Novel roles for phospholipase C in plant stress signalling and development

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Chapter 2

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

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Phospholipase C (PLC) is best known for its role in generating second messengers by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) 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$^{2+}$ 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 $^{32}$P$_1$-lipid labeling analyses, we found that ABA stimulated the formation of PIP$_2$ 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, IP₃ responses were reported, but none addressed IP₆ levels or other inositolpolyphosphates (IPPs) that are emerging as signalling molecules, i.e. IP₅, IP₇ and IP₈ (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 PIP₂ 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. *PLC*<sub>3</sub><sub>pro</sub>:*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), siliques (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. 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. 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](image)

(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) PLC3: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 PIP_{2} 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, PIP_{2} 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 PIP_{2}(27%) was found in wild-type seedlings upon ABA treatment, both plc3 mutants lacked this response (Fig. 6b).

Figure 6. PIP- 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}^{3-} 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 PIP_{2}, 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 PIP_{2} 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 PIP_{2} 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 PIP_{2}, 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 PIP₂, 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 ³²P₁-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 PIP₂, 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 PLC3-OEs was still significantly smaller than wild type. Above 1 µM, this difference was not observed (Fig. 8g).

Figure 8. Overexpression of PLC3 enhances drought tolerance.  
(a) PLC3 expression levels in wild-type and two homozygous PLC3 overexpression lines, PLC3-OE9 and PLC3-OE16 as measured by Q-PCR and based on the expression of the SAND reference gene. Values are means ± SD (n = 3) for one experiment. (b) Phenotype of wild type- and 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, PLC3 OE9 (left), 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. 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 IP₃ 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 PIP₂ (Fig. 6), which would be consistent with a loss of *PLC3* that would normally hydrolyze this lipid to produce IP₃. Unfortunately, the latter is very difficult to measure because seeds contain tiny amounts of IP₃ and huge amounts of IP₆, and are also extremely difficult to label with ³H-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 ³²P; see Methods). Seeds typically store high amounts of IP₆ during their development, where it is used as supply of phosphate (e.g. for DNA, ATP, membranes and sugars) and inositol (IPPs, PPIs,
Role for PLC3 in Arabidopsis

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 IP₄ 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
responsible 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 perusing 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_{6r} (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 \(^{32}\text{P}\)-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 IP3 and IP6 through IPK1 and IPK2. The IP6 can release Ca2+ 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. Ca2+ is a potential factor since it stimulates PLC activity in vitro (Munnik et al., 1998), but this would first require an influx of Ca2+ 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; ABI1: 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 PIP2 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 PIP2 and phosphorylation of DAG, which is readily picked-up by these 32P-labelling experiments. We also measured IPP levels with 3H-inositol labeling, but found no differences there between wt and PLC3-OE lines.

Besides guard-cell regulation, PA and PIP2 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, PIP2 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 IP6 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 PIP2 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 IP3 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). IP3 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 PIP2, then its competition with active PLCs might regulate PIP2’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 TUBLIα4 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 PLC3promHindIIIfw 5’- CCAAGCTTCAAGTCGCCGAACGAGACATC-3’ and PLC3promNheIrev 5’-CTGCTCTTCTTCTTACTTGTATG-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'-'TCCAGATTCTTCGTCAGATTGGA-3' (forward) and 5'-'TATAGGAAACCCTGATCGACAGC-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 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-β-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 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).
Role for PLC3 in Arabidopsis

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 Distefano 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).

32Pi-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 32PO4^3− (32P) (carrier free; Perkin-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 32P (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 CaCl2) 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 32P (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)] 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 12 h 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% phytagel) containing ³H-myo-inositol (80 µCi, Biotrend, ART-0261-5, Cologne, Germany) for 7 days. Seedlings were washed twice 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 1 ml/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: $[(\Sigma \text{cpms in peak}) / (\text{total IPP})] \times 100$.

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. Eigtheen plants from each genotype were used for measurement 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) 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 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>
</tr>
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<tbody>
<tr>
<td>PLC3_Foward</td>
<td>TGCTGAAGTTGTCATGCCG</td>
</tr>
<tr>
<td>PLC3_Reverse</td>
<td>GTCCACCCACATGAGGATCG</td>
</tr>
<tr>
<td>LBa*</td>
<td>TGGTCACGTAGGGCCATCG</td>
</tr>
<tr>
<td>TUBLINα4_Foward</td>
<td>CCAGCCACCAACAGTGTTTC</td>
</tr>
<tr>
<td>TUBLINα4_Reverse</td>
<td>CACAAGACGAGATTAGAGA</td>
</tr>
</tbody>
</table>

* LBa and PLC3_Foward 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).
Role for PLC3 in Arabidopsis

Supplemental Figure S2. Confocal analysis of PLC3pro:GUS-YFP 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 inositolphosphates (total IPs) 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 32P-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 32P-labeled for 3h and then treated with buffer or 100 µM ABA for 1 h. (e) Autoradiograph of TLC. (f-h) Quantification of PIP2, 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 ½MS 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.