0) T-DNA insertion mutants *plc3-2* (SALK_037453) and *plc3-3* (SALK_054406) were obtained from SALK collection (signal.salk.edu). Homozygous plants were identified by PCR in F2 generation by using gene-specific primers in combination with left border primer LBa (Supplemental Table1).

RNA extraction and RT-PCR

The expression levels of plc3 mutants were confirmed by RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (Pieterse, 1998). RNA (5 µg) was converted to cDNA using oligo-dT18 primers, dNTPs and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. *PLC3* and *TUBLINα4* were PCR amplified for 40 and 30 cycles respectively with gene specific primers (Supplemental Table1).

Cloning and plant transformation

To generate the *PLC3*<sub>pro</sub>:GUSYFP fusion, a 2437 bp *PLC3* promoter region was amplified from genomic DNA using *PLC3promHindIII-fw* 5'- CCAAGCTTCAAGTCGCCGAACGAGACATC-3' and *PLC3promNheI-rev* 5' -CTGCTCTTCTTCTTACTTTGTTAG-3' and cloned in HindIII/XbaI digested pJV-GUSYFP. The *PLC3*<sub>pro</sub>:GUSYF cassette was transferred to pGreen0179 using NotI. MultiSite Gateway Three-Fragment Vector Construction Kit (www.lifetechnologies.com) was used to generate *UBQ10*<sub>pro</sub>:*PLC3*, *PLC3* cDNA was cloned into pGreen0125 expression vector. The procedure followed MultiSite Gateway Three-Fragment Vector Construction Kit user guide (https://tools.thermofisher.com/content/sfs/manuals/multisite_gateway_man.pdf). Constructs were transferred into *Agrobacterium tumefaciens* strain GV3101, which was used to transform wild type plant by floral dip (Clough and Bent, 1998). Homozygous lines were selected in T3 generation and used for further experiments.
Real-time quantitative RT-PCR
The primer pairs used for conformation of \textit{PLC3} (At4g38530) expression level were: 5’-TCCAGTTTCTTCGTAAGATTGGA-3’ (forward) and 5’-TATAGGAACACTGATCGACAGC-3’ (reverse). 1µg total RNA from 10-day-old seedlings was used for cDNA synthesis as described before. Q-PCR was performed with ABI 7500 Real-Time PCR System (Applied Biosystem). The relative gene expression was determined by comparative threshold cycle value. Transcript levels were normalized by the level of \textit{SAND} (At2g28390; forward primer: 5’-AAC TCT ATG CAG CAT TTG ATC CAC T-3’, reverse primer: 5’TGA TTG CAT ATC TTT ATC GCC ATC-3’) (Han et al., 2013). Three biological replicates and two technical replicates were used for the values of means and standard deviations.

Histochemical analyses for GUS activity
GUS staining was performed according to the method described by Jefferson \textit{et al.} (1987) with minor modifications. Transgenic plants carrying \textit{PLC3}_{pro:GUSYFP} were grown for indicated times and specific tissues were taken and incubated in X-Gluc reaction solution containing 1mg/ml 5-bromo-4-chloro-3 indolyl-\textbeta-D-glucuronic acid (X-gluc), 50 mM phosphate buffer pH 7.0 and 0.1% TX-100. The materials were incubated overnight at 37°C. The next day, the solution was replaced by 70% ethanol to destain the tissue. Plant tissues were viewed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera).

Confocal microscopy
Arabidopsis \textit{PLC3}_{pro:GUSYFP} seedlings were grown 5 days and then transferred to object slides containing a fixed cover slide, separated by a spacer of approximately 0.32 mm. This allows seedlings to grow in liquid medium (½ MS and 1% sucrose, pH 5.8) for 1-2 days and could directly be used for microscopy. Microscopy was performed using a Zeiss LSM 510 CLSM (confocal laser scanning microscope) (Carl-Zeiss GMBH, Jena, Germany), implemented on an inverted microscope (Axiovert 100, Carl-Zeiss GMBH, Jena, Germany). For imaging YFP, we used confocal configurations as described before (Vermeer \textit{et al.}, 2006).

Seed germination
Mature seeds were harvested and stored at room temperature. Seeds were surface sterilized in a desiccator by using 20 ml thin bleach and 1ml 37% HCl for 3 hours and then were sown on square petri dish containing 30 ml medium consisting of ½ Murashi-Skoog (½ MS), 0.5% sucrose, pH 5.8, and 1.2 % daishin agar with or without ABA or GA at indicated concentrations under 4 °C in dark for two nights and transferred to long day condition (22 °C, 16 h of light and 8h of dark). Germination was scored as radical emergence at indicated time points by using a binocular microscope (Leica MZFLIII).
Root growth
Seeds were sterilized and stratified as described above. Plates were transferred to long day condition and placed vertically under an angle of 70°. Four-day-old seedlings with comparable size were transferred to ½ MS agar plate. The plates were scanned 12 days after germination (Epson Perfection V700 scanner). Primary root length and lateral root number from each genotype were quantified by ImageJ analysis software (National Institute of Health (NIH)). Lateral root density was expressed as the lateral number per primary length (LR number/ PR length). For gravitropic responses, seedlings were grown on ½ MS plates with 0.5% sucrose for 4 days. Plates were then rotated by 90° and scanned 2 days later. Bending was expressed as curvature angle, which was quantified by ImageJ analysis software (NIH) (Perera et al., 2006).

Stomatal aperture
The stomatal aperture measurement was performed according to Distéfano et al. (2012) with minor changes. The stomatal aperture treatments were performed on epidermal strips excised from the abaxial side of fully expanded Arabidopsis leaves. Epidermal peels from leaves of 3-week-old plants grown at 22°C under 16 h of light and 8 h of dark were stripped and immediately floated in opening buffer (5 mM MES-KOH, pH 6.1, and 50 mM KCl) for 3 h. The strips were subsequently maintained in the same opening buffer and exposed to different ABA concentration. After 90 min, stomata were digitized using a Nikon DS-Fi 1 camera coupled to a Nikon Eclipse Ti microscope. The stomatal aperture width was measured using ImageJ software (NIH).

$^{32}$Pi-phospholipid labelling, extraction and analysis
Different types of tissues were labelled. For germinating seeds: Seeds were sterilized and stratified on ½ MS (pH 5.8) as described and germinated under long day condition for around 20 h when testa ruptured. Germinating seeds were then transferred to 200 µl buffer (2.5 mM MES, pH 5.8, 1 mM KCl) containing 5-10 µCi $^{32}$PO$_4^{3-}$ ($^{32}$P$_i$) (carrier free; Perklin-Elmer) in 2 ml Eppendorf microcentrifuge tube for 24 h. Samples were then treated with 200 µl buffer with or without ABA for the times and concentrations indicated.

Epidermal leaf peels: Leaves of 3-week-old plants grown at 22°C under 16 h of light and 8 h of dark were stripped and immediately floated on 100 µl opening buffer (10 mM MES, pH 6.1 and 50 mM KCl) containing $^{32}$P$_i$ (5-10 µCi) in a 48-wells cell culture plate (Greiner bio-one) for 3 h. Samples were treated with 400 µl buffer (10 mM MES-KOH, pH 6.1, 2.5 µM CaCl$_2$) with or without ABA for the times and concentrations indicated.

Seedlings: Five-day-old seedlings were transferred to 200 µl labeling buffer (2.5 mM MES-KOH, pH 5.8, 1 mM KCl) containing $^{32}$P$_i$ (5-10 µCi) in 2 ml Eppendorf tubes and labeled overnight.
Samples were treated the next day by adding 200 µl labeling buffer with or without ABA or Sorbitol for times and concentrations indicated.

All treatments were stopped by adding perchloric acid at a final concentration of 5% (v/v) for 5-10 min, after which the material was transferred to 400 µl of CHCl₃/MeOH/HCl [50:100:1 v/v/v] to extract the lipids. After 15 min, 400 µl of CHCl₃ was added followed by 200 µl of 0.9 % (w/v) NaCl to separate the extract into two phases. Lipid fractions were washed and concentrated as described earlier (Munnik & Zarza, 2013). Lipids were separated by thin-layer chromatography (TLC) using an alkaline solvent system, containing: chloroform/methanol/28% ammonia/water [90:70:4:16 (v/v)] (Munnik et al., 1994). Radioactive phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA). Individual phospholipid levels are expressed as the percentage of the total ³²P-lipid fraction.

**Inositol phosphates labeling, extraction and HPLC Analyses**

For the measurement of inositol polyphosphates (IPPs), two different procedures were followed. The first is based on the method described in Laha et al. (2015) with minor modifications. Seedlings were grown under short day (22 °C, 12 h of light and 12h of dark) and sterile conditions in plant media (½ MS, 2% sucrose, pH 5.7, 0.6% phytagel) for 11 days and then 10 seedlings were transferred to 2ml liquid medium (½ MS, pH 5.7, 0.3% phytage) containing ³H- myo-inositol (80 µCi, Biotrend, ART-0261-5, Cologne, Germany) for 7 days. Seedlings were washed two times with water before harvesting and then snap-frozen into liquid N₂. IPPs were extracted (Azevedo and Saiardi, 2006) and resolved by strong anion exchange chromatography HPLC (using the partisphere SAX 4.6 x 125mm column; Whatman) at a flow rate of 0.5 mL/min, using a shallow gradient formed by buffer A (1 mM EDTA) and buffer B (1 mM EDTA and 1.3 M Ammonium Phosphate, pH 3.8 with H₃PO₄). Fractions were collected every minute and radioactivity quantified by liquid scintillation counting. The results are expressed as percentage of total. The latter was determined by counting all fractions from 13 min to the end of the run.

Alternatively, IPP were determined as described by Desai et al., (2014) with some modifications. Seedlings were grown in ½ MS with 0.8 % agar under long day condition (100 µE light with a 16 h day and 8 h night cycle) for 4 days. Fifteen seedlings were incubated with 50 µl medium (1x MS, 1% sucrose, pH 5.7) and 100 µl of aqueous myo [2-³H(N)]-inositol (100 µCi, American Radiolabeled Chemicals Cat. #ART 0116A, specific activity 20 Ci/mmol) was added to each tube. The tubes were incubated with supplemental light for 4 days. IPPs were extracted as Azevedo and Saiardi (2006) described, by vortexing the tissue with glass beads in extraction buffer (25 mM EDTA, 10 mg/ml IP₆ and 1M HClO₄). Samples were then neutralized to ~pH 6 to 8 with 250 mM EDTA, 1M K₂CO₃. Samples were dried to a volume of 70 µl and separated using a binary HPLC pump (Beckman Coulter) equipped with a Partisphere-SAX (4.6 x 125 mm) column, which was connected to a guard cartridge. The elution gradient was set up as described by Azevedo and Saiardi (2006) using the same
buffers as above at a flow rate of 1ml/min. An on-line IN/US radiation detector was used to generate chromatograms. Four ml of Ultima-Flo AP scintillation cocktail (Perkin Elmer, Waltham, MA, USA) was added to each 1 ml eluted fraction post-detector to quantify the radioactivity of the eluted fractions using the $^3$H window of a Beckman Coulter LS6500 Scintillation Counter. Scintillation counts were graphed using MicroSoft Excel. The $^3$H-myo-Ins cpm incorporated into total IPPs was calculated by taking the sum of cpm of all fractions and subtracting the peak of free $^3$H-Ins cpm. The amount of each IPP was calculated as follows: \[ \frac{[\sum \text{cpms in peak}]}{\text{total IPP}} \times 100 \].

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**Drought tolerance assays**

Determination of survival rates, fresh weight (FW) and dry weight (DW) under water deficit condition and water loss were performed as described previously (Hua et al., 2012; Osakabe et al., 2013) with some changes. Seeds were stratified under 4°C, dark for 2 nights and sown on soil pot (4.5 cm x 4.5 cm x 7.5 cm) directly. Nine plants were grown in each pot with certain amount of soil (80 g) under short day condition (22 °C with 12 h light/12 h dark) for 4 weeks and then subjected to dehydration by withholding them for water for 2 weeks, while control plants were normally watered. And then plants were photographed. The plants were re-watered for another week and photographed. The surviving green plants were counted and survival rate was determined by the percentage of green plants compared to total plants. Each experiment used 36 plants per genotype and experiments were repeated at least 3 times.

To determine the FW and DW under dehydration stress, plants were grown under short day conditions as described above for 4 weeks and experienced 1 week dehydration by water withholding, while control plants were normally watered. Rosettes FWs were scored immediately after detachment. After complete drying, dry DWs were also determined. Each experiment used 36 plants per genotype and experiments were repeated for 3 times.

To assay the water-loss, rosettes from 4-week-old plants were detached and FW determined every one hour by weighing. Water content was calculated as a percentage from the initial FW. Twenty plants were used for each experiment and each experiment was repeated at least 3 times.

**Soluble carbohydrates measurement in seeds**

Soluble carbohydrates were determined as described by (Ribeiro et al., 2014) with minor modifications. Three milligrams of dry seeds were transferred to a 2 mL Eppendorf tube and homogenized in 1 mL of methanol (80% v/v) with the addition of 40 µg of melezitose as internal standard. Samples were incubated in a water bath for 15 minutes at 76°C and dried by vacuum centrifugation. Then, 500 µL of milliQ water was added, thoroughly vortexed and centrifuged for 5 min at 17,000 g in an Eppendorf centrifuge. The supernatant was analyzed with a Dionex HPLC system (ICS 5000 + DC) using a CarboPac PA1, 4 x 250-mm column (Dionex) preceded by a guard column (CarboPac PA1, 4 x 50 mm). Mono-, di-, and trisaccharides were separated by elution in an increasing concentration of NaOH (20-350 mM) with a flow rate of 1 mL per minute. Peaks were identified by coelution of standards.
**Phloem sap soluble carbohydrates measurement**

Phloem exudates were extracted and analyzed as described earlier (Guelette et al., 2012; Tetyuk et al., 2013; modified from Roessner et al., 2000). The hydrophilic fraction (sugars, amino acids, small molecules) was dried and derivatized using methoxyamine in pyridine and BSTFA (N,O-bis-trimethylsilyl trifluoroacetamide) with 1% trimethylchlorosilane. The metabolites were subsequently separated using an Agilent 5890N GC system, coupled to a 5973 inert MSD using a DB-5MS column (J&W Scientific, 30m x 0.32mm ID x 0.25 µm film; temperature program: 70-5, 5/min-320-5). Identification of compounds occurred using the NIST library (NIST Standard Reference Database; [https://www.nist.gov/srd/nist-standard-reference-database-1a-v14](https://www.nist.gov/srd/nist-standard-reference-database-1a-v14)) in combination with co-elution of standards. Peak area determination was performed using QuanLynx, a quantification software within the MassLynx software (Waters). The experiment was repeated five times.

**ACKNOWLEDGEMENTS**

We thank Max van Hooren for critically reading this manuscript. This work was financially supported by a grant from the China Scholarship Council (CSC File No. 201206300058 to Q.Z.) and by the Netherlands Organization for Scientific Research (NWO/ALW 813.06.003, 864.05.001, and 867.15.020 to T.M.).

**REFERENCES**


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Supplemental Table S1. Primers for the identification of PLC3 T-DNA insertion mutants and for PLC3 RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tr>
<td>PLC3_Forward</td>
<td>TGCTGAAGTTCGTCATGGCAG</td>
</tr>
<tr>
<td>PLC3_Reverse</td>
<td>GTCCACCCAACATGAGGATCG</td>
</tr>
<tr>
<td>LBa*</td>
<td>TGGTTCACTGATGGGCCATCG</td>
</tr>
<tr>
<td>TUBLINα4_Forward</td>
<td>CCAGCCACCAACAGTTGTTC</td>
</tr>
<tr>
<td>TUBLINα4_Reverse</td>
<td>CACAAGACGAGATTATAGAGA</td>
</tr>
</tbody>
</table>

* LBa and PLC3_Forward combination is for T-DNA insertion identification

Supplemental Figure S1. PLC3pro-GUS-YFP expression in seedling grown at 45° angle.

To find a stronger correlation between lateral root formation and the segmented PLC3-GUS expression, seedlings were grown on ½MS agar plates at an angle of 45° for 10 days to force later root formation at the curved sites of the primary root. (a) Cartoon of the setup. (b) Cartoon of the curvy seedlings generated and the lateral root formation at the curved sites (blue circles). (c) Histological GUS analysis of PLC3pro-GUS-YFP seedlings grown for 10-d at a 45° angle. Using this setup, less segments without lateral root were found (red circles). These results also show that GUS activity is not homogenously expressed throughout the root vasculature, going from segmented (top), to complete GUS positive (middle) to no GUS activity (root tip, transition zone).
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**Supplemental Figure S2.** Confocal analysis of PLC3 expression. Confocal image of longitudinal section (a) and cross section (b) of 5-d old seedlings. (c) eFP browser database of PLC3 expression in old and young root tissues.

**Supplemental Figure S3.** Soluble carbohydrates content in the phloem sap of wild-type and plc3 mutants. Phloem was isolated from 6-week-old Arabidopsis plants and their carbohydrates analyzed and quantified by GC-MS. Values are the means of triplicates ± SD from 3 independent experiments.

**Supplemental Figure S4.** Inositolpolyphosphate levels in wild-type and plc3 mutants. (a) Inositolpolyphosphate levels in wild-type and plc3 mutants. (b) IP7 in wild type and plc3-mutant seedlings. Four-days old seedlings were labelled with [2-3H(N)]-inositol for 4 days after which IPPs were extracted and resolved by HPLC-SAX analysis. Fractions were collected each minute and analyzed by liquid scintillation counting. The ^3H-myo-Ins cpm incorporated into all inositol phosphates (total IP%) was calculated by taking the sum of cpm of all fractions and subtracting the ^3H-Ins peak cpm. The amount of each IP was calculated as follows: [(Σ cpm in peak) / (total InsPs)]*100%. Data shown are means ± SE (n=10) from three independent experiments.
Supplemental Figure S5. Effect of ABA on root development in wild-type and plc3 mutants.
(a) Seedling morphology of wild-type and plc3 under normal and ABA conditions. Seeds were germinated on ½MS with 0.5% sucrose for 4 days and then transferred to ½ MS with and without ABA (10 µM). Photographs were taken 12 d after germination. (b) Relative primary root (PR-) and lateral root (LR) growth were calculated as a percentage of the length under control condition. Three independent experiments were performed. Data shown are the means ± SD (n>10) for one representative experiment.
Supplemental Figure S6. Effect of ABA on phospholipid-signaling responses in *Arabidopsis* wt- and *plc3* seedlings.

(a-d) Time-course of ABA response in wt seedlings. Six-day-old seedlings were $^{32}$P-labeled for 3h and then treated with buffer with or without 100 µM ABA for different periods of time (0, 2, 4, 8, 16, 32 and 64 min). Lipids were extracted and separate by TLC. Radioactivity was visualized by autoradiography (a) and quantified by phosphoimaging (b-d). Lipids are expressed as fold-increase with respect to control. Values are the means of triplicates ± SD for one representative experiment. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).

(e-h) ABA response in *plc3* mutants. Five-day-old wt- and *plc3* seedlings were $^{32}$P-labeled for 3h and then treated with buffer or 100 µM ABA for 1 h. (e) Autoradiograph of TLC. (f-h) Quantification of PIP$_2$, PIP and PA. Data shown are the means ± SE of three independent experiments. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).
**Supplemental Figure S7.** Effect of GA on seed germination of wild-type and plc3 mutants

Seeds germination rate of wild-type and plc3 mutants in the absence (a) or presence of 1 µM GA (b). Seeds were germinated on ½ MS with 0.5% sucrose plates with or without GA at 22 °C after 2 days of stratification at 4 °C. Germination is defined by radical emergence and was scored at the indicated times. In (c), the relative effect of GA on seed germination is calculated. Data shown are the means ± SE of 3 independent experiments (n=55 seeds for each genotype). Asterisks (*) mark that plc3 value are significantly different from wild-type based on Student’s t-test (P< 0.05).

**Supplemental Figure S8.** Gravitropic response of roots from wild-type and plc3 mutant seedlings

(a,b) Seedling morphology of wild-type and plc3 mutants under gravitropic stimulation. Seedlings were grown on ½ MS with 0.5% sucrose plate for 4 days, plates were then rotated by 90°. Photographs were taken 2 days after plate turning (6-d old seedling). (c) Bending was expressed as curvature angle. Values are means ± SD for one representative experiment (n>20). Three independent experiments were performed.
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Functional characterization of PLC5 in *Arabidopsis thaliana* - knock-down affects lateral root initiation while overexpression stunts root hair growth and enhances drought tolerance


1Section Plant Physiology or 2Section Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, Amsterdam, 1098 XH, The Netherlands, 3Dept. of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland, 4Instituto de Investigaciones Biológicas (IIB-CONICET-UNMdP), Universidad Nacional de Mar del Plata, Mar del Plata, Argentina, 5Laboratory of Molecular Plant Biology, University of Leuven, Leuven, Belgium, 6Dept. of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, USA. #Current address: Dept. of Botany, University of Agriculture, Faisalabad, Pakistan
ABSTRACT

In animal cells, phospholipase C (PLC) is known to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to generate the second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG), which release intracellular Ca$^{2+}$ and activate protein kinase C (PKC), respectively, orchestrating a wide range of cellular- and physiological processes. Plants contain PLCs too but lack IP$_3$ receptors and PKC, and it still remains largely unclear what its physiological role is in plants and what molecular targets it has. The genome of Arabidopsis thaliana encodes 9 PLC genes. Earlier work on PLC2, PLC3 and PLC9 revealed roles for PLC in gametogenesis, ABA signalling, lateral root formation and heat stress tolerance. Here, we functionally characterised the role of PLC5. Promoter-GUS analyses revealed that this gene is predominantly expressed in vascular tissue, most likely the phloem, including roots, leaves and flowers, but expression was also detected in the root-apical meristem, in guard cells and in trichomes. We only managed to find one homozygous T-DNA insertion line, plc5-1, which turned out to be a knock-down mutant, suggesting that a KO mutant is probably lethal. Growth of plc5-1 plants on agar plates consistently exhibited a ~20% reduction in their lateral root formation. The latter was caused by a decrease in initiation rather than emergence of the lateral roots. PLC3 was found to be required for lateral root formation earlier (Chapter 2), but a double plc3plc5 mutant did not intensify the phenotype, indicating the involvement of possible additional redundant PLCs. Complementation of plc5-1 with the PLC5-wt gene, expressed behind its own promoter, restored growth and rescued the lateral root phenotype. UBQ10-Overexpression of PLC5 did not affect lateral root development, but was found to stunt root hair growth, to decrease the stomatal aperture and to increase their tolerance to drought stress. In vivo $^{32}$P$_i$-labeling analyses of PLC’s substrate/product lipids revealed no differences in plc5-1 seedlings, however, PLC5-overexpression lines clearly exhibited reduced levels of PIP- and PIP$_2$ and increased levels of phosphatidic acid (PA), the latter likely produced through phosphorylation of PLC-generated DAG. Inducible overexpression of PIP5K3 in PLC5 overexpressor lines recovered the stunted root-hair growth and restored PIP$_2$ level. These results provide independent evidence for PIP$_2$’s role in polar tip growth of root hairs, confirm PLC’s involvement in lateral root formation, and that overexpression of PLC seems to increase drought tolerance in general.

Key words: PLC5; lateral root formation; root hair; drought tolerance.
INTRODUCTION

Phospholipase C (PLC) signaling is implicated in various cellular events and plays crucial roles in all eukaryotic cells. The pathway is best known from animal systems where it generates the second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG), which are formed through PLC-catalyzed hydrolysis of the minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP$_2$). The water-soluble IP$_3$ diffuses into the cytosol where it triggers the release of Ca$^{2+}$ from the ER via a ligand-gated-Ca$^{2+}$ channel, while the lipid DAG, remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family or stimulates TRP (transient receptor potential) type-ion channels. Stimulation of the PLC pathway activates multiple downstream signaling cascades, regulating multiple cellular processes (Irvine, 2006; Michell, 2008; Balla, 2013).

Plants also contain PLCs, but the signaling pathway is likely different from animal systems, as plants lacks the primary targets for both IP$_3$ and DAG, i.e., the IP$_3$ receptor, PKC and TRP channels (Zonia & Munnik, 2006; Wheeler and Brownlee, 2008; Munnik, 2014). Also different, is that its potential substrate, PIP$_2$ is hardly present in plant plasma membranes, while its precursor, phosphatidylinositol 4-monophosphate (PIP) is relative abundant (Munnik and Testerink, 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Munnik, 2014; Heilmann, 2016). In vitro, plant PLCs do hydrolyze both PIP and PIP$_2$ but in vivo it is still unknown what the normal substrate is (Munnik, 2014). Biochemical subfractionation analyses showed that most (>90%) PLC activity is present in the plasma membrane fraction (Munnik et al., 1998a).

IP$_3$ was initially linked to the release of intracellular Ca$^{2+}$ (Gilroy et al., 1990; Blatt et al., 1990; Allen and Sanders, 1994; Hunt and Gray, 2001), but later this was shown to be caused by IP$_6$ that was produced by phosphorylating IP$_3$ (Lemtiri-Chlieh et al., 2000, 2003; Munnik and Vermeer, 2010). Similarly, not DAG, but its phosphorylated product, phosphatic acid (PA) has been emerging as the plant lipid-second messenger (Munnik, 2001; Testerink and Munnik, 2005; Arisz et al., 2009; Pokotylo et al., 2014; Munnik, 2014; Heilmann 2016). In plants, PA can be further phosphorylated into diacylglycerolpyrophosphate (DGPP) by PA kinase, an enzyme that is lacking from animals but is present in fungi, oomycetes and trypanosomes (van Schooten et al., 2006a). Whether the formation of DGPP reflects an attenuation of the PA signal or the formation of a new signal (this lipid is normally not there either), remains unknown. Meanwhile, various other inositolpolyphosphates (IPPs) than IP$_6$ are emerging as signaling molecules, also in fungi and animals, where they have been implicated in ion channel binding, phosphate sensing, transcription and embryonic development etc. In plants, IP$_4$ has been proposed to regulate a chloride channel (Zonia et al., 2002), while IP$_5$ and IP$_6$ were discovered in the crystal structure of TIR1 and COI1, which are receptors for auxin- and jasmonate signaling, respectively(Tan et al., 2007; Sheard et al., 2010). Gle1, an mRNA export factor, is an IP$_6$-binding protein that has recently been identified as a key activator of the ATPase/RNA helicase, LOS4 (low expression of osmotically responsive genes 4), similar to the Gle1-IP$_6$-Dbp5 (a LOS4 homolog).
paradigm in yeast (Lee et al., 2015). Recently, SPX-domain containing proteins were also identified as interactors with IP₆ and many of these proteins are involved in phosphate homeostasis (Kuo et al., 2014; Puga et al., 2014; Wild et al., 2016). Besides Ca²⁺ release in guard cells upon ABA stimulation (Lemtiri-Chlieh et al., 2003), IP₆ production has also been linked to disease resistance (Murphy et al., 2008). Recently, it has been discovered that IP₆ can also be pyrophosphorylated to IP₇ and IP₈, which have also been implicated in signaling, including JA and plant defense (Laha et al., 2015, 2016; Williams et al., 2015). Besides the lipid-generated pathway via PLC, IPPs can also be synthesized by the conversion of glucose 6-phosphate into inositol 3-phosphate, which is then stepwise phosphorylated by various IPP kinases (Munnik and Vermeer, 2010).

Plant PLCs have been implicated in various abiotic stress responses. PLC gene expression is typically induced by various stresses, including salt, drought, heat, and cold stress (Hirayama et al., 1995; Hunt et al., 2004; Lin et al., 2004; Das et al., 2005; Vergnolle et al., 2005; Zhai et al., 2005; Skinner et al., 2005; Liu et al., 2006a; Tasma et al., 2008; Sui et al., 2008). Some of these have been correlated with changes in IP₃ (DeWald et al., 2001; Takahashi et al., 2001; Ruelland et al., 2002; Zheng et al., 2012; Gao et al., 2014), but we currently know that these measurements are strongly subject to artifacts and to changes in the flux of IP₆, the most abundant IPP (for discussion, see Munnik and Vermeer, 2010; Munnik 2014). Increases in PA have also been reported and some of these were indeed generated (or at least partly) by DAG kinase (DGK) rather than phospholipase D (PLD), which is another important PA generator (Arisz et al., 2009, 2013; Arisz and Munnik, 2013).

Increases in PIP₂ upon salt- or heat stress have been reported (DeWald et al., 2001; van Leeuwen et al., 2007; Mishkind et al., 2009; Simon et al., 2014), and these are mainly due to activation of a PI4P 5-kinase (PIPK) rather than inhibition of a PLC (Mishkind et al., 2009; Zarza, 2017; Munnik Lab, unpublished). Decreased PIP levels have been reported in response to osmotic- and temperature stresses (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., 2016). While many of these responses have been correlated to PLC signaling, none of them have ever been functionally linked.

Overexpression of Arabidopsis PLC3 increased the plant’s tolerance to drought stress (Chapter 2; Zhang et al., 2017), which was consistent with earlier studies on tomato, canola and maize (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011).

PLC has also been associated to biotic-stress responses (Luit et al., 2000; Hartog et al., 2003; De Jong et al., 2004; Vossen et al., 2010; Gonorazky et al., 2014, 2016), some in relation to nitric oxide (NO) signaling (Laxalt et al., 2007; Lanteri et al., 2011; Raho et al., 2011). Genetic evidence for PLC’s involvement in disease resistance has been obtained for tomato (Vossen et al., 2010; Gonorazky et al., 2014, 2016; Abd-El-Haliem et al., 2016) and recently for Arabidopsis PLC2 (D’Ambrosio et al., 2017). In both cases, PLC seems to be involved in the production of reactive oxygen species (ROS); for AtPLC2 this involved RBOHD (D’Ambrosio et al., 2017).
While plant-PLC signaling is often linked to stress, there is also evidence for its involvement in growth- and developmental responses. For example, PLC is important for pollen tube growth in petunia and tobacco (Dowd et al., 2006; Helling et al., 2006) and affects male- and female gametophyte development in Arabidopsis and Torenia fournieri (Song et al., 2008; Li et al., 2015; Di Fino et al., 2017). In Physcomitrella, PLC is involved in cytokinin- and gravity responses (Repp et al., 2004). In higher plants, IP$_3$ changes have been correlated to gravitropism and Ca$^{2+}$ signaling too (Perera et al., 1999, 2006; Stevenson et al., 2000; Boss et al., 2010) but it is unclear whether this involves a PLC.

Loss-of-PLC3 in Arabidopsis did not alter gravitropism response (chapter 2; Zhang et al., 2017). The latter did, however, reveal reduced lateral root formation, reduced seed germination, and reduced sensitivity to ABA with respect to stomatal aperture and the inhibition of seed germination (chapter 2; Zhang et al., 2017). Arabidopsis plc3- and plc9 mutants also exhibit reduced thermotolerance responses (Zheng et al., 2012; Gao et al., 2014).

The Arabidopsis genome encodes 9 PLC genes (Hunt et al., 2004; Tasma et al., 2008; Munnik, 2014; Pokotylo et al., 2014). While AtPLC3 is mainly expressed in the vasculature, most likely in the phloem and companion cells (Chapter 2; Zhang et al., 2017), we searched for other PLC genes specifically expressed in the phloem, and this resulted in the identification of AtPLC5, which belongs to a different subfamily than AtPLC3 (Hunt et al., 2004; Tasma et al. 2008). Here, we provide evidence that AtPLC5 plays a role in lateral root development and that its overexpression increases the plant’s tolerance to drought. Interestingly, this overexpression led to a stunted root-hair phenotype, which is likely caused by the increased hydrolysis of PIP$_2$ at the tip of the root hair, required for its growth.
RESULTS

Knockdown of PLC5 affects root development

Previously, we found that PLC3 knock-down mutants were affected in their primary- and lateral root development, and that this was linked to specific PLC3 expression in the phloem/companion cells within the root (Chapter 2; Zhang et al., 2017). In the eFP browser, we found that Arabidopsis PLC5 is also predicted to be expressed in the phloem/companion cells (eFPbrowser At5g58690). To functionally characterize PLC5, we tried to obtain homozygous T-DNA insertion mutants, but uncovered only one, i.e. plc5-1 (SALK_144469) (Fig. 1a). Q-PCR analysis revealed that it was a knock-down and not a knock-out mutant (Fig. 1b). As no other T-DNA insertion mutants were found, it may be that PLC5-KO mutants are actually lethal.

Figure 1. Phenotypic analysis of plc5-1 seedlings and complementation by wild-type PLC5 gene.

(a) Representation of the PLC5 gene and T-DNA insertion position of plc5-1. Filled boxes and lines represent exons and introns, respectively. Open boxes and triangle represent untranslated regions and T-DNA insertion, respectively. (b) Q-PCR analysis of PLC5 expression level in wild type, plc5-1 and two complementation lines, PLC5#2 and #4 (in plc5-1 background using SAND as a reference gene. Values are means ± SD (n=3). (c) Seedling morphology of wild type, plc5-1 and complementation lines. Seeds were germinated on ½MS with 0.5% sucrose for 4 days, and then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d) Primary root (PR) length and lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n>20). Asterisk (*) indicate significance at P<0.05 compared to wild type, based on Student’s t test.
Analyzing plc5-1 mutant seedlings revealed shorter primary roots (10 %) and contained less (19 %) lateral roots than wild type (Fig. 1c-e), a phenotype that was also found for plc3 mutants (Chapter 2; Zhang et al., 2017). Expression and phenotype could, however, be complemented by expressing the PLC5 gene driven by its own promoter in the plc5-1 mutant background in independent T3 lines (Fig. 1c-e), confirming PLC5's role in root development.

Detailed analyses of the different lateral root stages indicated that the plc5-1 phenotype concerned lateral root initiation rather than their emergence (Supplemental Fig. S1). To analyze plc3plc5-double mutants, plc3-2 (Chapter 2; Zhang et al., 2017) was crossed with plc5-1, and homozygous T3 lines generated, but as shown in Supplemental Figure S2, the lateral root phenotype was only marginally enhanced (22 % fewer lateral roots), indicating other bottle necks, or that additional PLCs are involved. In the eFP browser, we additionally found PLC2 and PLC7 also to be expressed in the phloem/companion cells, however, plc3plc5plc7-triple mutants as well as plc2-single mutants were found to be homozygous lethal (Munnik lab, unpublished; Di Fino et al., 2017).

Expression of PLC5 during plant development

Earlier, analyses of PLC5 expression by Q-PCR revealed some variation in the different organs and upon hormone- or stress treatments (Tasma et al., 2008). To investigate this locally in more detail, a PLC5-promoter β-glucuronidase- (GUS-) reporter line, kindly provided by Dr. Julie Gray (Hunt et al., 2004) was analysed. As shown in Figure 2a, the pPLC5-GUS expression was already apparent during germination (28h after transfer to 22°C) in the cotyledon, hypocotyl and root of the embryo (Fig. 2a). During further development, GUS activity was mainly found in the vasculature throughout all stages, i.e. root, cotyledons, leaves, hypocotyl, flower, incl. stamen, style, receptacle and pedicel (Fig. 2b-l). Interestingly, GUS activity was also visualized in the whole trichome (Fig. 2i), which is different from PLC3 where it is only expressed at the trichome base. Expression in the root was not homogenous. Like PLC3 (Chapter 2, Zhang et al., 2017), it tended to be 'segmented' at the distal side of the root maturation zone, while the expression was continuous in the apical maturation zone, and stopped near the transition zone, but appeared again in the root tip (Fig. 2d-g). The latter is again different from PLC3, which was never found to be expressed at the root tip. Strikingly, lateral roots were always found to emerge from a colored segment, but not every segment led to a lateral root (Fig. 2c; Chapter 2, Zhang et al., 2017). Moreover, both segmented- and root tip expressions were also observed during the formation of tertiary roots (Fig. 2e). Lastly, we also found GUS activity in guard cells (Fig. 2m).

Together, these results confirm that PLC5 is expressed throughout the plant (Hunt et al., 2004; Tasma et al., 2008), but that expression is clearly restricted to the vasculature, trichomes and guard cells.
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Figure 2. pPLC5::GUS expression analyses in Arabidopsis seedlings and mature tissues.
(a) GUS activity was present in embryo cotyledons and roots during seed germination, from testa rupture until radical emergence (28 hrs after transfer from 4°C to 22°C). GUS staining was observed in the vasculature of 2-d old- (b) and 10-d old seedlings, including leaf (c) and roots (d-g). GUS activity was also found in vascular tissue of mature 3 weeks old plants (b), trichomes (i), hydathodes (indicated by arrows) (j), guard cells (m), siliques (l) and in different parts of the flower (k), including style, filament, receptacle and pedicel.

Analysis of PPI- and PA levels in plc5-1 mutant Arabidopsis seedlings
To determine whether the knockdown-of-PLC5 caused any changes in the levels of PLC's substrates (i.e. PIP and PIP₂) or -product (i.e. the conversion of DAG into PA) (Munnik et al., 1998b; Ruelland et al., 2002; Arisz et al., 2009, 2013), seedlings were ³²P-labeled O/N and their lipids extracted and analyzed. As shown in Figures 3a and 3b, no significant differences in PIP₂, PIP and PA levels were found between wt and plc5-1 seedlings.

Figure 3. PPI- and PA levels in wild type- and plc5-1 seedlings.
Five-day old seedlings were labelled with ³²PO₄⁻ overnight and the next day their lipids extracted and separated by TLC. (a) Autoradiograph of a typical experiment, each lane representing 1/5th of the extract of three seedlings. (b) Quantification of ³²P-labelled PIP₂, PIP- and PA levels in wild type and plc5-1. Values are calculated as the percentage of total ³²P-labelled phospholipids and are represented as means ± SD (n=3). The experiment was repeated twice with similar results.
Overexpression of *PLC5* increases tolerance to drought

Previous results revealed that overexpression of *PLC3* enhanced drought tolerance (Chapter 2; Zhang et al., 2017). Similar phenotypes were obtained earlier, when *PLC* was overexpressed in maize, canola or tobacco (Wang *et al.*, 2008; Georges *et al.*, 2009; Tripathy *et al.*, 2011). It is unknown whether specific PLCs are required for this, or whether overexpression of any *PLC* could achieve this. Hence, we generated transgenic plants overexpressing *PLC5* under the control of the ubiquitin (UBI10) promoter. Transgenic plants were selected from T0 to T3, and independent homozygous lines were obtained.

Two homozygous lines, *PLC5*-OE2 and *PLC5*-OE3, were selected for further studies, overexpressing *PLC5* for 300- and 100-fold, respectively (Fig. 4a). In general, overexpression of *PLC5* caused a slight inhibition of their growth. Soil grown *PLC-OE* plants exhibited a 13.9% - 17.2% shorter inflorescence lengths and smaller rosette size (31.4%). Accordingly, the fresh weight of rosettes was less too (~46%) (see Supplemental Fig. S3). Nonetheless, *PLC5*-OE lines were consistently (at least three independent experiments) found to be more drought tolerant than wild type (Fig. 4b) and when the water loss of detached rosettes of 4-week-old plants was analysed, it was clear that the *PLC5*-OE lines lost less water that wt (Fig. 9c).

ABA plays a key role in the plant’s response to dehydration, including the induction of stomatal closure to reduce the water loss through evaporation (Sean *et al.*, 2010). Previously, we found that the stomatal aperture of *PLC3*-OE lines was strongly reduced compared to wt in the absence of ABA, but responded similarly to increasing ABA concentrations (Chapter 2; Zhang *et al.*, 2017).

To check the stomatal response in *PLC5*-OE lines, leaf peels were isolated as before and the ABA sensitivity analyzed. As shown in Figure 4d, stomatal opening in both *PLC5*-OE lines was significantly reduced compared to wt under control conditions, like *PLC3*-OE lines (Chapter 2, Fig 5). However, upon ABA treatment (0.1 µM), the stomates closed rapidly for all genotypes, but the aperture of the two *PLC5*-OE lines was significantly smaller than in wild type. Above 1 µM, this difference was maintained in *PLC5*-OE3, but was lost in *PLC5*-OE2 (Fig. 4d).
Figure 4. Overexpression of PLC5 increases tolerance to drought.
Overexpression lines were generated and the expression level of PLC5 determined by Q-PCR, relative to the expression of SAND. Values are means ± SD (n = 3) for one representative experiment. At least three experiments have been repeated with similar result. (b) Phenotype of 4-weeks old wild type- and PLC5 OE plants, grown on soil and exposed to drought by withholding water for 2 weeks. (c) Water loss of detached rosette. Water loss was measured at indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment (n=36). At least three experiments has been repeated with similar result. (d) ABA-induced stomatal closure in wild type, PLC5 OE2 (left), PLC5 OE3 (right) plants. Leaves from 3-weeks old plants were striped and incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentration of ABA for 90 mins, after which stomata were digitized and the aperture width measured. Values are means ± SE of at least three independent experiments (n > 100). Asterisk (*) marks that PLC5-OE levels are significantly different from wild-type, based on Student’s t-test (P< 0.05).

PLC5-overexpressing plants exhibit increased PPI responses
To determine whether overexpression of PLC5 caused any changes in PPI- and/or PA levels, 32P-labeling experiments were performed on seedlings (3h labeling) and the effect of sorbitol tested to mimic water stress. Interestingly, under control conditions, PLC5-OE lines exhibited a clear reduction in PIP2- and PIP levels, by about 80- and 20% respectively, and an increase in PA by 30% (Fig. 5), indicating a constitutively higher PLC activity in vivo. O/N 32P-labeling conditions gave similar results (Supplemental Fig. S4). Upon sorbitol treatment, however, a much stronger relative increase in PIP2 was observed in the OE lines. While PIP2 levels increased about 4-fold in wt, in the OE lines a massive, 12-fold increase was witnessed, although the absolute levels of both PIP2 and PIP remained below that of wild type. The relative increase in PA and PIP was similar, however, for both wt and OE lines (Fig. 5b).
Figure 5. PLC5 OE lines have altered PIP-, PIP2- and PA responses, both in control conditions and upon osmotic stress. Six-day-old seedlings were 32P-labeled for 3h and then treated with buffer ± 600 mM sorbitol for 30 min. Lipids were extracted, analyzed by TLC and quantified by phosphoimaging. (a) Typical TLC profile with each lane representing 1/5th of the extract of 3 seedlings. (b) 32P-levels of PIP2, PIP and PA of wild-type and PLC5 OE lines #2 and #3 under control conditions and with sorbitol. Data shown are the means ± SE (n=3) from three independent experiments. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05).

Overexpression of PLC5 stunts root hair growth by hydrolyzing essential PIP2 at the tip

Overexpression of PLC5 resulted in slightly smaller shoots in terms of inflorescence- and rosette sizes. Also the root system was smaller, i.e. a shorter primary root length, fewer lateral roots, and shorter lateral roots (see Supplemental Figs. S3 e-h). Analyzing the root architecture in more detail, led to another interesting discovery, namely that root hairs were found to be stunted in the OE lines (Figs. 6a, b). Measuring individual root hairs revealed a ~90 to ~80% reduction in root hair length for PLC5-OE2 and PLC5-OE3, respectively (Fig. 6c).

Earlier, PIP2 was found to promote root hair elongation at the tip of growing root hairs, and that PIP5K3 was involved in its generation(van Leeuwen et al., 2007; Kusano et al., 2008; Stenzel et al., 2008; Grierson et al., 2014). Since the PLC5-OE lines had reduced PIP2 levels, i.e 83% in PLC5 OE2 and 77% in PLC5 OE3, which corresponded to the severity of the root hair phenotype (Fig. 6a) and phenocopied the reduced root hair length of four independent T-DNA insertion pip5k3 mutants, we
hypothesized that the root hair phenotype in PLC5-OE lines was caused by the continuous removal of PIP₂ from the tip that is essential for its growth. To confirm that PIP₂ is a key regulator for root hair elongation, we crossed PLC5-OE2 with an estradiol-inducible over-expressor of PIP5K3 (ER8-PIP5K3) that is known to increase PIP₂ and to induce massive root hair formation (Kusano et al., 2008). T3 transgenic of PLC5-OE2 x ER8-PIP5K3 were selected and grown together with wild type, and the individual mutant lines, PLC5-OE2 and ER8-PIP5K3, for four days on ½MS plates and then transferred to ½MS plates with and without 10 µM estradiol for another three days. Without estradiol, the ER8-PIP5K3 lines showed similar root hair growth as wild type, while, the crossed line, PLC5-OE2 x ER8-PIP5K3 clearly exhibited the reduced root hair phenotype described above (Figs. 7a and 7b). After estradiol induction, the root hair length significantly increased in both ER8-PIP5K3 and PLC5-OE2 x ER8-PIP5K3 lines, but did not change in wild type or PLC5-OE2 (Figs. 7a and 7b). Determining the PIP₂ levels in all above lines, revealed that without estradiol PLC5-OE2 and PLC5-OE2 ER8-PIP5K3 lines showed PIP₂ levels that were significantly lower than wild type and ER8-PIP5K3 line. However, upon induction by estradiol, PIP₂ levels in both ER8-PIP5K3 and PLC5-OE2 ER8-PIP5K3 went up sharply, while remained the same for wild type and PLC5-OE2 (Fig. 7c and 7d). Increasing PIP₂ levels by estradiol-induced overexpression of PIPK3 clearly recovered root hair growth in the PLC5-OE2 background.

**Figure 7.** Root hair phenotype in PLC5-OE lines is rescued by inducible overexpression of PIP5K3.

(a) Root hair phenotypes of wild type, PLC5-OE2, ER8-PIP5K3 and PLC5-OE2 x ER8-PIP5K3 after estradiol induction. ER8-PIP5K3 is an estradiol-inducible overexpression line (ref). Seeds were first germinated on ½MS plates supplemented with 0.5% sucrose for 4 days, and then transferred to plates containing ± 10 µM estradiol. Seedlings were scanned three days after transferring. Bar = 0.5 mm. (b) Quantification of root hair length after estradiol induction. (c) PIP₂ levels after estradiol induction. For the latter, seedlings were grown on ½MS plates with 0.5% sucrose for 4 days and then transferred to the plates containing ± 10 µM estradiol for three days, after which they were labelled overnight with ³²P to measure the changes in PIP₂. (d) Values are calculated as the percentage of total ³²P-labeled phospholipids and represented as means ± SD (n=3). The experiment was repeated twice with similar results.
DISCUSSION

Earlier, we found that loss-of-function mutants of the phloem/companion cell-specific Arabidopsis PLC3 were affected in seed germination, root development, and ABA sensitivity, and that ectopic overexpression resulted in plants with increased drought tolerance (Chapter 2, Zhang., et al, 2017). In this study, PLC5 that is also expressed in phloem/companion cells, but belongs to a different subclade of the Arabidopsis PLC family was analyzed. Knock-down of PLC5 affected primary- and lateral root development and overexpression again enhanced the plant's tolerance to drought. In this case, PLC5 overexpression led to an additional phenotype, i.e. a strong inhibition of root hair growth. Together, these findings underline the fact that PLCs play subtle roles in plant development and stress signaling.

How PLC achieves this, is still largely unknown, but there are several possibilities. Plants lack the primary targets for IP3 and DAG, so these molecules are unlikely to fulfill a second messenger role, even though we cannot completely rule this out, as plants may have evolved distinct targets than animals. Nonetheless, there is accumulating evidence that the phosphorylated products of IP3 and DAG, which includes various IPPs and PP-IPPs and the lipids, PA and DGPP, are acting act the plant signaling molecules (van Schooten et al., 2006b; Arisz et al., 2009; Munnik and Vermeer, 2010; Testerink and Munnik, 2011; Gillaspy, 2013; Hou et al., 2016). The reason for this remains unknown, but it is striking since seedlings are quite active in PA signaling (Wang et al., 2006; Testerink and Munnik, 2011; Hou et al., 2016).

Overexpression of PLC5 resulted in decreased PIP- and PIP2 levels and increased levels of PA, however not of DGPP. While DGPP responses are relatively abundant in algae and cell suspensions (Wissing et al., 1994, 1995, 1992, 1993; Wissing and Behrbohn, 1993; Munnik et al., 1996, 2000; Pical et al., 1999; van der Luit et al., 2000; Munnik and Meijer, 2001; Meijer and Munnik, 2003; Meijer et al., 2017), in Arabidopsis seedlings this lipid is hardly detectable, even under stress (Arisz et al., 2013).

While PIP2 is the authentic PLC substrate in animal systems, in plants, PIP2 concentrations are extremely low and hardly detectable in plasma membranes where most of the PLC activity is believed to reside (Munnik et al., 1998a, 1998b; Meijer and Munnik, 2003; van Leeuwen et al., 2007; Munnik, 2014; Simon et al., 2014; Tejos et al., 2014). In contrast, PI4P is 30-100 times more abundant, highly enriched in plasma membranes (Munnik et al., 1994; Vermeer et al., 2009; Vermeer and Munnik, 2013; Simon et al., 2014, 2016), and is hydrolyzed equally in vitro (Munnik et al., 1998a; Munnik, 2014). Hence, in vivo, PI4P may actually be the common PLC substrate for plants. Obviously, this may be very different in stressed cells, where PIP2 levels do go up, e.g. in response to ABA, salt stress or heat (Takahashi et al., 2001; DeWald et al., 2001; van Leeuwen et al., 2007; Mishkind et al., 2009; Darwish et al., 2009; Zhang et al., 2017) or during events where local PIP2 turnover is high, but levels too low to be visualized and measured. Indeed, PI4P levels have been reported to drop in response to stimuli, e.g. upon salt and cold stress (Cho et al., 1993; Pical et al., 1999; DeWald et al., 2001; Ruelland et al.,
2002; Vermeer et al., 2009; Zarza et al., 2016), even though it still remains to be shown whether this reflects hydrolysis by PLC or PI4P phosphatase or an activation of PIPK, for example. Nonetheless, PLC hydrolysis of PI4P would still generate DAG and IP$_2$ that can be converted into PA and IPPs through DGK, IPK2 and IPK1, respectively (Munnik and Vermeer, 2010), and into PP-IPPs via VIP2 (Laha et al., 2015, 2016), to activate downstream-signaling responses.

While basal levels of PIP$_2$ in plant cells are extremely low, this lipid is clearly emerging as a second messenger itself, involving various stress and developmental responses, including tip growth in pollen and root hairs, vascular differentiation, salt- and heat stress, ion-channel regulation, cytoskeletal organization, and membrane trafficking, including endo- and exocytosis (Ischebeck et al., 2010; Gillaspy, 2013; Rodriguez-Villalon et al., 2015; Heilmann, 2016). Potential targets include small G-proteins like, K$^+$ channels, clathrin-adaptor proteins, and Exo70 (Ischebeck et al., 2010; Munnik and Nielsen, 2011; Gillaspy, 2013; Munnik, 2014; Heilmann, 2016). Similarly, PI4P has been emerging as a lipid second messenger too under certain conditions, and in particular cells (Stevenson et al., 2000; Meijer and Munnik, 2003; Vermeer et al., 2009; Munnik and Nielsen, 2011; Heilmann, 2016; Simon et al., 2016). In such cases, PLC could also function as an attenuator of signaling. However, whether PLC attenuates second messengers or produces them (or likely both!), clearly requires more research and will be difficult to address anyway because of the huge redundancy of both PPI/PA pathways (e.g. Arabidopsis has 9 PLC, 11 PI4P, 12 PIK, 7 DGK and 12 PLD genes), but also due to the highly localized events, such as within the vascular system (phloem, companion cells) in this case.

**Role for PLC5 in root development and auxin signaling?**

Knock-down of PLC5 led to shorter primary roots and fewer lateral roots, which was functionally complemented by expressing PLC5 behind its own promotor. Promotor-GUS analyses indicated that PLC5 is predominantly expressed in the root vasculature, which was not homogenous but revealed some form of segmentation. Moreover, lateral roots always emerged from a segment, even though not every segment gave a lateral root, which was similar to what we found for PLC3 (Chapter 2; Zhang et al., 2017). Interestingly, the emergence of tertiary roots revealed the same segmentation pattern, indicating that tertiary roots initiate from PLC segments in lateral roots (Figs. 2d,e). That the lateral root phenotype is quite mild in either plc3 (knockout) or plc5 (knockdown) mutants pointed to gene redundancy. We therefore created plc3plc5-double mutants, but they displayed a similar reduction in primary-root length and lateral-root number as the single mutants (Supplemental Fig. S2). These results indicate that more PLCs are involved in this phenotype. Re-analyzing the eFP browser further, we found two other PLCs expressed in the phloem vasculature, i.e., PLC2 and PLC7 (Chapter 4; Di Fino et al., 2017; Van Wijk, Laxalt and Munnik, unpublished). Unfortunately, plc3plc5plc7-triple mutants or plc2-single mutants were found to be homozygous lethal (Di Fino et al., 2017; Chapter 4; Munnik lab, unpublished). Inducible-silencing should offer new perspectives here.
Root growth and -branching are important events in root development and the phytohormone, auxin plays a key role in this (Péret, De Rybel, et al., 2009; Péret, Larrieu, et al., 2009; Benková and Bielach, 2010). Auxin regulates massive changes in gene expression by promoting the degradation of the transcriptional repressor, Aux/IAA through its receptor, TIR1. The latter is an F-box protein that forms an SCF complex that functions as a multi-protein E3 ubiquitin-ligase complex, which catalyzes the ubiquitination of Aux/IAAs destined for proteasomal degradation (Kepinski and Leyser, 2005). Interestingly, the crystal structure of TIR1 was found to contain IP_6, which is anticipated to regulate auxin binding and TIR1 activity (Tan et al., 2007; Chapter 3; Zhang et al., 2017). So far, it has remained unknown where this IP_6 would be coming from, but earlier we proposed that it could be formed though a PLC3-generated IP_3/IP_2 response, with subsequent phosphorylation into IP_6 at the PLC segments from which the lateral roots emerge (Chapter 3; Zhang et al., 2017). Our data here, indicates a similar role for PLC5. Double-plc3plc5 mutants were expected to produce less PLC-generated IP_6 and hence, exhibit reduced auxin responsiveness and root development, but likely redundant PLCs, such as PLC2 and PLC7, take over, even though it should be noted that plc5-1 is a knock-down mutant. Alternatively, chemical redundancy could play a role, e.g. by making IP_6 via de novo synthesis (Munnik and Vermeer, 2010; Gillaspy, 2011; 2013). To functionally couple IP_6 with TIR1, it will be important to determine the amount of IP_6 in TIR1 in both wt and plc-mutant backgrounds. For the latter, we may require induced PLC-silencing lines in combination with KD- and KO mutants.

An alternative explanation for the root phenotype in plc5 could be related to the metabolism of inositol-based Raffinose Family Oligosaccharides (RFOs). RFOs are derived from sucrose to which a galactosyl unit is added via galactinol (Gol). Gol is synthesised from myo-inositol and UDP-galactose, catalyzed by galactinol synthase (GolS). Since RFOs are important for carbohydrate transport- and storage, they could be involved in loading sugars to sink organs such as lateral roots (Van den Ende, 2013; Sengupta et al., 2015). The free myo-inositol that RFOs synthesis requires is normally generated via glucose 6-phosphate (G6P), which forms inositol-3-phosphate (Ins3P) by myo-inositol-3-phosphate synthase (MIPS), which is subsequently dephosphorylated by inositol mono-phosphatase (InsPase). Theoretically, inositol could also be generated via dephosphorylation of PLC-generated IPPs (Munnik and Vermeer, 2010), especially since PLC3 and PLC5 are both expressed at phloem companion cells. However, like the phloem-sap analyses of plc3 mutants (Chapter 2, Chang et al., 2017), we could not detect significant differences in plc5-1, even though myo-inositol levels tended to be a bit lower (Supplemental Fig. S5). Nonetheless, with all this PLC redundancy and the fact that our plc5-1 mutant is not a full KO, we cannot completely rule out this pathway yet, even though biochemically, in terms of amounts, it is less likely. CRISPR-Cas9 generated full KO lines of PLC3, 5 and 7 could help solving this puzzle.
Overexpression of PLC5 enhances drought tolerance

Plants have developed many strategies to cope with drought stress (Zhu, 2002, 2016; Osakabe et al., 2013; Mickelbart et al., 2015) and lipid signaling has been implicated in its responses (Munnik and Meijer, 2001; Zhu, 2002; Meijer and Munnik, 2003; Wang et al., 2007; Munnik and Vermeer, 2010; Hou et al., 2016). Earlier, overexpression of PLC has been shown to improve drought tolerance in maize, tobacco and canola (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011). We confirmed this for Arabidopsis by overexpressing AtPLC3 (Chapter 2, Zhang et al., 2017) and found it again here for PLC5. The latter are both phloem-expressed PLCs, but we don’t know whether this is a requirement or whether any PLC could achieve this. Overexpression of a non-phloem specific AtPLC will shed light on this.

Under control conditions, PLC5-OE plants grew slightly slower, and eventually developed smaller in size and produced less biomass (Supplemental Fig. S4). However, upon drought stress, they performed much better (Figs. 4b,c), also better than PLC3-OE lines (not shown). PLC5-OE plants loose significantly less water than wild type, which is likely related to the increased number of closed stomata that we found under normal growth conditions (Fig. 4d), as there was no difference in the number of stomata (not shown). The reduction in stomatal aperture could also be the reason why PLC5-OE plants tend to be smaller, as CO₂ uptake and sugar production could be affected (Cornic and Massacci, 1996). Similarly, PLC5-OE lines led to a stunted root-hair growth (Fig. 6) (see discussion below), which could be another explanation for their smaller size, as nutrient uptake could be affected (Leitner et al., 2009). To validate this, we measured glucose-, fructose- and sucrose levels in shoots and roots of 10-day old seedlings but found that PLC5-OE lines even contained slightly higher carbohydrate concentrations (Supplemental Fig. S6). Whether this is related to their drought tolerance or a consequence of their lower water content remains unclear.

PLC5-OE lines displayed reduced PIP- and PIP₂ levels and increased PA levels, likely reflecting increased PLC activity. Earlier, PIP₂ has been proposed to be important for stomatal opening by lowering the water potential through inhibition of SLAC1 (Lee et al., 2007) and K⁺-efflux channels (Ma et al., 2009), which would favor the influx of water and open the stomata. Hence, the closed-stomata phenotype of PLC5-OE lines could be due to the strongly reduced PIP₂ levels.

In an attempt to mimic drought stress in seedlings and to analyze the effect on the PA- and PPI levels, we used sorbitol. Interestingly, PIP₂ levels dramatically increased upon this osmotic stress, and the accumulation was much stronger in the PLC5-OE lines (Fig. 5). The latter may reflect the increased turnover of PIP₂ due to enhanced PLC5 hydrolysis that would readily be picked-up by these ³²P-labeling experiments. Since UBQ10 drives PLC5 expression in all cells, which is totally different from the endogenous, limited PLC5 expression in the vasculature, enhanced PLC-signal formation (PA and IPPs) or PIP₂ attenuation may affect many cells, tissues and processes relevant to control responses to osmotic stress (Munnik and Vermeer, 2010).
Similarly, *PLC5-OE* lines led to a stunted root-hair growth (Fig. 6) (see discussion below), and this could be another explanation for their smaller size, as nutrient uptake could be affected (Leitner et al., 2009). Strikingly, these lipid changes were not observed in the *PLC3-OE* lines, which did not reveal a root-hair phenotype either. It is not clear from the amino-acid sequence why *PLC5* would be more active than *PLC3*. All *AtPLCs* contain the same domain structure, i.e. two EF-hands, a catalytic and CalB/C2 domain. So there must be subtle changes in the enzymatic properties, caused by Ca\(^{2+}\) sensitivity, K\(_m\) or V\(_{max}\) values, for example, or by interacting other proteins.

### PLC-dependent PIP\(_2\) generation is essential for root hair tip growth

Overexpression of *PLC5* also resulted in a very-short-root hair phenotype (Fig. 6), which is very likely due to the low amounts of basal PIP\(_2\) required for tip growth (Fig. 5b; supplemental Fig. 6). Interestingly, these lipid changes and root-hair phenotype were not observed in the *PLC3-OE* lines, indicating differences in PLC3/5 activity or regulation. Evidence that this phenotype was coupled to PIP\(_2\) came from the induced-overexpression of *PIP5K3* in *PLC5-OE2* line, which restored PIP\(_2\) levels and rescued the root hair phenotype (Fig. 7). T-DNA insertion mutants of *PIP5K3* exhibit a similar short-root hair phenotype as *PLC5-OE* lines, and this lipid kinase has been shown to be responsible for generating the PIP\(_2\) at the plasma membrane of the growing tip (van Leeuwen et al., 2007; Kusano et al., 2008; Stenzel et al., 2008; Ischebeck et al., 2008).

Similar results have been found in tobacco pollen tubes (Ischebeck et al., 2008), whose tip growth resembles that of root hairs (Ovečka et al., 2005; Zonia and Munnik, 2008; Ischebeck et al., 2010; Grierson et al., 2014). Reduced PLC1 activity in petunia pollen tubes led to arrested- and depolarized pollen tubes, which was accompanied by a disorganization of the actin cytoskeleton (Dowd et al., 2006). On the other hand, overexpression of *NtPLC3* was shown to reduce pollen tube length (Helling et al., 2006). FP-tagged *NtPLC3* was localized at the flanks of the growing tip, while the PIP\(_2\), visualized by a biosensor, accumulated at the apex (Helling et al., 2006), similar to what was found in growing root hairs (van Leeuwen et al., 2007; Kusano et al., 2008). We found no aberrant root hair morphology in the *plc5-1* mutant, which could be due to the fact that this is not a KO mutant and/or due to *PLC* redundancy.

How PIP\(_2\) is exactly involved in this polar tip growth remains unknown, but is likely to involve a complex signaling network between membrane trafficking and cytoskeletal dynamics (Ovečka et al., 2005; Ischebeck et al., 2010; Munnik and Nielsen, 2011; Grierson et al., 2014). Tip growth is sustained by exocytosis of vesicles containing growth materials, like polysaccharides and proteins, for growing cell wall and membrane (Grierson et al., 2014). In animal cells, PIP\(_2\) is involved in priming exocytosis and vesicle fusion by binding EXO70, a subunit form the exocyst complex (Aikawa and Martin, 2003; Munson and Novick, 2006; Liu et al., 2007). Arabidopsis contains 23 EXO70s, and the exocyst complex is essential for pollen tube germination and growth (Synek et al., 2006; Hála et al., 2008)(Synek et al., 2006; Hála et al., 2008). The local accumulation of PIP\(_2\) has also been shown to
correlate with vesical secretion (Ischebeck et al., 2008). Apart from exocytosis, endocytosis is also important for cell growth and regulation and PIP\(_2\) has been implicated in clathrin-mediated endocytosis (CME) as clathrin-adaptor proteins bind and are recruited by PIP\(_2\) (Zhao et al., 2010; Baisa et al., 2013; Ischebeck et al., 2013). To deliver and return vesicles to and from the cell expanding areas, the actin cytoskeleton also plays crucial roles and PIP\(_2\) has been suggested to participate too (Gungabissoon et al., 1998; Wasteneys and Yang, 2004; Logan and Mandato, 2006). An important PIP\(_2\) target in regulating this is Rho GTPases (Ras-related monomeric small G proteins), which in plant are called Rop (Rho of plants). They are crucial regulators of tip growth (Kost, 2008) proposed to control the actin cytoskeleton and membrane trafficking (Lee et al., 2008) and there is evidence that PIP\(_2\) regulates Rop in organizing this (Kost et al., 1999; Klahre et al., 2006; Kost, 2008; Ischebeck et al., 2011). In summary, it is clear that PIP\(_2\) can regulate root hair growth via various cellular processes and it is not unlikely that PLC(s), plays a role it its attenuation and/or additional second messenger formation. Clearly, further studies are required to decipher the molecular downstream components of PPIs, IPPs and PA, and what the role of PLC is herein. Characterization of KO, KD and OE mutants provides a start at least.

**MATERIALS AND METHODS**

**Plant material**

*Arabidopsis thaliana* (Columbia-0) T-DNA insertion mutant *plc5-1* (SALK_144469), was obtained from SALK collection (signal.salk.edu). Homozygous plants were identified by PCR in F2- and F3 generations using gene-specific primers (forward primer 5'-TGGAAACTCGCAGGATATGTC-3'; reverse primer 5'-TTGCGTCTTTTGTATATTGCAGG-3') and by the combination between reverse primer and left border primer LBb1.3 (5'-ATTTTGCCGATTTCGAAC-3'). A double mutant, *plc3plc5* (*plc3/5*) was created by crossing *plc3-2* (SALK_037453) and *plc5-1* (SALK_144469) and selecting homozygous lines in F2- and F3 generations. The pPLC5::GUS line was kindly provided by Dr. Julie E. Gray (Hunt et al., 2004).

**Root growth**

Seeds were surface sterilized in a desiccator by using 20 ml thin bleach and 1 ml 37% HCl for 3 hours, and then sowed on square petri dishes containing 30 ml ½-strength Murashi-Skoog (½MS) medium (pH 5.8), supplemented with 0.5 % sucrose, and 1.2 % Daishin agar (Duchefa Biochemie). Plates were stratified at 4 °C in the dark for two nights and then transferred to long-day conditions (22 °C, 16 h of light, 8h of dark), placed vertically under an angle of 70°. Four-day-old seedlings with comparable size were then transferred to ½MS-ager plates without sucrose and scanned 10 - 12 days after germination (Epson Perfection V700 scanner). Primary root length, lateral root number, and average lateral root length was quantified for each genotype through imageJ-analysis software (National Institutes of
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Health). For root hair visualization, seedlings were grown on ½MS with 0.5% sucrose for seven days and viewed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera). More than 200 root hairs per seedling were quantified and ~10 seedlings for each genotype were used for the measurement. For inducible overexpression, seedlings were grown on ½MS with 0.5% sucrose plate for four days and then transferred to agar medium supplemented with 10 µM β-estradiol for another three days.

Cloning and plant transformation

MultiSite Gateway Three-Fragment Vector Construction Kit (www.lifetechnologies.com) was used to generate \textit{pPLC5::PLC5}. Oligonucleotide primers (5'-GGGGACAACCTTTGTATAGAAAAAGTTGCTTTTATAATAGATTAAGAGCTTCATATC-3' and 5'-GGGGACTGCTTTTTTGTACAAAACCTTGGCTTTCAAAAAAGTTTCTGCAATTAG-3'), including attB4 and attB1r sites, were used to PCR amplify a region of approximately 770-bp upstream of the predicted \textit{PLC5} ATG start codon. The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX1. Oligonucleotide primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGAGAGATATGGGGAGTTAC-3' and 5'-GGGGACCACCTTTGTACAAGAAAGCTGGGTTAAGAAAGTGAAACCGCATGAGA-3'), including attB1 and attB2 sites, were used to PCR amplify \textit{PLC5} CDS. The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX2. BOX3 was pGEM-TNOS entry clone containing attR2 and attL2 sites, which was obtained from university of Amsterdam Plant Physiology published construct resources. The three entry clones and a destination vector (pGreen0125) were used in MultiSite Gateway LR recombination reaction to create expression clone (Multi gateway protocol).

To generate \textit{PLC5} overexpression line, \textit{pUBQ10::PLC5} was constructed. The PLC5 CDS was amplified from cDNA using the following primers: \textit{AtPLC5-BsrGI-fw} (5'-GAGCTGTACAATGAAGAGAGATATGGGG-3') and \textit{AtPLC5-T-BamHI}(5'-CGGGATCCTTTAAGAAGTGAAACCACGATGAG-3'). The PCR product was transformed into pJET1.2, sequenced and digested with \textit{BsrGI} and \textit{BamHI}. After gel extraction the \textit{BsrGI-AtPLC5-BamHI} fragment was cloned into the \textit{BsrGI/BamHI} digested pGreenII0029JV-pUBQ10 mcs vector.

All constructs were transformed into \textit{Agrobacterium tumefaciens} strain GV3101, which was used to transform either Arabidopsis (Col-0) wild type plants or the \textit{plc5-1} mutant background by floral dip (Clough and Bent, 1998). Homozygous lines were selected in T3 and used for further experiments.

RNA extraction and Q-PCR

The primer pairs to measure the \textit{PLC5} (At5g58690) expression level were: 5'-CTTCAACATGCAGGGCTATGGAAG-3’ and 5'-GAGATTATTGTTTATCATAAAAGTCCGG-3’.

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) as described (Pieterse, 1998).
One- and a half µg of total RNA from 10-day-old seedlings was converted to cDNA using oilgo-dT18 primers, dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. Q-PCR was performed with ABI 7500 Real-Time PCR System (Applied Biosystem). The relative gene expression was determined by comparative threshold cycle value. Transcript levels were normalized by the level of SAND (At2g28390; forward primer: 5’-AAC TCT ATG CAG CAT TTG ATC CAC T-3’, reverse primer: 5’-TGA TTG CAT ATC TTT ATC GCC ATC-3’). Three biological replicates and two technical replicates were used for the values of means and standard deviations (Han et al., 2013).

Histochemical analyses for GUS activity
Transgenic plants carrying pPLC5::GUS were grown for the times indicated in the legend. Specific tissues were taken and incubated in X-Gluc reaction solution containing 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM phosphate buffer pH 7.0 and 0.1% TX-100 and incubated overnight at 37°C as described before (Chapter 2; Zhang et al., 2017). The next day, solutions were replaced by 70% ethanol to de-stain the tissue after which the material was analysed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera).

Stomatal aperture
Stomatal-aperture measurements were performed according to Distefano et al., (2012) with minor changes. Epidermal strips were excised from the abaxial side of fully expanded Arabidopsis leaves of 3-week-old plants grown at 22°C under 16 h of light and 8h of dark, and immediately floated in opening buffer (5 mM MES-KOH, pH 6.1, and 50 mM KCl) for 3 h. Strips were then treated with (0-10 µM) Stomata were digitized using a Nikon DS-Fi 1 camera, coupled to a Nikon Eclipse Ti microscope. The stomatal-aperture width was subsequently measured using ImageJ software (National Institute of Health).

$^{32}$P-phospholipid labelling, extraction and analysis
Different types of tissues were used, whole seedlings and epidermal leaf peels. For Seedlings: Five-day-old seedlings were transferred to 200 µl labeling buffer (2.5 mM MES-KOH, pH 5.8, 1 mM KCl) containing $^{32}$P$_{i}$ (5-10 µCi) in 2 ml Eppendorf tubes and labeled overnight (~16 h). Samples were treated the next day by adding 200 µl labeling buffer with or without sorbitol for the times and concentrations indicated. For epidermal leaf peels: Leaves of 3-week-old plants grown at 22°C under 16 h of light and 8 h of dark were stripped and immediately floated on 100 µl opening buffer (10 mM MES, pH 6.1 and 50 mM KCl) containing $^{32}$P$_{i}$ (5-10 µCi) in a 48-wells cell culture plate (Greiner bio-one) for 3 h. All treatments were stopped by adding perchloric acid at a final concentration of 5% (v/v) for 5-10 min, after which the material was transferred to 400 µl of CHCl$_3$/MeOH/HCl [50:100:1 v/v/v] to extract the
lips. After 15 min, 400 µl of CHCl₃ was added, followed by 200 µl of 0.9 % (w/v) NaCl to induce two phases. The organic lipid fractions were washed and concentrated as described earlier (Munnik & Zarza, 2013). Lipids were separated by thin-layer chromatography (TLC) using an alkaline solvent system, containing: chloroform/methanol/28% ammonia/water [90:70:4:16 (v/v)] (Munnik et al., 1994). Radioactive phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA). Individual phospholipid levels are expressed as the percentage of the total ³²P-lipid fraction.

**Drought tolerance**

Drought assays were performed as described earlier (Hua et al., 2012; Osakabe et al., 2013) with some modifications. Seeds were stratified at 4°C in the dark for 2 nights and sown in soil. Each pot (4.5 x 4.5 x 7.5 cm) contained 80 g of soil and nine plants, which were grown under short day conditions at 22 °C with 12 h light/12 h dark for 4 weeks, and then subjected to dehydration by withholding them for water for 2 weeks, while control plants were normally watered. Each experiment used 36 plants per genotype and experiments were repeated at least 3 times.

To assay the water-loss, rosettes from 4-week-old plants were detached and the fresh weight (FW) determined by weighing them every hour. Water content was calculated as a percentage from the initial FW. Twenty plants were used for each experiment and each experiment was repeated at least 3 times.

**Soluble carbohydrates measurement in seedlings**

Soluble carbohydrates were determined as described before (Vergauwen et al., 2000) with some minor modifications. Ten-day old Arabidopsis seedlings, grown on ½MS plates without sucrose, were separated in shoot- and root parts and immediately frozen in liquid nitrogen. Samples were then grinded and their fresh weights recorded for further analysis. Soluble sugars were extracted in water and immediately boiled in a water bath. Carbohydrates were separated by anion-exchange chromatography and quantified by pulsed-amperometric detection (Dionex, Sunnyvale Ca, USA). Mannitol was used as an internal standard. Factors for Glc, Fru, Suc were obtained by injecting pure compounds. A Carbopac™ PA-100 guard and Carbopac™ PA-100 (4×250) in series were equilibrated with 90 mM NaOH for 24 min. Regeneration was 5 min with 500 mM Na-acetate and 10 min with 500 mM NaOH. Values shown are the means ± SD (n=3) for one representative experiment that was repeated twice.
Phloem sap soluble carbohydrates measurement

Phloem exudates were extracted and analyzed as described earlier (Roessner et al., 2000; Greco et al., 2012; Tetyuk et al., 2013). The hydrophilic fraction (sugars, amino acids, small molecules) was dried and derivatized using methoxyamine in pyridine and BSTFA (N,O-bis-trimethylsilyl trifluoroacetamide) with 1% trimethylchlorosilane. The metabolites were subsequently separated using an Agilent 5890N GC system, coupled to a 5973 inert MSD using a DB-5MS column (J&W Scientific, 30m x 0.32mm ID x 0.25 µm film; temperature program: 70-5, 5/min-320-5). Identification of compounds occurred using the NIST library (NIST Standard Reference Database; https://www.nist.gov/srd/nist-standard-reference-database-1a-v14) in combination with co-elution of standards. Peak area determination was performed using QuanLynx, a quantification software within the MassLynx software (Waters). The experiment was repeated five times.

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SUPPLEMENTAL DATA

Supplemental Figure S1. Developmental stages of lateral root formation in plc5-1 and wild-type seedlings. Developmental stages of lateral root primordia from wild type and plc5-1 seedlings. Values are means ± SE of three independent experiments (n>20).

Supplemental Figure S2. Root development in seedlings of plc3plc5-double mutant. (a, b) Q-PCR analysis of PLC3- and PLC5-expression levels in wild type and plc3plc5. Relative expression is based on the expression of SAND. Values are means ± SD (n = 3) for one representative experiment. (c) Seedling morphology of wild-type and plc3plc5. Seeds were germinated on ½MS medium supplemented with 0.5% sucrose for 4 days, then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d, e) Primary root (PR) length and lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n>20). Asterisk (*) indicates significance at P<0.05 compared to wild type, based on Student’s t test.
Supplemental Figure S3. Phenotypic analysis of wild type- and PLC5 OE lines grown on soil or agar plates.

Eight-weeks old wild type- and PLC5 OE plants were grown on soil under long day condition and the whole plants (a) and rosettes without inflorescences (b) were photographed. The inflorescence length was measured (c), and the fresh weight of rosette was determined (d). (e) Seedling morphology of wild type and PLC5-OE lines grown on agar plates. Seeds were germinated on ½ MS with 0.5% sucrose for 4 days, then transferred to ½ MS plates without sucrose. Photographs were taken 10 days after germination (DAG). (f) Primary root (PR) length, lateral root (LR) number and average lateral root (ALR) length at 10 DAG. All experiments were repeated at least three times. Values are means ± SD for one representative experiment (n=36). Asterisk (*) marks that PLC5 OEs value is significantly different from wild-type based on Student’s t-test (P< 0.05)
**Supplemental Figure S4.** PPI- and PA levels in wild type and PLC5-OE lines.

Five-days old seedlings were \(^{32}\text{P}\)-labelled overnight, and the next day their lipids extracted, separated by TLC and quantified by phosphoimaging. (a) Autoradiograph of a typical TLC, each lane representing \(1/5\)th of the extract of 3 seedlings. (b) Quantification of \(^{32}\text{P}\)-labeled PIP\(_2\), PIP- and PA levels in wild type or PLC5 OE lines. Values are calculated as the percentage of total \(^{32}\text{P}\)-labeled phospholipids, and are represented as means ± SD (n=3). This experiment was repeated twice with similar results.

**Supplemental Figure S5.** Soluble carbohydrates content in phloem sap of wild type and plc5-1.

Phloem sap was isolated from 6 weeks-old Arabidopsis plants and their carbohydrates analyzed and quantified by GC-MS. Values are the means of triplicates ± SD from 3 independent experiments.

**Supplemental Figure S6.** Sugar content in seedlings of wild-type and PLC5 OE lines.

Soluble carbohydrates were extracted from 10-day old Arabidopsis seedlings, including shoot (a) and root (b), analyzed by anion-exchange chromatography, and quantified by pulsed-amperometric detection. Mannitol was used as an internal standard. Values are the means ± SD (n=3) for one representative experiment. The experiment was repeated twice with similar outcome.
Chapter 4

Role for Arabidopsis PLC7 in stomatal closure, mucilage adherence, and leaf serration

Qianqian Zhang,1,2 Ringo van Wijk,1,2 Mart Lamers,1,* Francisca Reyes Maarquez,3 Aisha Guardia,4 Denise Scuffi,4 Carlos Garcia-Mata,4 Wilco Ligterink,3 Michel A. Haring,1 Ana M. Laxalt,4 & Teun Munnik1,2

1Section Plant Physiology or 2Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, Amsterdam, 1098 XH, The Netherlands, 3Lab. of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands, 4Instituto de Investigaciones Biológicas (IIB-CONICET-UNMdP), Universidad Nacional de Mar del Plata, Mar del Plata, Argentina.
ABSTRACT

Phospholipase C (PLC) signaling in plants is likely different from the well-established animal paradigm as the prime targets for IP$_3$ and DAG are absent, and plants contain very small amounts of the putative PLC substrate, phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Nonetheless, the genome of *Arabidopsis thaliana* encodes 9 PLC genes. To increase our understanding of PLC signaling in plants, we have started to analyze various knock-out (KO), knock-down (KD) and overexpression lines of Arabidopsis. Here, we functionally characterized Arabidopsis *PLC7*. Promoter-GUS analyses revealed that *PLC7* is specifically expressed in the phloem of roots, leaves and flowers and also in trichomes and hydathodes. Two T-DNA insertion mutants were obtained, with *plc7-3* being a KO- and *plc7-4* a KD line. In contrast to earlier-characterized phloem-expressed *PLC* mutants, *plc3* and *plc5*, *plc7* mutants revealed no defects in primary- or lateral root development. Nonetheless, like *plc3* mutants, *plc7* mutants were found to exhibit a reduced sensitivity to ABA during stomatal closure. Double-knockout *plc3 plc7* lines were found to be lethal, whereas *plc5 plc7* (*plc5/7*) mutants were viable, and revealed several new phenotypes, not observed earlier in the single mutants. These include a defect in seed mucilage, enhanced leaf serration, and an increased tolerance to drought. *PLC7* overexpression lines showed an enhanced drought tolerance, similar to what was found for *PLC3* and *PLC5* overexpression lines. *In vivo* $^{32}$P$_i$-labeling of seedlings treated with sorbitol, to mimic drought stress, revealed increased PIP$_2$ responses in both drought tolerant *plc5/7* and *PLC7-OE* mutants. Together, these results reveal several novel functions for *PLCs* in plant stress and development. Potential mechanisms for this are discussed.

*Key words*: PLC; seed mucilage; leaf serration; ABA sensitivity; drought tolerance.
INTRODUCTION

Phospholipase C (PLC) plays a key role in mammalian signal transduction. Its intracellular activation through transmembrane receptor occupation leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce two second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ triggers the release of Ca$^{2+}$ from an intracellular store via ligand-gated Ca$^{2+}$ channel, while DAG recruits and activates protein kinase C (PKC) and stimulates TRP- (transient receptor potential-) channels. The subsequent increase in Ca$^{2+}$ and change in phosphorylation status of numerous targets regulates multiple cellular processes that orchestrate the cell's response to the initial stimulus (Irvine, 2006; Michell, 2008; Balla, 2013).

The PLC signaling pathway in plants is still enigmatic (Munnik, 2014). While higher plant genomes encode all genes required for the metabolism of this pathway, they all lack the prime targets for IP$_3$ and DAG, i.e. the IP$_3$ receptor, PKC and TRP channels (Wheeler and Brownlee, 2008; Munnik and Testerink, 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Munnik, 2014; Heilmann, 2016). Instead, plants seem to phosphorylate IP$_3$ and DAG into inositolpolyphosphates (IPPs) and phosphatidic acid (PA), respectively, which are believed to function as second messengers (Gillaspy, 2013; Munnik, 2014; Heilmann, 2016). IP$_3$ was initially thought to release Ca$^{2+}$ from intracellular stores when microinjected (Gilroy et al., 1990; Blatt et al., 1990; Allen and Sanders, 1994; Hunt and Gray, 2001), but later it was shown that this IP$_3$ is phosphorylated to IP$_6$ within seconds, and that the latter compound was 10-100 fold more effective in releasing Ca$^{2+}$ (Lemtiri-Chlieh et al., 2000, 2003).

Meanwhile, various other signaling functions for IP$_6$ and other IPPs have been emerging, including the pyro-phosphorylated IP$_7$ and IP$_8$. In yeast and mammalian cells, these IPP molecules play important roles in various nuclear processes, including gene transcription, chromatin remodeling, mRNA export and DNA repair, and are involved in a wide range of cellular processes, including osmoregulation, phosphate homeostasis, vesicular trafficking, apoptosis, insulin- and immune signaling, cell cycle regulation, and ribosome synthesis (Monseratte and York, 2010; Thota and Bhandari, 2015; Williams et al., 2015). In plants, besides releasing Ca$^{2+}$ in guard cells, IP$_6$ has also been shown to bind the auxin receptor, TIR1 (Tan et al., 2007), which is proposed to functionally regulate the SCF$^{TIR1}$ ubiquitin-ligase complex to control downstream auxin mediated-gene expression (Chapter 2; Chapter 3; Zhang et al., 2017). Similarly, COII, the receptor for jasmonate signaling was found to bind IP$_5$ (Sheard et al., 2010), with functional significance for plant immunity (Mosblech et al., 2008, 2011; Murphy et al., 2008), even though it could be that the pyrophosphorylated form of IP$_5$, i.e. PP-IP$_5$ (= IP$_7$), is biologically responsible for this (Laha et al., 2015, 2016). GLE1, an mRNA export factor, was recently identified as an important IP$_6$ target in Arabidopsis involved in P$_i$ homeostasis (Lee et al., 2015). SPX domain-containing proteins have recently been identified to bind IPPs, including IP$_6$ and many of these proteins are involved in P$_i$ signaling (Kuo et al., 2014; Puga et al., 2014; Wild et al., 2016). For PA, several plant targets have been identified over the years, including protein kinases, proteins
phosphatases, small G-proteins, RBOH (NADPH oxidase), GAPDH, ion channels and actin-binding proteins (Wang et al., 2006; Mosblech et al., 2011), and PA has been implicated to regulate many cellular processes, including vesicular trafficking, cytoskeleton dynamics, and ion-channel regulation (Li et al., 2009, 2012, Pleskot et al., 2010, 2017; Testeink and Munnik, 2011; Thomas and Staiger, 2014). Note that PA is not only generated via PLC and DAG kinase (DGK), it can also be indirectly formed via other DAG-generating enzymes, like non-specific PLCs (NPC; Munnik, 2014), or directly, through phospholipase D (PLD) hydrolysis of structural phospholipids (Wang et al., 2006; Li et al., 2009; Liu et al., 2013).

How, when, and whether PLC signaling is involved in generating PA and IPPs in the above events is still largely unknown and mostly based on correlations and indirect evidence. Hence, tools to genetically manipulate PLC levels will be very helpful to functionally characterize its role(s). In this way, silencing of PLC revealed its importance in plant defense in tomato and Arabidopsis (Vossen et al., 2010; D’Ambrosio et al., 2017), in cytokines- and gravity signaling in Physcomitrella (Repp et al., 2004), in ABA signaling and stomatal control in tobacco and Arabidopsis (Sanchez and Chua, 2001; Hunt et al., 2003) but also in development, where Arabidopsis PLC2 plays a key role in gametogenesis, reproduction and root development (Li et al., 2015; Laxalt et al., 2016; R. van Wijk and T. Munnik, In prep.). In petunia and tobacco, PLC regulates pollen tube-tip growth (Dowd et al., 2006; Helling et al., 2006). Alternatively, several Arabidopsis T-DNA insertion mutants have meanwhile revealed several roles for PLC3, PLC5 and PLC9 in seed germination, (lateral) root development, ABA signaling and heat stress tolerance (Chapter 2 and 3; Zheng et al., 2012; Gao et al., 2014). On the other hand, overexpression of PLC has been shown to increase drought tolerance in maize, canola, tobacco and Arabidopsis (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011; Chapter 2 and 3). Even though it is still unknown how PLC exactly achieves all the above, it is very important that these molecular tools become available for physiological and biochemical studies.

The Arabidopsis genome encodes 9 PLC genes, which are subdivided into four clades (Hunt et al. 2004; Tasma et al., 2008; Pokotylo et al., 2014). Earlier, we found that knock-out (KO) mutants of PLC3 or a knock-down (KD) mutant of PLC5, exhibited small defects in root development, i.e. 5-10% reduced growth of the primary root and a 10-20% reduction in the number of lateral roots. Interestingly, even though these PLCs come from different subfamilies, plc3 plc5-double mutants did not intensify the phenotype, indicating other PLCs could be involved. Since PLC3 and PLC5 were specifically expressed in phloem-companion cells and revealed a segmented expression pattern in the root form which lateral roots emerged, we search for other Arabidopsis PLCs specially expressed in the phloem, and found PLC7. This gene belongs to a different subfamily than PLC3 and PLC5. Promotor-GUS analyses confirmed its expression in all vascular tissues, but also in trichomes and hydatodes, however plc7-3 KO- or plc7-4 KD mutants revealed no defects in root or shoot development. Creating double mutants revealed that the combination plc3/7 was lethal and that plc5/7 did not exhibit defects in root development either. Nonetheless, several new phenotypes appeared for the latter mutant, including non-
adherent mucilage, enhanced leaf serration and increased drought resistance. In addition, like PLC3 and PLC5, overexpression of PLC7 also leads to an increase in drought tolerance.

RESULTS

Expression of PLC7 during plant development
Histochemical assays on pPLC7-GUS-SYFP reporter lines indicated that PLC7 was mainly expressed in the vasculature throughout all developmental stages, including root, cotyledons, leaves, hypocotyl, flower (stamen, style, petal, sepal, receptacle and pedicel) and silique septum (Fig. 1a-j, n and o), which is similar to the expression pattern of PLC3 (Zhang et al., 2017; Chapter 2) and PLC5 (Chapter 3). However, there are also some differences. During seed germination PLC7 expression was mainly observed in the hypocotyl (28h after transfer to 22°C, Fig. 1a), which remained like this in 2-day old seedlings (Fig. 1b) and then spread to the vasculature throughout the plant upon further development (Fig. 1c-j). Interestingly, GUS expression was quite abundant in hydathodes, in young seedling, but also mature plants (Fig. 1b-e, l, arrows). Unlike PLC3 and PLC5, PLC7 did not display the characteristic, segmented expression in the root vasculature. Instead, expression was homogenous in both main root- and lateral root vasculature (Fig. 1f, g). The expression stopped near the transition zone of the root (Fig. 1h). Strong GUS staining was visible in trichomes (Fig. 1j, k), which is similar to that of PLC5, but stronger and different from PLC3, that is only expressed in trichome basal cells. No GUS activity was detected in guard cells, as was found for pPLC3::GUS-YFP and pPLC5::GUS. These results confirm that PLC7 is expressed throughout the plant (Tasma et al., 2008), but the expression is mostly restricted to the vasculature.
**Figure 1.** \textit{pPLC7::GUS-SYFP} expression in seedlings and mature tissues of Arabidopsis.

GUS staining was found in: Embryo, 28 hrs after stratification (a), vascular tissue of 2-d old seedlings (b) and in 10-d old seedlings, including, cotyledons and roots (c-h), mature plant (i), trichomes (i), hydathodes (indicated by arrows) (c-e, i), silique (o) and flower (n), including style, filament, receptacle and pedicel. No staining was detected in guard cells (m).

**No change in root system architecture in plc7 and plc5/7 mutants**

Knock-out/down mutants of \textit{PLC3} or \textit{PLC5} were affected in their primary- and lateral root growth formation. However, a double \textit{plc3/5} mutant did not reveal a stronger effect, indicating that there might be a third \textit{PLC} involved. (Zhang et al., 2017; Chapters 2, 3). To investigate the role of \textit{PLC7} we identified two homozygous T-DNA insertion lines, \textit{plc7-3} (SALK\_030333) and \textit{plc7-4} (SALK\_148821) (Fig. 2a). Reduction of \textit{PLC7} expression in \textit{plc7-3} and \textit{plc7-4} mutants was confirmed by Q-PCR (Fig. 2b) and revealed that the former is a KO- and the latter a KD mutant. However, the root architecture of 12 days old seedlings of these \textit{plc7} mutants did not differ from the wild-type (Fig. 2c, d).
Figure 2. In contrast to plc3- and plc5 mutants, KO- or KD mutants of PLC7 are not affected in root development. (a) T-DNA insertion positions (triangles) in the PLC7 gene of plc7-3 and plc7-4 lines. Filled boxes and lines represent exons and introns, respectively, while open boxes represent untranslated regions. (b) PLC7 expression levels in wild-type, plc7-3 and plc7-4 measured by Q-PCR using SAND as a reference gene. Values are the means ± SD (n = 3) of a representative experiment that was independently repeated at least three times. (c) Seedling morphology of wild-type, plc7 mutants. Seeds were first germinated on ½MS with 0.5% sucrose for 4 days and then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d) Primary root (PR) length and (e) lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n>20). Asterisk (*) indicate significance at P<0.05 compared to wild-type, based on Student’s t test.

To analyze gene redundancy, we tried to generate plc3/5/7-triple mutants by crossing plc7-3 with the plc3/5-double mutant. However, the plc3/7 combination turned out to be lethal and only homozygous plc5/7-double mutants could be obtained (Fig. 3a). As shown in Figures 3b-d, no significant difference was found between the root system of plc5/7- and wild-type seedlings.

Figure 3. A plc5/plc7 double mutant is viable and is not affected in root architecture. (a) PLC5 (left) and PLC7 (right) expression in wild-type and plc5/7-double mutant. Relative expression is based on the expression of SAND measured by Q-PCR. Values are means ± SD (n = 3) of a representative experiment that was repeated at least three times. (b) Seedling morphology of wild-type and plc5/7. Seeds were germinated on ½MS with 0.5% sucrose for 4 days and then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d) Primary root (PR) length and (e) lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n=20). Asterisk (*) indicate significance at P<0.05 compared to wild-type, based on Student’s t test.

**plc5/7 mutant displays loose seed coat mucilage and reduced cellulose rays**

While imbibing seeds for stratification, we noticed that the volume of the seed pellet of plc5/7 seeds was always smaller than wild type after overnight incubation (Fig. 4a). Upon imbibition, the seed coat-epidermal cells normally extrude a mucilage that forms two transparent layers (adherent and non-
adherent layers) around the seed. To examine whether the smaller volume of the plc5/7 mutant was caused by a mucilage defect, we stained imbibed seeds with Ruthenium red (Fig. 4b), which stains pectins, the main component of mucilage (Western et al., 2000; Macquet et al., 2007). Compared to wild-type, the adherent and non-adherent layers were more expanded in the plc5/7 mutant (Fig. 4b, top panel) and when seeds were mildly shaken to remove the non-adherent layer, or treated with EDTA, plc5/7 seeds lost the adherent layer completely (Fig. 4b, middle and lower panel, respectively).

Increased solubility of the pectins has been linked to perturbation of cellulose deposition (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011; Yu et al., 2014; Ben-Tov et al., 2015; Basu et al., 2016; Hu, Li, Wang, et al., 2016; Hu, Li, Yang, et al., 2016). To test this, wild-type and plc5/7 seeds were stained with Calcofluor White (CFW, for cellulose and other β-glucans staining; Fig. 4c, left panel) and Pontamine S4B (cellulose-specific dye; Fig. 4c, right panel) (Willats et al., 2001; Anderson et al., 2010; Wallace and Anderson, 2012). In wild-type seeds, the primary cell wall remnants, and rays extending from the columella were stained by both dyes (Fig. 4c). The plc5/7 seeds showed a similar staining pattern, but the CFW intensity was lower and the rays were clearly reduced compared to wild-type. These results point to a role for PLC5 and PLC7 in cellulose ray formation, which is a completely novel function for PLC.

**Figure 4.** Seeds of plc5/7 double mutant exhibit mucilage phenotype. (a) Seeds of plc5/7 swell less during imbibition. Equal amounts of dry seeds of wild type and mutant were immersed in water overnight and photographed the next day. (b) Ruthenium red staining of wild type- and plc5/7 seeds, without shaking (top), with shaking (middle) and EDTA treatment. With shaking shows adherent mucilage layer; without shaking displays both adherent and non-adherent mucilage layers. (c) Cellulose staining by Calcofluor white (left panel) or Pontamine B (right panel) in wild type- and plc5/7 seeds. Confocal images of whole seeds, cross section, and close-up views (top, middle and bottom, respectively) are shown. Bars represent 2 mm (a), 0.1mm (b), 0.1mm (top and middle rows) or 0.025 mm (bottom row) (c).

**PLC5 and PLC7 expression in developing seed**

To further investigate the expression pattern of PLC5 and PLC7 during seed development, pPLC5::GUS and pPLC7::GUS-SYFP lines were used for additional histochemical analyzes (Fig. 5). At 4 day after pollination (DAP), some GUS activity was found in pPLC5::GUS (Fig. 5a, left panel)
while a strong staining in the seed coat and chalazal area of \textit{pPLC7::GUS-SYFP} was observed (Fig. 5b). Later in development (at 8 and 10 DAP), GUS activity became stronger, with \textit{PLC5} expression appearing in the seed coat and funiculus (Fig. 5a) and \textit{PLC7} staining became stronger in seed coat and chalazal (Fig. 5b).

\textbf{Figure 5.} \textit{PLC5} and \textit{PLC7} expression during seed development. (a) GUS activity analysis in \textit{pPLC5::GUS} developing seeds. Expression was found in the funiculus at 8 days after pollination (DAP) and seed coat at 10 DAP. (b) GUS activity analysis in \textit{pPLC7::GUS} developing seeds. Staining was found in the chalazal and in the seed coat. Bar = 0.1 mm

\textbf{Analysis of PPI- and PA levels in developing and germinating seeds}

To analyze the levels of the potential PLC substrates (i.e. PIP and \textit{PIP}$_2$) and product (PA conversion of PLC-generated DAG), $^{32}$P$_4$-labelling (24h) of wild-type- and \textit{plc5/7} mutant seeds at 10 DAP were compared with germinating, mature seeds. As shown in Figure 6, wild-type and \textit{plc5/7} seeds were found to contain similar amounts of \textit{PIP}$_2$, PIP and PA in both stages. Interestingly, PIP- and PA levels were much higher in developing seeds while \textit{PIP}$_2$ levels were similar in both stages.

\textbf{Figure 6.} PPI- and PA levels in developing- and germinating (mature) seeds of wild type and \textit{plc5/7}.

(a) Developing seeds, ~200 seeds at 10 DAP of wild type and \textit{plc5/7} were labelled with $^{32}$PO$_4$~$^3$~ for 24 h and their lipids extracted, separated by TLC and quantified by Phosphoimaging. (b) Mature seeds (~200) of wild type and \textit{plc5/7} were pre-germinated on ½MS with 0.5% sucrose plates until testa ruptured, then labelled with $^{32}$PO$_4$~$^3$~ for 24 h. Lipids were then extracted, separated and quantified as above. $^{32}$P-levels of \textit{PIP}$_2$, PIP and PA are expressed as percentage of total $^{32}$P-phospholipids. Three independent experiments were performed; data shown are means ± SD (n=3) from one representative experiment.
Leaves of *plc5/7* plants display enhanced level of leaf serration

Growing *plc5/7* mutants on soil for longer periods of time revealed a novel phenotype in leaf-edge patterning (serration). Overall, the level of serration in successive rosette leaves was significantly increased in *plc5/7* mutant, which appeared to be stronger in the proximal part of the blade than in the distal part (Fig. 7a, b). To quantify this in more detail, we measured various parameters of the 8th leaf (Fig. 7c-e) of 4-weeks old rosettes of both genotypes. No changes in blade length were observed between wild-type and *plc5/7*, but the blade width, -perimeter and -area were slightly, although not significantly, bigger in *plc5/7* (Fig 7d). The serration number was not changed in the *plc5/7* mutant, but the serration level (indicated by the ratio between the distance from midvein to tip and the distance from midvein to sinus, see Fig. 7c) was significantly higher in three successive teeth (Fig. 7f).

Recent studies have indicated that Arabidopsis leaf-margin development is controlled by a balance between *microRNA164A* (MIR164A) and *CUP-SHAPED COTYLEDON2* (CUC2) (Nikovics et al., 2006). Hence, we compared the expression of MIR164A and CUC2 in wild-type and *plc5/7* leaves. As shown in Figure 7g, *plc5/7* leaves were consistently (3 independent experiments) found to contain higher levels of CUC2 and lower levels of MIR164A, resulting in a significant increase in the CUC2/MIR164A ratio.
Figure 7. Leaves of plc5/7 plants display enhanced leaf serration
(a) Phenotype of wild type- and plc5/7 rosette. Rosettes of 4-week old plants grown in short day conditions, were cut and photographed immediately. Bar = 1 cm. (b) Leaf series of 4-week old wild type and plc5/7 plant. (c) Cartoon to demonstrate the leaf parameters measured of the 8th leaf. (d) Quantification of blade size including, length, width, perimeter and area. (e,f) Quantification of leaf serration number (e) and level (f) in wild type and plc5/7 mutant. (g) Expression of CUC2 and MIR164A and their ratio in wild type and plc5/7 mutant. Expression is relative to the expression of OTC. Three independent experiments were performed. Data in (g) represents the means ± SD (n=3) from one representative experiment that was repeated twice with similar results. Asterisk (*) indicate significance at P<0.05 compared to wt, based on Student’s t test.

The plc5/7 mutant displays enhanced tolerance to drought

When plants were left in the greenhouse without watering, we noticed that plc5/7 mutants appeared to be more drought tolerant while the single mutants behave like wt (data not shown). Performing multiple drought tolerance assays indeed confirmed this (Fig. 8a), and detached rosettes of 4-week-old plc5/7 plants were found to loose less water than wild-type (Fig. 8b).

ABA plays a key role during the response to dehydration stress and is known to induce stomatal closure to reduce the loss of water (Sean et al., 2010). Hence, we checked the stomatal-closure of plc5/7 and wild-type in response to ABA. As shown in Figure 8c, plc5/7 has less-opened stomata compared to...
wild-type without ABA, but when ABA was applied, stomata closed in genotypes, although the plc5/7 mutant appeared to be less responsive.

Previously, we found that overexpression of PLC3 or PLC5 enhanced drought tolerance in Arabidopsis (Zhang et al., 2017; Chapters 2, 3), which was earlier also revealed for maize, canola and tobacco (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011). We therefore wondered whether the increase in drought tolerance in plc5/7 was a result of the overexpression of any of the other (redundant) 7 PLCs. However, no strong overexpression of any PLC was found in plc5/7 (Fig. 8d). In fact, PLC1, PLC2 and PLC4 were found to be slightly down-regulated (Fig. 8d).

Figure 8. Soil-grown plc5/7 mutant are more tolerant to drought stress.
(a) Six-weeks old plants of wild type- or plc5/7 mutant plants, grown on soil and exposed to drought by withholding water for the last two weeks and then were photographed. (b) Water loss of detached rosette of normally watered, 4-weeks old plants. Water loss was measured at indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment (n=36). (c) Effect of ABA on the stomatal aperture in leaf strips of wild type and plc5/7 plants. Leaves from 3-weeks old plants were stripped and the peels were incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentrations of ABA for 90 mins, after which stomata were digitized and aperture widths measured. Values are means ± SE of at least three independent experiments (n >100). Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method). (d) PLC1- PLC9 expression level in wild type and plc5/7 mutant plants as measured by QPCR, relative to the amount of SAND. Values are means ± SD (n = 3) for one representative experiment that was repeated two times with similar results.
Overexpression of PLC7 increases drought tolerance

As mentioned above, overexpression of Arabidopsis PLC3 and PLC5, which are from different subfamilies than PLC7, resulted in enhanced drought tolerance (Zhang et al., 2017; Chapters 2 and 3). To check the effect of PLC7 overexpression, homozygous T3 plants were generated. Two lines, PLC7-OE9 and PLC7-OE12, which overexpressed PLC7 around 80- to 100-fold, respectively, were selected for further studies, (Fig. 9a). Both lines were indeed found to be more drought tolerant than wild-type (Fig. 9b), and lost slightly less water when rosettes of 4-week-old plants were detached (Fig. 9c). The stomatal aperture and their response to ABA was found to be similar to wild-type (Fig. 9d), which is different to PLC3- and PLC5-OE plants that had stomata that opened less wide than the wt.

Figure 9. Overexpression of PLC7 enhances drought tolerance.
(a) PLC7 expression levels in wild type and two homozygous PLC7 overexpression lines, PLC7-OE9 and PLC7-OE12 as measured by Q-PCR and based on the expression of the SAND reference gene. Values are means ± SD (n = 3) for one representative experiment. (b) Phenotype of six week old plants from wild type and PLC7-overexpression lines, #OE9 and #OE12, after two weeks of drought stress. (c) Water loss of detached rosette of four weeks old plants. Water loss was measured at indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment (n=36). (d) Stomatal aperture in leaf peels of wild type, PLC7-OE9 (left), PLC7-OE12 (right) and the effect of ABA. Leaves from 3-weeks old plants were stripped and peels were incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentrations of ABA for 90 mins, after which stomata were digitized and the aperture width measured. Values are means ± SE of at least three independents (n > 100). Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).
Phospholipid responses upon osmotic stress

To analyze the phospholipid responses in plc5/7 and the PLC-OE lines, $^{32}$P-labeling experiments (3 hrs labeling) were performed on seedlings, and the effect of sorbitol treatment was tested to mimic osmotic stress. Both plc5/7 and wild-type showed similar levels of PPI- and PA in absence of sorbitol (Figs. 10a, b). Upon sorbitol treatment, a stronger PIP$_2$ response was typically observed in the plc5/7 lines though numbers appear not to be statistically different. While PIP$_2$ levels increased by ~4 times in wild-type, plc5/7 seedlings typically exhibited a 6-times increase. No difference in PA- or PIP response was observed (Fig. 10b).

PLC7-OE lines revealed no difference in PPI or PA levels compared to wild-type in control conditions (Fig. 10c, d). However, with sorbitol, again a stronger PIP$_2$ response was observed in PLC7-OE lines, ~6-times vs ~4-times for wild-type, while PA- and PIP responses were similar (Fig. 10c, d). These results suggest that both plc5/7 and PLC7-OE plants boost more PIP$_2$ in response to osmotic stress than wild-type, similar to what we found earlier for PLC3- and PLC5-OE lines (Chapters 2, 3).

![Figure 10](image_url)

**Figure 10.** Both plc5/7 and PLC7 over-expressers displayed stronger PIP$_2$ response upon osmotic stress. Six-day-old seedlings were $^{32}$P-labeled for 3h and then treated with buffer ± 600 mM sorbitol for another 30 min. Extracted lipids were analyzed by TLC and quantified through phosphoimaging. (a and c). Typical TLC profile with each lane representing the extract of 3 seedlings. (b, d) $^{32}$P-levels of PIP$_2$, PIP and PA of wild-type and plc5/7 (b) or PLC7-OEs (line #9 and #16) (d) with and without sorbitol. Three independent experiments were performed. Data shown are the means ± SE (n=3) from three independent experiments. Data was analyzed by 2-way ANOVA. Statistical significant differences between normal and sorbitol conditions in wild type and plc5/7 or PLC7-OEs are indicated by letters (P<0.05).
DISCUSSION

Knockout of PLC7 does not affect root architecture

Previously, we demonstrated that Arabidopsis PLC3 and PLC5 were both involved in lateral root formation and that a plc3/5-double mutant did not exhibit a more severe phenotype than single mutants (Chapters 2, 3). We therefore speculated that another PLC must be involved. Since promotor-GUS analyses revealed specific expression in phloem companion cells and a typical segmented pattern in the vascular of the primary root from which lateral roots emerged, we specifically searched for other phloem-specific PLCs. Using the eFP browser, we found two potential candidates, PLC2 and PLC7. Since T-DNA insertion mutants of PLC2 were lethal (D’Ambrosio et al., 2017; Di Fino et al., 2017; R. van Wijk and Munnik, unpublished), we focused on PLC7. Two independent homozygous T-DNA insertion mutants were obtained, with plc7-3 being a KO- and plc7-4 a KD line. Both mutants exhibited normal root architecture. In an attempt to create double- and triple mutants of the redundant PLCs, we crossed the plc3-2/plc5-1 (plc3/5; Chapter 3) with plc7-3, but this only resulted in viable plc5/7 double mutants, as the combination plc3/plc7 turned out to be lethal (not shown). The plc5/7 double mutant, however, did not reveal any significant changes in root morphology (Figs. 2, 3). While pPLC7-GUS analyses confirmed the vascular expression of PLC7, the typical segmented pattern found for PLC3 and PLC5 was not found. These results may indicate that PLC2 is the redundant PLC in root development, and preliminary results from this laboratory, using induced silencing (R. van Wijk and T. Munnik, Unpublished), confirm this. Interestingly, PLC7 was also expressed in hydathodes and in seeds, which correlates well with the two new phenotypes that were found for plc5/7-double mutants, which have never been observed before. These include a mucilage phenotype in seeds and a serration phenotype in leaves. The latter may correlate with PLC7’s expression at hydathodes. PLC7 was also strongly expressed in trichomes. PLC5 is also expressed there, although less, and PLC3 is typically expressed only in the trichome basal cells of developing leaves (Chapters 2, 3). We checked for trichome phenotypes in individual plc3, plc5 or plc7 and plc3/5- and plc5/7- mutants, but found no obvious effect (number, shape) was visible. Again, this could be due to redundancy, or PLC is involved in content of, or transport to the trichomes, which would not lead to an obvious phenotype.

Strikingly, and new to PLC loss-of-function lines, is that plc5/7 mutants were more drought tolerant, a phenotype that is typically found when PLC is overexpressed (Chapter 2 and 3; Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011). However, we found no upregulation of redundant PLCs in the plc5/7-mutant background. If anything, some down regulation of redundant PLCs in the plc5/7-mutant background. If anything, some down regulation of redundant PLC1, PLC2 and PLC4. RNASEq analyses of the plc mutant and OE lines may shed light on potential pathways that are up- or down-regulated to explain various phenotypes. Most importantly, altering expression of PLC genes results in clear phenotypes and this will further help elucidating the role of PLC in plant signaling and development.
While several new pieces of the PLC-signaling puzzle (new phenotypes) in plants have been found, it remains unclear how this is achieved at the cellular and molecular level. It is clear that plants lack the prime targets for IP$_3$ and DAG, and there are several indications that responses are coupled via inositolpolyphosphates (IPPs) and/or PA (Wang et al., 2006; Arisz et al., 2009, 2013; Munnik and Vermeersch, 2010; Munnik and Nielsen, 2011; Testerink and Munnik, 2011; Gillaspy, 2013; Munnik, 2014; Laha et al., 2015; Heilmann, 2016; Hou et al., 2016). Alternatively, since PIP or PIP$_2$ are emerging as second messengers themselves, PLC could function as an attenuator of signaling too. In that respect, it is interesting to notice that the increased drought-tolerant phenotype in plc5/7 and the PLC3, -5, and -7 overexpression lines correlates with a stronger PIP$_2$ response in seedlings osmotically stressed with sorbitol. How this would work remains unclear, but these lines could somehow be primed for an enhanced PIP$_2$ turnover.

**Role for PLC5 and PLC7 in seed mucilage**

Mucilage is a complex of polysaccharides, which is from seed coat epidermal cells when seeds are exposed to an aqueous environment (Western et al., 2000). The major component of mucilage is pectin, of which polygalacturonic acid (PGA) and rhamnogalacturonan I (RGI) are the most common compounds found in cell walls and mucilage (Carpita and Gibeaut, 1993; Cosgrove, 1997). In addition to pectin, mucilage also contains some minor monosaccharides, i.e. arabinose, galactose, glucose, xylose and mannose, which are equally important in controlling mucilage properties (Voiniciuc, et al., 2015). The extruded mucilage contains two layers, a non-adherent outer layer and an adherent inner layer. The former is easily removed from the seed (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). The latter is hard to detach, even chemically (Macquet et al., 2007). Our result showed that inner mucilage of plc5/7 seed coats was easily lost (Fig. 4b). Many factors are involved in maintaining the adherence of the inner mucilage, with cellulose being the most important (Harpaz-Saad et al., 2011; Sullivan et al., 2011; Yu et al., 2014; Ben-Tov et al., 2015; Voiniciuc, Schmidt, et al., 2015; Hu, Li, Wang, et al., 2016; Hu, Li, Yang, et al., 2016). Staining with Calcofluor white and Pontamin S4B indicated that certain cellulose rays were missing from plc5/7 seed-coat mucilage (Fig. 4c). We checked for sugar-composition aberrations in whole seeds, but found no changes compared to wild-type (Supplemental Fig. S1). Possibly, the distribution between non-adherent and adherent mucilage layers might be different, which needs to be further determined. The adherence of mucilage by cellulose is mainly caused by its crystallization by self-association via hydrogen bonding (Sullivan et al., 2011), and several genes have been recently reported to regulate cellulose crystallization, i.e. COBRA-LIKE2, IRX14 and IRX7 etc (Ben-Tov et al., 2015; Hu, Li, Wang, et al., 2016; Hu, Li, Yang, et al., 2016).

Histochemical analysis of the GUS reporter lines indicated that both PLC5 and PLC7 are expressed during seed development (Fig. 5). Until now, no mucilage deficiency has been linked to PLC, and we only observed the mucilage phenotype in the plc5/7-double mutant of all our PLC knockout
mutants. The defect in both PLC5 and PLC7 probably breaks the balance for mucilage maintenance by altering cellulose deposition and/or crystallization in the inner mucilage. How the enzyme PLC could be involved in all this will require additional research. Potentially, PLC could be required for mucilage secretion or for the localization or activity of cellulose synthases, for example. PA, PIP and PIP2 have been implicated to play essential roles in vesicular trafficking, -fusion and -fission. Even though no difference was found in PPI- or PA levels in either developing or mature seeds (Fig. 6), reduced amounts of PLC5 and lack of PLC7 might cause crucial local changes in lipid or IPP molecules.

**Role for PLC in leaf serration**

Leaf shape is defined by the pattern and degree of indentation at the margin area, thus distinguishing plant species (Tsukaya, 2006). The patterning involves a complex crosstalk between hormone signaling and genetic regulators (Byrne, 2005; Fleming, 2005). Leaf serration is an important patterning event and its development involves auxin maxima at the protrusion of each serrated section (Hay and Tsiantis, 2006). Based on recent genetic studies, the auxin efflux carrier PIN-FORMED 1 (PIN1) and CUP-SHAPED COTYLEDON 2 (CUC2) have been identified as two key factors required for the formation of leaf serration (Hay et al., 2006; Nikovics et al., 2006). PIN1 asymmetrically localizes on plasma membranes and directionally transports auxin, creating auxin maxima that direct the outgrowth of the serrations (Hay et al., 2006; Scarpella et al., 2006). CUC2 is a transcription factor that is post-transcriptionally downregulated in leaves by MIR164A (Nikovics et al., 2006). CUC2 expression is limited to the sinus where the serration starts, and the promotion of serration outgrowth is through cell division, not by suppression of sinus growth (Kawamura et al., 2010). CUC2 has also been indicated to regulate the polarized localization of PIN1 in convergence points at the leaf margin, playing a role in establishing, maintaining and/or enhancing auxin maxima that result in leaf serration (Kawamura et al., 2010; Bilsborough et al., 2011). In a feedback loop, auxin downregulates CUC2, both transcriptionally and post-transcriptionally through activation of MIR164A (Bilsborough et al., 2011).

The *plc5/7*-double mutant revealed a mildly-enhanced leaf-serration phenotype (Fig. 7) Subsequent measurement of CUC2 and MIR164A expression revealed a an up-regulation of CUC2 and down-regulation MIR164A, which is consistent with the enhanced serration (Kawamura et al., 2010; Bilsborough et al., 2011). Promotor-GUS analyses showed that both PLC5 and PLC7 were expressed at leaf hydathodes, a secretory tissue that secretes water through the leaf margin that is also associated with leaf serration (Tsukaya and Uchimiya, 1997) and auxin response maxima (Scarpella et al., 2006). Hence, it is possible that PLC5 and PLC7 together contribute to the regulation of leaf serration, also because there was no such phenotype found in single mutants. We also measured PPI-and PA levels in *plc5/7* rosette leaves but, like seeds and seedlings, found no changes (data not shown; Figs. 6 and 10). It is possible that differences are limited to particular cells or tissues and that most of the cells have normal levels/responses. How PLC5 and PLC7 are involved in leaf serration requires further investigation. IP₆ could play a positive role in auxin signaling through activation of TIR1 (Chapter 2).
However, increased leaf serration would then be expected to result from increased IP$_6$ formation, which not what we would directly expect from a plc5/7 mutant. On the other hand, PIP$_2$ has been suggested to play a role in the polarized localization of PIN1 (Ischebeck et al., 2013; Tejos et al., 2014), in which PLC could also play a role. As mentioned earlier, down-regulation of these two PLCs (plc5 KO is likely lethal; Chapter 3) could result in a local upregulation of another PLC, even though overall PLC expression levels (Fig. 8d) do not confirm such a model.

**Both PLC7 overexpression and plc5/7 down-regulation enhance drought tolerance**

Overexpression of PLC in maize, tobacco and canola improved drought tolerance (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011). Similarly, we found that overexpression of PLC3 or PLC5 increased the drought tolerance of Arabidopsis. To investigate the effect of PLC7 overexpression, homozygous pUBQ10::PLC7 lines were generated. Meanwhile, we took plc5-1, plc7-3 and plc5/7-double mutants along to see how they would behave under drought stress. While plants appeared normal under control conditions, except for plc5/7 showing more serrated leaves, upon drought stress both plc5/7- and PLC7-OE lines performed better (Figs. 8a, 9b), and lost less water than wild type (Figs. 8b, 9c), even though the latter was not statistically significant for PLC7-OE lines (Fig. 9c).

ABA synthesis is stimulated by dehydration stress and known to induce stomatal closure to reduce the water loss (Sean et al., 2010). Previous result indicated that PLC3- and PLC5-OE lines showed a 'less-open stomata' phenotype in the absence of ABA, and that they exhibited a reduced closing response to ABA compared to wild-type (Chapters 2 and 3). Overexpression of PLC7 did not show 'less-open stomata' under control conditions, and their response to ABA was like wild type. Stomata of plc5/7 plants were less open but their response to ABA appeared normal, maybe slightly decreased, which is in contrast to plc5- and plc7-single mutants, which have a normal opening at control conditions, and plc7 is less sensitive to ABA (Supplemental Fig. S2; Chapter 3). Interestingly, PLC7 itself was not expressed in guard cells (Fig. 1), but the gene is rapidly upregulated in guard cells upon ABA treatment (Bauer et al., 2013). Hence, it could be that the drought tolerant phenotype in the plc5/7 is a consequence of a local upregulation (for instance in the guard cells) of one or more redundant PLCs, which may remain undetectable when whole seedling levels are measured (Fig. 9).

Osmotic stress-induced phospholipid signaling responses have been reported in many studies (Munnik and Vermeer, 2010; Meijer et al., 2017). We measured PPI- and PA levels in response to sorbitol to mimic drought stress in plc5/7, PLC7-OE and wild-type seedlings using $^{32}$P-labeling. Under control conditions, no difference in PPI- and PA levels were found among the genotypes (Fig. 10). However, in response to osmotic stress, the PIP$_2$ response in both plc5/7 and PLC7-OE lines were stronger than wild-type (Fig. 10b and 10d). Apart from second messenger precursor, PIP$_2$ could also play a role as signaling molecule itself, for example in the reorganization of the cytoskeleton, in endo- and exocytosis, or ion channel regulation (Stevenson et al., 2000; Martin, 2001; van Leeuwen et al., 2007; Heilmann, 2016). Identification and characterization of some of PIP$_2$’s main targets will be
essential to start unraveling the molecular mechanisms involved. Whether the above response in plc5/7 is a consequence of enhanced, local expression of another PLC, or a consequence of differential gene expression and hence drought tolerant response, requires further studies.

MATERIALS AND METHODS

Plant material

*Arabidopsis thaliana* (*Col-0*) was used throughout. T-DNA insertion mutants *plc7-3* (SALK_030333) and *plc7-4* (SALK_148821) were obtained from the SALK collection (signal.salk.edu). Homozygous plants were identified by PCR in F2 generation using gene-specific primers. For the identification of *plc7-3*, we used forward primer 5’- GATTTGGTGATAAAAGAAGTTTG -3’; reverse primer 5’-CTCCACACAATCTCAGCATTAC-3’) and left border primer LBb1.3 (5’-ATTTTGCCGATTTCGAAC-3’, in combination with the forward primer). For *plc7-4* identification, forward primer 5’-TCCTTCCTGTATCCATGACG-3’; reverse primer 5’-TTGAAGAAAGCATCAAGGTGG-3’ and left border primer LBb1.3 (5’-ATTTTGCCGATTTCGAAC-3’, in combination with the reverse primer) were used. To generate double mutant *plc5/7*, *plc5-1* and *plc7-3* were used for crossing.

Root growth

Seeds were surface sterilized in a desiccator using 20 ml thin bleach and 1ml 37% HCl for 3 hours, and then sowed on square petri dishes containing 30 ml of ½ strength of Murashige and Skoog (½MS) medium (pH 5.8), 0.5% sucrose, and 1.2 % Daishin agar (Duchefa Biochemie). Plates were stratified at 4 °C in the dark for two days, and then transferred to long day conditions (22 °C, 16 h of light and 8h of dark) in a vertical position, under an angle of 70°. Four-day-old seedlings of comparable size were then transferred to ½MS ager plates without sucrose and allowed to grow further for another 6-8 days. Plates were then scanned with an Epson Perfection V700 scanner and primary root length, lateral root number and average lateral root length from each genotype quantified through ImageJ software (National Institutes of Health).
Cloning and plant transformation

To generate the pPLC7::GUS-SYFP reporter line, the PLC7 promoter was amplified from genomic DNA using the following primers: PLC7proH3fw (5’-CCCAAGCTTGATCCTATCAATATTCCTAATTCAGC-3’) and PLC7proNheIrev (5’-CTAGCTAGCTTGAACAATTCCTCAAGTG-3’). The PCR product was cloned into pGEM-T easy and sequenced. A HindIII-pPLC7-Nhel fragment was then ligated into pJV-GUS-SYFP, cut with HindIII and Nhel. A pPLC7::GUS-SYFP1 fragment, cut with NotI and transferred to pGreenII-0229.

The MultiSite Gateway Three-Fragment Vector Construction Kit (www.lifetechnologies.com) was used to generate PLC7 overexpression lines driven by the UBQ10 promoter (pUBQ10::PLC7). Oligonucleotide primers (5’-GGGGACAACGTTTGTACAAAAAAGCAGGCTATGTCGAAGCAAACATACAAAGT-3’ and 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTACAAACTCCAACCGCACAAGAA-3’) including attB1 and attB2 sites, were used to PCR from PLC7 cDNA and was cloned into the donor vector (pDONR207) by using BP Clonase II enzyme mix to create entry clone BOX2. BOX1 was pGEM-pUBQ10 entry clone containing attL4 and attR1 sites. BOX3 was pGEM-TNOS entry clone containing attR2 and attL2 sites. Both entry clone were obtained from university of Amsterdam plant physiology publish construct source. The three entry clones (BOX1, BOX2 and BOX3) and a destination vector (pGreen0125) were used in MultiSite Gateway LR recombination reaction to create the expression clone. For details, check: (Multi gateway protocol).

All constructs were transformed into Agrobacterium tumefaciens, strain GV3101, which was subsequently used to transform Arabidopsis plants by floral dip (Clough and Bent, 1998). Homozygous lines were selected in T3 generation and used for future experiments.

RNA extraction and Q-PCR

The primer pairs to check for PLC1- to PLC9-expression levels were obtained from Tasma et al. (2008). Similarly, identification of CUC2- and MIR164A-expression levels were performed as described by Bilsborough et al. (2011). Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) as described by Pieterse (1998). Total RNA (1.5 µg) from 10-day-old seedlings, or 4-week old rosette leaves, were converted to cDNA using oligo-dT18 primers, dNTPs and SuperSccript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. Q-PCR was performed with ABI 7500 Real-Time PCR System (Applied Biosystem). The relative gene expression was determined by comparative threshold cycle value. Transcript levels were normalized by the level of SAND (At2g28390; forward primer: 5’-AACTCTATGCAGCATTTGATCCACT-3’, reverse primer: 5’-TGAAGGGGACAAGGTGTTGATCGT-3’), or OTC (At1g75330; forward primer: 5’-TGAAGGGGACAAGGTGTTGATCGT-3’, reverse primer: 5’-CGCAGACAAAGTGGATGGA-3’) (Bilsborough et al., 2011; Han et al., 2013). Three biological- and two technical replicates were performed to obtain the values for means and standard deviations (Han et al., 2013).
**Histochemical analyses for GUS activity**

GUS staining was performed as described previously (Chapter 2; Zhang et al., 2017). Briefly, transgenic plants carrying pPLC7::GUS-SYFP were grown for indicated times, after which specific tissues were taken and incubated in an X-Gluc reaction solution containing 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM phosphate buffer (pH 7.0), and 0.1% TX-100. Material was incubated overnight at 37°C and the next day, cleared by 70% ethanol, and kept in that solution. GUS staining was visualized under a stereo microscope (Leica MZFLIII) and digitalized with a ThorLabs, CCD camera.

**Seed staining and microscopy**

To visualize seed coat mucilage, mature dry seeds were stained as described in Macquet et al. (2007). Seeds were directly incubated in 0.03% (w/v) Ruthenium red, or after imbibition in 0.5 M EDTA, pH 8.0, for 90 min. For the latter, seeds were washed with water to remove the EDTA and then stained for 20 min with Ruthenium red. Stained seeds were routinely observed with a light microscope (Aristoplan; Leitz). To visualize the seed surface and the adherent mucilage (AM) layer by confocal microscopy, Calcofluor white (0.01%) and Pontamine S4B were used as staining solutions (Western et al., 2000; Saez-Aguayo et al., 2014). Optical sections were obtained with an Olympus LX81 spectral confocal laser-scanning microscope. A 405 nm diode laser line was used to excite Calcofluor white and the fluorescence emission was detected between 412 and 490 nm. For Pontamine S4B a 561 nm diode laser line was used and the detection was done between 570 and 650 nm. For comparisons of the signal intensity within one experiment, the laser gain values were fixed. Three different batches of seeds were analyzed and all of them showed the same phenotype. LUT green fire blue filter and LUT fire filter (Image J) were applied to the Calcofluor white and Pontamine S4B images, respectively.

**Determination of sugar composition by HPAEC-PAD**

Sugar composition of wild-type and mutant seeds were measured as described previously (Saez-Aguayo et al., 2017). 20 mg of seeds were ground in liquid nitrogen and extracted twice in 80% ethanol with agitation for 1 h at room temperature followed by removal of lipids by washing twice with methanol:chloroform (1:1) and twice with acetone. The final AIR was dried overnight at RT and two mg was hydrolyzed for 1 h with 450 µL 2 M trifluoroacetic acid (TFA) at 121 °C, using inositol as an internal control. TFA was evaporated at 60 °C with nitrogen, samples washed two times with 250 µL of 100% isopropanol, and dried in a speed-vac. Samples were dissolved in 200 µL Ultrapure water, clarified with a syringe filter (0.45 µm) and transferred to a new tube for analysis by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).
The sugar quantification was done as described by Sáez-Aguayo et al. (2017). A Dionex ICS3000 ion-chromatography system, equipped with a PAD detector, a CarboPac PA1 (4mm x 250mm) analytical column, and a CarboPac PA1 (4mm x 50mm) guard column were used. The separation of neutral sugars was performed at 40ºC with a flow rate of 1 mL/min using an isocratic gradient of 20 mM NaOH for 20 min followed by a wash with 200 mM NaOH for 10 min. After every run, the column was re-equilibrated in 20 mM NaOH for 10 min. Separation of acidic sugars was performed by using 150 mM NaOAc and 100 mM NaOH for 10 min at a flow rate of 1 mL/min at 40°C. Standard curves of neutral sugars (D-Fuc, L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl, and D-Man) or acidic sugars (D-GalA and D-GlcA) were used for quantitation.

**Leaf-shape analysis**

Rosettes from 4-week old plants grown under long day condition were detached and photographed immediately. Leaves were removed from the rosette and adhered to white paper and then scanned (Epson Perfection V700 scanner). The 8th leaf was used for calculation. Blade length, width, perimeter, area, serration number and serration levels were calculated from silhouettes using ImageJ software. Leaf-serration levels are expressed as the distance from tip-to-midvein divided by the distance from sinus-to-midvein, for indicated tooth (2nd-4th) (Kawamura et al., 2010).

**Stomatal aperture**

The stomatal aperture measurement was performed according to Distéfano et al., (2012) with minor modifications. The stomatal aperture treatments were performed on epidermal strips excised from the abaxial side of fully expanded Arabidopsis leaves. Epidermal peels from leaves of 3-week-old plants, grown at 22°C under 16 h of light and 8h of dark, were stripped and immediately floated in opening buffer (5 mM MES-KOH (pH 6.1), 50 mM KCl) for 3 h. The strips were subsequently maintained in the same opening buffer and exposed to different ABA concentration. After 90 min, stomata were digitized using a Nikon DS-Fi 1 camera, coupled to a Nikon Eclipse Ti microscope. The stomatal-aperture width was measured using ImageJ software (National Institute of Health).

**32Pi-phospholipid labelling, extraction and analysis**

Developing seeds at 10 DAP were carefully removed from silique. Mature seeds were sterilized and stratified on ½MS (pH 5.8) as described and germinated under long day conditions for around 20 h when testa ruptured. Both developing and germinating seeds were then transferred to 200 µl labeling buffer (2.5 mM MES, pH 5.8, 1 mM KCl) containing 5-10 µCl $^{32}$PO$_4^{3-}$ ($^{32}$P$_i$) (carrier free; Perklin-Elmer) in a 2 ml Eppendorf, Safelock microcentrifuge tube for 24 h.

Five-day-old seedlings were labeled similarly, except for 3 h labeling time. Samples were treated by adding 200 µl labeling buffer with or without 600 mM sorbitol for 30 mins.
Labeling an subsequent treatments were stopped by adding perchloric acid (final concentration, 5% by vol.) for 5-10 min, after which lipids were extracted and the phosphoinositides and PA separated from the rest of the phospholipids by thin-layer chromatography (TLC) as described in detail by Munnik & Zarza (2013). Radioactive phospholipids were visualized by autoradiography and quantified by phoshoimaging (Molecular Dynamics, Sunnyvale, CA, USA). Individual phospholipid levels are expressed as the percentage of the total $^{32}$P-lipid fraction.

**Drought tolerance assays**

Drought assay were performed as described previously (Hua et al., 2012; Osakabe et al., 2013) with some changes. Seeds were stratified at 4°C in the dark and sowed in soil pots (4.5 cm x 4.5 cm x 7.5 cm) containing equal amounts (80 g) of soil. Nine plants per pot were grown under short-day conditions (22 °C with 12 h light/12 h dark) for 4 weeks, and then subjected to dehydration by withholding them for water for 2 weeks, while control plants were normally watered. At this point, plants were photographed. Each experiment used 36 plants per genotype and experiments were repeated at least 3 times.

To assay water-loss, rosettes from 4-week-old plants were detached and FW determined every hour by weighing. Water content was calculated as the percentage from the initial FW. Twenty plants were used for each experiment, which was repeated at least 3 times.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Chapter 4


SUPPLEMENTAL DATA

**Supplemental Figure S1.** Sugar composition of wild type- and plc5/7-mutant seeds. Sugars were extracted from dry seeds and quantified by HPAEC-PAD. Quantities were corrected through internal standards, and transformed into mg of sugar per gram of dry material. Values represent the means of triplicates ± SE of three independent experiments.

**Supplemental Figure S2.** Decreased ABA sensitivity of plc7 mutants in stomatal closure. Leaves from 3-weeks old plants were stripped and peels were incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentrations of ABA for 90 mins, after which stomata were digitized and the aperture width measured for wild type and plc7-3 (left) or plc7-4 (right). Values are means ± SE of at least three independents (n >100). Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05, Dunn’s method).
Chapter 5

General discussion

Qianqian Zhang,¹,² Michel A. Haring,¹ & Teun Munnik¹,²

¹Section Plant Physiology or ²Section Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, Amsterdam, 1098 XH, The Netherlands
Comparison of Arabidopsis PLC3, PLC5 and PLC7 -- similarities and differences

In this thesis, we explored the roles of three PLC genes - PLC3, PLC5 and PLC7 - in Arabidopsis development and stress responses at the molecular, physiological and biochemical level. Arabidopsis contains 9 PLCs, which share several conserved protein domains, including an EF-hand domain, the catalytic X- and Y-domains, and a C2 domain (Tasma et al., 2008; Munnik, 2014). In addition, the genes for PLC3, PLC5 and PLC7 localize to chromosome 3, 4 and 5 respectively and may have evolved differently (Hunt et al., 2004; Tasma et al., 2008). The difference in protein structure and evolutionary history is likely to result into functional differences between the nine PLC homologs of Arabidopsis.

The discussion below will focus on the similarities and differences between PLC3, PLC5 and PLC7 in terms of their function in plant development and stress. This will help to put the results presented in thesis, in a broader perspective.

Expression

The PLC-expression analyses in this thesis were based on promoter-GUS analysis. The expression patterns of PLC3, PLC5 and PLC7 have similarities, but also some differences (see Table 1). The most striking communality in expression is that they are all vascular-expressed PLCs in both vegetative (shoot and root) and reproductive organs (flower). Cell-specific gene profiling analyses in roots revealed that these PLCs are expressed in phloem-related cells, e.g. companion cells (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Phloem is responsible for transferring soluble organic compounds, especially sucrose made by photosynthesis in the leaves, to tissues where it is required, such as roots (Turgeon and Wolf, 2009; De Schepper et al., 2013). PLC expression detected in phloem suggests a potential role in plant development. A closer look at the expression in root vasculature revealed a typical "segmented-expression" pattern for PLC3 and PLC5 in roots, but not for PLC7. This could be linked to the lateral root formation phenotype in plants that have lost the function of PLC3 or PLC5, but not PLC7 (see discussion below).

Table 1. Promoter-GUS analysis of PLC expression.

<table>
<thead>
<tr>
<th>expression</th>
<th>vascular tissue</th>
<th>segment expression in root</th>
<th>developing seed chalazal</th>
<th>developing seed coat</th>
<th>mature seed embryo</th>
<th>trichome</th>
<th>guard cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC3</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes (base only)</td>
<td>yes</td>
</tr>
<tr>
<td>PLC5</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>PLC7</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
PLC is expressed throughout the whole life cycle, based on current knowledge (Hunt et al., 2004; Tasma et al., 2008; Munnik, 2014; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We found all three PLCs were expressed in the chalazal during seed development. This is the tissue that serves as a channel for passing nutrients from the mother plant to the embryo (Xu et al., 2016). Therefore, PLCs might exert the same function here as they do in the vasculature. PLC5 and PLC7 were expressed in the developing seed coat but PLC3 was not expressed in this tissue. The plc5plc7 double mutant exhibited a non-adherent seed coat mucilage phenotype, which might have to do with the expression of both PLC5 and PLC7 in the seed coat. The expression of all three genes was also detected in germinating seeds. pPLC3::GUS-YFP embryo was intensively stained by X-Gluc (Chapter 2, Figure 4b), this was much less obvious for PLC5::GUS (Chapter 3, Figure 2a) and PLC7::GUS-SYPF (Chapter 4, Figure 1a), which could be a link to the slower germination rate of the plc3 mutants only.

Interestingly, all three PLCs were expressed in trichomes. The expression of PLC5 and PLC7 was detected in the whole trichome body, but PLC3 only in the basal cells of the trichome, which connect to the leaf-vascular tissue and seems to be part of the vasculature. In Arabidopsis, trichomes are non-secreting epidermal cells, which protect plants from adverse conditions and also serve as a source of phytochemicals (Schwab et al., 2000; Pattanaik et al., 2014). A numbers of genes have been identified to be involved in trichome development. The consistent expression of PLC5 and PLC7 in trichomes hints at a possible role in morphogenesis and/or function, even though we did not observe any phenotype in the single- and double plc mutants that were viable; obviously the PLCs may be redundant. An inducible-amiPLC3 line in a plc5/7 background may reveal more on this.

In guard cells, both PLC3 and PLC5 were expressed, which confirms data provided by the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). However, PLC7::GUS-SYPF staining was not detected in guard cells. According to online database from eFP browser, PLC7 could be induced in the guard cells after exogenous ABA application. We performed GUS staining on the leaf peel from pPLC7::GUS-SYPF plants after ABA treatment, but found no GUS signal (data not shown). The online data is based on microarray technique and guard cell protoplasts were used as plant material, which is different with our GUS staining analysis using seedlings, or freshly peeled leaf epidermis.

The promoter-GUS analysis and light microscopy studies cannot provide information about the PLC localization at the cellular level. The precise location in the cell will help to understand how plant PLCs work in various biological events. Thus, making transgenic plants that express PLC-GFP fusions driven by their own promoters is essential and will be our next step. We attempted by complementing the plc5-1 mutant with an N-terminal YFP-PLC5, driven by the PLC5 promoter, but no fluorescence could be observed anymore in T2- and T3 generations while antibiotic resistance, genotyping and phenotyping (root architecture) suggested that the wild-type gene was there. We suspect that the N-terminal FP-fusion is lethal/interferes too much and that we select for plants that have been able to loose the FP, as similar results were obtained for PLC2 (R. van Wijk and T. Munnik, unpublished).
Currently, the lab is testing this for new N- and C-terminal FP-PLC fusions (M. van Hooren, T. Munnik).

Role for PLCs in seed germination

We discovered a number of phenotypes in plc3, plc5 and plc7 single mutants, but also double plc3/5 and plc5/7 mutants (table 2). Among the mutants studied, only plc3 exhibited a delayed seed germination phenotype (Chapter 2, Figure 4a). Seed germination is a very complex process, involving many factors, such as light, hormones and sugars etc. (Bentsink and Koornneef, 2008). Among these, abscisic acid (ABA) and gibberellin (GA) are best known for their crucial roles. ABA inhibits seed germination (Nambara et al., 2010; Nakashima & Yamaguchi-Shinozaki, 2013) while, GA acts stimulates germination (Yamaguchi and Kamiya, 2001). We compared the responses of plc3 mutants to both ABA (Chapter 2; Fig.5) and GA (Chapter 2; Supplemental Fig. S7) with that of wild-type seeds. plc3 mutant seeds germinated faster than wild-type when treated with exogenous ABA, indicating an increased ABA insensitivity. Therefore, the slower germination of plc3 compared to wild type under normal condition cannot be caused by hypersensitivity to ABA. In response to GA, plc3 mutants seemed to be hypersensitive, which again does not explain their slower germination phenotype. One possibility is that the endogenous GA level in plc3 mutants is lower. Similarly, plc3 mutants’ insensitivity to ABA might correspond to a higher level of ABA in seeds. Hence, checking hormone level in plc3 mutant seeds will be essential. Apart from hormones, sugar is another important factor during seed germination. The link between sugar metabolism and PLC signaling could be the Raffinose Family Oligosaccharides (RFOs), whose synthesis might require PLC-dependent inositol (Chapter 2 discussion). However, the analysis of soluble carbohydrate composition in seeds showed no significant differences between wild type and plc3 mutants. Of course, changes could be very local, so it is also possible that these differences remain unobserved (Chapter 2; Fig. 4c).

A role for PLC in ABA signaling

Both plc mutants and PLC-overexpression lines of PLC3, 5 and 7 appear to have changes in ABA related physiological responses. In addition, PLC3 and PLC5 are strongly expressed in guard cells. Both plc3 (Chapter 2; Fig. 5c) and plc7 (Chapter 4; Supplemental Fig. 3) KO mutants did not close their stomata as much as wild type did during ABA treatment of epidermal leaf strips, which indicates an insensitivity for ABA. Furthermore, in both PLC3 and PLC5 overexpressor lines stomata were more closed in control conditions and while the guard cells in epidermal peels appeared to be less responsive to ABA. PLC7-OE lines did not show these changes. Together, these results hint to an involvement of PLC in ABA-induced stomatal closure. The T-DNA insertion plc mutants provide a tool to investigate the link between PLC and IPPs. According to our observation, using ^3H-Inositol pre-labeled seedlings and HPLC analyses, plc3 mutants did not any show change in IP3 or IP6 levels, but a small decrease in either IP7 or IP8, was found depending on the seedling age. Plants contain plenty of IP6, which is predominantly formed from MIPS-generated Ins3P (Munnik and Vermeer, 2010). The possible changes
in PLC-derived IP₆ in guard cells will be difficult to observe in a huge background other cells. Nevertheless, our results suggest that higher IPP and PP-IPP levels could be involved in PLC signaling. The measurement of IPPs and PP-IPPs will be continued in other plc mutants with ABA and other stresses application, in this way we will get closer to the real PLC signaling pathway in plant! Alternatively, fluorescent biosensors expressed in guard cells could help studying changes in PIP₂ or PIP levels during ABA induced stomatal closure.

Table 2. Phenotypes that are exhibited in plc knock-out or knock-down mutants

<table>
<thead>
<tr>
<th>plc mutant phenotype</th>
<th>delayed seed germination</th>
<th>insensitive to ABA</th>
<th>shorter primary root</th>
<th>fewer lateral roots</th>
<th>loose seed coat mucilage</th>
<th>stronger leaf serration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>seed germination</td>
<td>stomatal closure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plc3</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc5</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc7</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc3/5</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc5/7</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

The role for PLC in lateral root formation

PLC3::GUS-YFP and PLC5::GUS displayed a segmented-expression pattern in roots, which could be associated with the decrease in lateral root formation in plc3- and plc5 mutants. PLC7::GUS-SYFP did not have this expression pattern, and plc7 mutants did not display the lateral root phenotype either. The double mutant plc3/5 had a similar root phenotype as the single mutants, with only a minor increase in severity (Chapter 3; Fig. S2), whereas no changes were found in the root architecture of the plc5/7 mutant (Chapter 4; Fig. 3), suggesting redundancy of other PLC family members. The combination of plc3- and plc7-KO mutations was not viable, as we could not obtain homozygous lines from this cross. Generation of induced silencing lines for PLC3 and PLC7 could help to overcome this problem.

The possible role of PLC in lateral root formation has been discussed in Chapter 2 and 3. Two options were considered: PLC might affect the root architecture through the generation of IP₆ to influence auxin-TIR1 signaling, or via the metabolism, transport or storage of raffinose oligosaccharides (RFOs). One function for RFOs is loading sucrose to lateral roots, which could either facilitate their development or functions to store sugars. In plc3 mutants, phloem sap sucrose had increased and inositol was slightly reduced in plc3 mutants compared to wild type. However, plc5 only displayed a slightly lower inositol level (had no change in phloem sucrose). Further investigation of the phloem sap sugar composition in plc3/5 and multiple plc mutants is required to confirm the PLC/inositol/RFOs/LR hypothesis. Alternatively, PLC might regulate lateral root formation by PLC-dependent IP₆, which could affect TIR1 activity, resulting in less efficient auxin perception and fewer lateral root initiation. To confirm this hypothesis, an FP-tagged version of TIR1 in a tir1 mutant

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background (\textit{pTIR1::gTIR-mVENUS}) will be crossed with \textit{plc3-} and \textit{plc5} mutants. TIR-mVENUS will then be immunoprecipitated and IP$_6$ presence will be validated by its conversion into $^{32}$P-IP$_7$ by incubating shortly-boiled fractions with an IP$_6$ kinase (yeast KCS1, fused to GST and expressed in \textit{E.coli}) and $^{32}$P-$\gamma$ATP. Radioactive labeling is very sensitive, and this might allow us to detect the decreased IP$_6$ levels in the \textit{plc} mutant backgrounds that may not be detected by other methods. In this way, we can link PLC activity directly with IP$_6$ production and auxin signaling through TIR1.

\textit{Overexpression of PLC enhances drought tolerance}

Drought stress is a major threat that limits plant productivity in agriculture. It does not only cause hyperosmotic stress, but also oxidative stress on cells (Zhu, 2016). Plant responses to drought stress are complex, including the sensing and transduction of the stress signal, but also the adaptation to the adverse environment, which involves numerous physiological-, structural-, morphological-, and biochemical changes. PLC signaling is activated under drought stress (Munnik and Meijer, 2001; Munnik and Vermeer, 2010; Hou et al., 2016). Both PLC’s substrates (PIP, PIP$_2$) and products (IPP, PA converted by DAG) have been reported to be implicated in important cellular events, such as the reorganization of the cytoskeleton, endo- and exocytosis, vesicular trafficking, and ion-channel regulation, including intracellular Ca$^{2+}$ release (Stevenson et al., 2000; Martin, 2001; van Leeuwen et al., 2007; Heilmann, 2016). Recently, overexpression of a \textit{PLC} in maize, tobacco and canola have been shown to improve drought tolerance (Wang et al., 2008; Georges et al., 2009; Tripathy et al., 2011).

Overexpression of \textit{PLC3}, \textit{PLC5} or \textit{PLC7} were all found to enhance the drought tolerance of Arabidopsis, with \textit{PLC5-OE} showing the strongest phenotype (Table 3). Similarly, overexpression of \textit{PLC2} and \textit{PLC4} showed an increased drought-tolerant phenotype (Munnik lab, unpublished), indicating that overexpression of any \textit{PLC} might improve the plant's tolerance to drought. When plants experience drought, several physiological changes happen: e.g stomata close, root systems become denser and grow deeper, compatible solutes accumulate, photosynthesis decreases, etc. (Comas et al., 2013; Singh et al., 2015; Basu et al., 2016; Joshi et al., 2016). \textit{PLC3-OE} and \textit{PLC5-OE} exhibited a smaller stomatal aperture than wild type, which could be a reason for their enhanced drought tolerance. However, two \textit{PLC7-OE} lines #9 and #12 showed no difference in stomatal aperture, but they did exhibit the drought tolerance phenotype. It is possible though, that the difference in stomatal opening in \textit{PLC7-OE} lines #9 and #12 is too small to be observed. Therefore, more independent \textit{PLC7-OE} lines should be used for further confirmation, and preferably, we would like to measure stomatal conductance \textit{in vivo}, on a growing plant that experiences drought stress (Andrés et al., 2014). The closure of stomata is induced by the phytohormone, ABA and this signaling pathway is central to the drought-stress response. How \textit{PLC} could be involved in ABA signaling has been well summarized in Chapter 2 (Figure 10).
Table 3. Phenotypes that are exhibited in PLC-OE lines.

<table>
<thead>
<tr>
<th>PLC-OE phenotype</th>
<th>drought tolerance</th>
<th>stunted root hairs</th>
<th>smaller stomatal aperture</th>
<th>ABA sensitivity stomata</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC3</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>less</td>
</tr>
<tr>
<td>PLC5</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>less</td>
</tr>
<tr>
<td>PLC7</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no change</td>
</tr>
</tbody>
</table>

The root system of PLC-OE lines appears to be neither denser nor larger that would account for absorbing more water, and thus more drought tolerant. PLC5-OE lines even grow a smaller root system with very short root hairs, which was not found in other PLC-OE lines. Thus, the increased tolerance of the PLC-OE lines to drought is probably not through alteration of their root systems. In PLC5-OE lines, an increase in the accumulation of sugars was detected, which could be another possibility for drought resistance. Sugar content of the phloem should be carried out with other PLC-OE lines. The drought response in plants involves many genes with potential cross-talk (Basu et al., 2016; Zhu, 2016) and PLC could be one of them. Knowing the transcripts of drought-responsive genes in PLC-OE plant will help to discover the link between PLC and other genes in plant's response to drought stress.

An overview of PLC3, PLC5 and PLC7 phenotypes

All phenotypes found in manipulated PLC lines (KO mutants and OE lines) are summarized in Figure 1. We discovered several novel roles for PLC in plant stress and development, which provides a step forward in understanding how PLC functions in plants. Here, we emphasize some new scenarios for plant PLC signaling.

Firstly, apart from second messengers (IPPs and DAG/PA) producer, PLC could also function as PIP2 and PIP signaling attenuator (Munnik and Nielsen, 2011). PIP2 and PIP are emerging as second messenger themselves, involved in various stress- and developmental responses (Stevenson et al., 2000; Meijer and Munnik, 2003; Vermeer et al., 2009; Ischebeck et al., 2010; Munnik and Nielsen, 2011; Gillaspy, 2013; Rodriguez-Villalon et al., 2015; Heilmann, 2016; Simon et al., 2016).

Secondly, PLC follows the traditional signaling pathway to generate second messengers, except that IP3 is either phosphorylated to IP6 to release Ca2+, or dephosphorylated to inositol, which is the precursor for RFO sugar metabolism. The DAG that originates from PLC activity is converted into PA to activate downstream targets (Munnik and Nielsen, 2011; Testerink and Munnik, 2011; Munnik, 2014).

Thirdly, higher IPPs and PP-IPPs (IP4, IP5, IP6, IP7 and IP8) function as signaling molecules independently of Ca2+ signaling, such as ion-channel regulation (Zonia et al., 2002), hormone
perception (Tan et al., 2007; Sheard et al., 2010; Laha et al., 2015, 2016), mRNA transport (Lee et al., 2015) and phosphate homeostasis (Kuo et al., 2014; Puga et al., 2014; Wild et al., 2016).

Last but not the least, PI4P exerts great potential to be PLC’s substrate in non-stressed cells. The authentic PIP2 is missing from most plant plasma membranes, whereas PI4P is much more abundant (30-100 times), and most (>90%) of the PLC activity is associated with the plasma membrane fraction. In vitro, PI4P is hydrolyzed equally well as PIP2. The formed IP2 can still be further phosphorylated to IP3 by the same two IPKs, and the DAG be converted into PA (Munnik et al., 1998a, 1998b; Meijer and Munnik, 2003; van Leeuwen et al., 2007; Vermeer et al., 2009; Vermeer and Munnik, 2013; Munnik, 2014; Simon et al., 2014; Tejos et al., 2014; Simon et al., 2016). Hence, we should keep our eyes open for everything!

Figure 1. Summary of phenotypes in plc knockout and PLC overexpression lines. Left: plc knockout mutants exhibit delayed germination; fewer lateral roots; insensitivity to ABA during seed germination and ABA-induced stomatal closure; non-adherent seed coat mucilage and enhanced leaf serration phenotypes. Middle: proposed PLC signaling in plant development and stress responses. Right: PLC-OE display increased drought tolerance; less opened stomata and stunted roothair growth phenotypes.
REFERENCES


Summary

For many years, efforts have been made to explore PLC signaling in plants. Compared to the classical eukaryotic, mammalian PLC signaling pathway, a different picture is emerging for plants. Several roles for PLC in plant development and stress responses have been claimed but genetic evidence for this is mostly missing.

In chapter 2, we functionally characterized the role of PLC3 and found it mainly expressed in vascular tissue, but was also present in guard cells and trichomes. Loss-of-function mutants showed different phenotypes, including delayed seed germination, slightly less-elongated primary roots (~5%), fewer lateral roots (15%), and decreased sensitivity to ABA in terms seed germination inhibiting and stomatal-closure induction. ABA triggered an increase in PIP2 in various Arabidopsis tissues, however, no difference in PLC substrate- or product levels in two independent plc3 KD mutants were found. Overexpression of PLC3 increased the drought tolerance of plants and decreased their stomatal aperture.

In chapter 3, similar analyses were processed for PLC5, which was also predominantly expressed in the vasculature, but also in the root apical meristem, guard cells and trichomes. Knock-down mutant, plc5-1 displayed shorter primary roots (~10%) and exhibited fewer lateral roots (~20%), which could be restored by the expression of the endogenous PLC5-wt gene, driven by its own promoter. Double-plc3plc5 mutant did not intensify the root phenotype, indicating the involvement of (a)other PLC(s). Overexpression of PLC5 enhanced drought tolerance and reduced stomatal aperture, like PLC3, but in this case also led to a new phenotype, i.e. a stunted root-hair growth. 32P-phospholipid labeling analyses revealed no differences between wt and plc5-1 seedlings, however, decreased levels of PIP and PIP were found with increased levels of PA in PLC5 over-expressor lines. Inducible overexpression of PIP5K3 in PLC5-OE line rescued the root hair phenotype and restored the level of PIP2, providing independent evidence for PIP2’s crucial role in tip growth.

In chapter 4, we characterized the role of PLC7, another phloem-expressed PLC. Expression was throughout the plant vasculature, including roots, leaves and flowers, and also appeared in trichomes and hydatodes. We obtained a plc7-3 KO- and plc7-4 KD mutant but found no affects in root development, which is different from the plc3- and plc5 mutants. However, like plc3 mutants, plc7 mutants exhibited a reduced sensitivity to ABA-dependent stomatal closure. Double-knockout mutants of plc3plc7 were lethal, whereas plc5plc7 (plc5/7) mutants were viable, and revealed several new phenotypes not observed earlier. These include a defect in seed coat mucilage, enhanced leaf serration, and an increased tolerance to drought. The latter phenotype was previously found when PLCs were overexpressed. Overexpression of PLC7 also led to an enhanced drought tolerant phenotype. In vivo 32P-labeling of seedlings treated with sorbitol to mimick drought stress revealed increased PIP2 responses in both drought tolerant plc5/7 and PLC7-OE mutants. Together, these results reveal several novel functions for PLC in plant stress and development.
De rol van PLC in signaaltransductieprocessen in planten is nog grotendeels onbekend. Het is in ieder geval duidelijk dat dit anders moet zijn dan de klassieke, dierlijke, PLC-signaleringsroute, voornamelijk omdat planten de targets missen voor zowel IP₃ als DAG. Dit roept veel vragen op zoals, dienen er soms andere moleculen als second messengers, en hoe werkt dat dan? Is PIP₂ het enige substraat voor PLC of wordt standaard het meer abundante PIP gebruikt? En is de functie van PLC om PPI signalering te dempen of om second messengers te produceren? Meer bewijs is verzameld voor rollen van PLC in de ontwikkeling van de plant en stressreacties, langzaam waardoor meer en meer stukken van deze puzzel op tafel.

In hoofdstuk 2 wordt de rol van PLC3 functioneel gekarakteriseerd. Het gen komt vooral tot expressie in vaatweefsel, maar is ook aanwezig in sluitcellen en trichomen. Mutanten met een defect PLC3 gen hebben een vertraagde zaadkieming, minder lange hoofdwortel (~ 5%), minder zijwortels (15%), en een verminderde gevoeligheid voor ABA tijdens zaadkieming en bij het sluiten van huidmondjes. ABA stimuleert de toename in PIP₂ hoeveelheden in verschillende weefsels, maar er wordt geen verschil gevonden in PLC's substraat- of productniveaus van plc3 mutanten. Overexpressie van PLC3 in Arabidopsis planten blijkt de droogtetolerantie te verhogen en de opening van huidmondjes te verlagen.

In hoofdstuk 3 werden soortgelijke analyses uitgevoerd voor PLC5, welke ook overwegend in het vaatstelsel tot expressie komt, maar ook werd gevonden in het meristeem van de worteltop, in sluitcellen en in trichomen. Een knock-down, plc5-1 mutant heeft kortere hoofdwortels (~ 10%) en minder zijwortels (~ 20%), welke weer kan worden hersteld door expressie van het wild-type PLC5 gen, aangedreven door de eigen promotor. Dubbel plc3plc5-mutanten gaven geen sterker fenotype, mogelijke doordat andere redundante PLCs de rol overnemen. Overexpressie van PLC5 verhoogd de droogtetolerantie en vermindert de opening van huidmondjes. Daarnaast werd nog een nieuw fenotype gevonden, namelijk een remming van de wortelhaargroei. ³²P₁-fosfolipidenlabelinganalyses lieten geen verschil zien tussen wt and plc5-1 zaailingen, echter een aanzienlijk verminderde PIP₁ en PIP₂ niveau en verhoogde PA niveau werden in PLC5 over-expressie mutanten gevonden. Induceerbare overexpressie van PIP5K3 in deze PLC5-overexpressielijn, kon de wortelhaargroei en de PIP₂ niveaus herstellen, welke onafhankelijk bewijs levert voor de cruciale rol die PIP₂ speelt in tip groei.

In Hoofdstuk 4 werd de rol van PLC7, weer een andere floëem-specifieke PLC gen gekarakteriseerd. Het gen komt in het gehele vaatstelsel tot expressie, inclusief wortels, bladeren, en bloemen, maar ook in trichomen en in de waterporiën van de bladeren (hydratoden). Mutanten, i.e. plc7-3 KO en plc7-4 KD, bleken geen defect in de wortelontwikkeling te hebben, anders dan in de plc3- en plc5 mutanten. Echter, net als plc3 mutanten, bleken plc7 mutanten ook een verlaagde
gevoeligheid voor ABA te hebben voor het sluiten van huidmondjes. Dubbel-KO mutanten van plc3plc7 bleken letaal te zijn, maar plc5plc7 (plc5/7) mutanten waren wel levensvatbaar. Deze laatste lieten echter verschillende nieuwe fenotypes zien die nog nooit niet eerder waren waargenomen, te weten: een defect in het slijm van de zaadhuid, een toename in de gezaagdheid van de bladeren, en een verhoogde tolerantie voor droogtestress. Dit laatste fenotype werd eerder gevonden wanneer juist PLCs to overexpressie werden gebracht. En ook overexpressie van PLC7 laat wederom zien de droogtestolerantie te verbeteren. In vivo $^{32}$P$_{i}$-labeling van zaailingen die behandeld werden met sorbitol om droogtestress na te bootsen, blijken sterkere PIP$_{2}$ responsen te vertonen in zowel de droogtestolerante plc5/7- als de PLC7-OE mutanten. Samen onthullen deze resultaten een aantal nieuwe functies voor planten PLCs in stressreacties en ontwikkeling.
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List of publications

A role for Arabidopsis phospholipase C3 (*PLC3*) in seed germination, lateral root formation and stomatal closure. (In preparation)

Functional characterization of *PLC5* in Arabidopsis thaliana - knock-down affects lateral root initiation while overexpression stunts root hair growth and enhances drought tolerance. (In preparation)

Role for Arabidopsis *PLC7* in stomatal closure, mucilage adherence, and leaf serration. (In preparation)