Arabidopsis Phospholipase C3 is Involved in Lateral Root Initiation and ABA Responses in Seed Germination and Stomatal Closure


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Arabidopsis Phospholipase C3 is Involved in Lateral Root Initiation and ABA Responses in Seed Germination and Stomatal Closure


Phospholipase C (PLC) is well known for its role in animal signaling, where it generates the second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), by hydrolyzing the minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), upon receptor stimulation. In plants, PLC’s role is still unclear, especially because the primary targets of both second messengers are lacking, i.e. the ligand-gated Ca2+ channel and protein kinase C, and because PIP2 levels are extremely low. Nonetheless, the Arabidopsis genome encodes nine PLCs. We used a reverse-genetic approach to explore PLC’s function in Arabidopsis, and report here that PLC3 is required for proper root development, seed germination and stomatal opening. Two independent knock-down mutants, plc3-2 and plc3-3, were found to exhibit reduced lateral root densities by 10–20%. Mutant seeds germinated more slowly but were less sensitive to ABA to inhibit reduced lateral root densities by 10–20%. Mutant seeds germinated more slowly but were less sensitive to ABA to prevent germination. Guard cells of plc3 were also compromised in ABA-dependent stomatal closure. Promoter–β-glucuronidase (GUS) analyses confirmed PLC3 expression in guard cells and germinating seeds, and revealed that the majority is expressed in vascular tissue, most probably phloem companion cells, in roots, leaves and flowers.

Introduction

In animals, phospholipase C (PLC) plays a crucial role in cellular signaling. It is activated by hundreds of receptors, which causes the minor plasma membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP2) to be hydrolyzed into the second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). While IP3 diffuses into the cytosol where it triggers the release of Ca2+ from an intracellular Ca2+ store via a ligand-gated Ca2+ channel, DAG remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family. Subsequent changes in Ca2+ and phosphorylation status affect multiple protein targets and downstream cellular processes (Irvine 2006, Michell 2008, Balla 2013).

Keywords: ABA • Arabidopsis • Drought tolerance • Lateral root formation • Seed germination • Stomatal closure.

Abbreviations: DAG, diacylglycerol; GUS, β-glucuronidase; IP3, inositol 1,4,5-trisphosphate; IP6, inositol hexakisphosphate and phytate; IPK, inositol polyphosphate kinase; IPP, inositol polyphosphate; MS, Murashige and Skoog; MS, Murashige and Skoog; MS, Murashige and Skoog; PA, phosphatidic acid; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIPK, PIP kinase; PKC, protein kinase C; PLC, phospholipase C; PP2C, type 2C protein phosphatase; PP-IPP, pyrophosphorylated inositol polyphosphate; Q-PCR, quantitative real-time PCR; RFO, rafinose family oligosaccharide; RT–PCR, reverse transcription–PCR; SLAC1, slow anion channel 1; SnRK2, SNF1-Related kinase; TLC, thin-layer chromatography; WT, wild type; YFP, yellow fluorescent protein.
Much less is clear about the PLC signaling system in plants (Ischebeck et al. 2010, Munnik 2014, Heilmann 2016, Heilmann and Ischebeck 2016, Gerth et al. 2017). Initially, it was thought to be equivalent to the animal paradigm since most of the components driving the pathway were thought to be present (Munnik et al. 1998a, Stevenson et al. 2000, Meijer and Munnik 2003), especially when microinjected IP$_3$ was shown to release Ca$^{2+}$ from an intracellular store and to induce stomatal closure (Blatt et al. 1990, Gilroy et al. 1990, Allen and Sanders 1994, Hunt and Gray 2001). However, 20 years later, Brearley’s lab provided evidence that it was not IP$_3$, but its subsequent conversion into IP$_6$, that caused these effects (Lemtiri-Chlieh et al. 2000, Lemtiri-Chlieh et al. 2003). Similarly, not DAG but its phosphorylated product, phosphatidic acid (PA), has been emerging as the plant lipid second messenger (Munnik 2001, Laxalt and Munnik 2002, Testerink and Munnik 2005, Ariz et al. 2009, Testerink and Munnik 2011, McLaughlin and Testerink 2013, Pokorylo et al. 2014, Munnik 2014, Hou et al. 2016, Vermeer et al. 2017). Moreover, genome sequencing has meanwhile confirmed that flowering plants lack homologs of both the IP$_3$ receptor and PKC (Wheeler and Brownlee 2008, Munnik et al. 2006b, Munnik and Vermeer 2017). That PLC is important for plants, however, has come from several studies. Silencing of PLC1 in Arabidopsis and tobacco has indicated a role for PLC in ABA signaling in seed germination and stomatal closure, respectively (Sanchez and Chua 2001, Hunt et al. 2003). ABA has been shown to trigger IP$_6$ responses minutes after application in potato guard cell protoplasts and in duckweed turions (Lemtiri-Chlieh et al. 2000, Lemtiri-Chlieh et al. 2003, Flores and Smart 2000), but it is unknown whether this requires PLC. Interestingly, of the nine Arabidopsis PLC genes, six are induced upon ABA treatment (Hirayama et al. 1995, Tasma et al. 2008, Pokorylo et al. 2014).

PLC signaling has been linked to other plant abiotic stress responses, including salt, drought (mimicked by sorbitol, mannitol or polyethylene glycol) and heat stress (Liu et al. 2006a, 2006b, Munnik and Vermeer 2010, Zheng et al. 2012, Abd-El-Haleem et al. 2012, Gao et al. 2014, Munnik 2014, Pokorylo et al. 2014). Interestingly, these stresses have also been shown to trigger an increase in IP$_6$ levels (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). In some cases, IP$_3$ responses were reported but none of these studies addressed IP$_6$, or any of the other inositol polyphosphates (IPPs) that are emerging as signaling molecules, i.e. IP$_4$, IP$_5$, and the phosphorylated inositol phosphates (PP-IPPs), IP$_2$, and IP$_4$ (Takahashi et al. 2001, Zonia et al. 2002, Liu et al. 2006a, Liu et al. 2006b, Zheng et al. 2012, Gillaspy 2013, Laha et al. 2015, Laha et al. 2016). Decreases in IP$_6$ and IP$_3$ have also been reported (Cho et al. 1993, Pical et al. 1999, DeWald et al. 2001, Vermeer et al. 2009) and, theoretically, PLC could use IP$_4$P as a substrate. In fact, IP$_4$P and IP$_3$ are hydrolyzed equally well in vitro, but whether this occurs in vivo is still not clear (Munnik 2014). Overexpression of PLC has been shown to increase drought tolerance in maize, canola and tobacco (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011, Nokhrina et al. 2014). The molecular mechanism for this still remains unknown (Das et al. 2005, Georges et al. 2009).

PLC has also been implicated in plant–microbe interactions (Laxalt and Munnik 2002, Abd-El-Haleem and Joosten 2017), both symbiotic and pathogenic (van der Luit et al. 2000, Hartog et al. 2003, De Jong et al. 2004). For some of these interactions, nitric oxide signaling has been shown and is required (Laxalt et al. 2007, Raho et al. 2011, Lanteri et al. 2011). Genetic evidence for PLC’s role in disease resistance has been obtained for tomato (Vossen et al. 2010, Gonorazky et al. 2014, Gonorazky et al. 2016) and recently for Arabidopsis PLC2 (D’Ambrosio et al. 2017).

Apart from stress, PLC signaling has also been linked to pollen tube growth (Dowd et al. 2006, Helling et al. 2006, Ischebeck et al. 2010, Heilmann and Ischebeck 2016) and gametophyte development (Song et al. 2008, Li et al. 2015, Di Fino et al. 2017). In Physcomitrella, PLC1 has been shown to play a role in cytokinin and gravity responses (Repp et al. 2004).

Arabidopsis contains nine PLC genes (Tasma et al. 2009, Munnik 2014, Pokorylo et al. 2014). So far, no developmental disorders other than the gametophyte development defects mentioned above were reported for Arabidopsis mutants, presumably due to strong genetic redundancy. Ordering T-DNA insertion mutants of various PLC genes, we discovered that plc3 mutants exhibited a lateral root phenotype. From there, we investigated PLC3’s function further, and found genetic evidence for its involvement in ABA responses in seed germination and stomatal closure, and that overexpression of PLC3 leads to increased drought tolerance.

## Results

### Knock-down of PLC3 affects lateral root architecture density

To be able to investigate PLC3’s function, two independent homozygous T-DNA insertion mutants were isolated, i.e. plc3-2 (SALK_037453) and plc3-3 (SALK_054406), exhibiting T-DNA inserts in exon 3 and intron 3 in the X-domain, respectively (Fig. 1A). Genotyping was verified by reverse transcription–PCR (RT–PCR) (Fig. 1B) and PLC3 expression by real-time quantitative PCR (Q-PCR) (Fig. 1C), which showed that both insertion lines were knock-down mutants having lost approximately 90% of the PLC3 transcript.

Growing seedlings on agar plates revealed that plc3 mutants exhibited a small but significant difference in root system architecture compared with the wild type (WT), i.e. shorter primary roots (~5–10%), fewer lateral roots (~10–20%) and reduced lateral root densities (Fig. 1D, E).

### Expression of PLC3 during plant development

RT–PCR and Q-PCR analyses have shown that Arabidopsis PLC3 is expressed in all major plant tissues (Hunt et al. 2004, Tasma et al. 2008). To investigate this in more detail, PLC3 promoter–β-glucuronidase–yellow fluorescent protein (GUS–YFP) reporter lines were generated, using a 2.4 kb PLC3 promoter fragment (PLC3$_{2400}$GUS–YFP). Histochemical analyses...
revealed GUS activity at all stages of plant development, but expression was very much restricted to the vasculature, including root, shoot, cotyledons, leaves, different flower parts as well as in developing seeds (Fig. 2A–N). The trichome bases also revealed expression (Fig. 2J–K), but subsequent phenotypic analysis of WT and plc3 trichomes revealed no differences in terms of shape or density (not shown).

Strikingly, the vascular PLC3 expression in the main root was not homogenous. On the distal side of the maturation zone, the expression was often found to be discontinuous, i.e. ‘segmented’ (Fig. 2C, D; Supplementary Fig. S1). In the apical maturation zone, it was continuous until the transition zone (Fig. 2F), and there was no PLC3 expression at the root tip (Fig. 2I). Interestingly, lateral roots always emerged from a segment, but not every segment led to a lateral root (Fig. 2C, D).

To investigate the correlation between PLC3 expression and the vasculature and lateral root formation in more detail, GUS expression was analyzed in seedlings grown on agar plates positioned at a 45° angle, which forces lateral roots to emerge specifically at the curved sites (Ditengou et al. 2008). Under these conditions, fewer segments were found, and those present were always located at a curved position (Supplementary Fig. S2; red and blue circles) from most of which a lateral root emerged (Supplementary Fig. S2; blue circles). Moreover, every lateral root came from a segment (Supplementary Fig. S2). Interestingly, a similar discontinuous, segmented pattern was found for tertiary root formation (Fig. 2E).

To obtain more detailed information about PLC3 expression, optical cross- and longitudinal sections were made by confocal
microscopy to visualize the YFP expression. These analyses suggest that PLC3 is specifically expressed in the phloem (Supplementary Fig. S3), which correlates with data on the eFP browser, showing predominant expression in the phloem companion cells (Winter et al. 2007).

Together, these results confirm that PLC3 is expressed throughout the plant (Hunt et al. 2004, Tasma et al. 2008), and show that this expression is predominantly restricted to the vasculature, particularly in the phloem. The data also show that the segmented PLC3 expression pattern positively correlates with lateral root formation.

PLC3 is involved in seed germination

When growing seedlings on agar plates, we observed that plc3 mutants germinated slightly more slowly than WT seeds (Fig. 3A). Seeds were imbibed on half-strength Murashige and Skoog (1/2 MS) plates in the dark at 4°C for 48 h, after which they were transferred to light. After 24 h in the light, plc3-2 and plc3-3 mutants were found to germinate 54% and 60% less than the WT, respectively, and after 28 h this difference was 17% and 34% (Fig. 3A). In the end, all seeds germinated. Subsequent analysis of PLC3

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Fig. 2 Expression pattern of PLC3. cDNA for GUS was cloned in-frame in the PLC3 promoter and introduced into Arabidopsis. GUS was expressed throughout Arabidopsis plants, predominantly in the vasculature of 2-day-old (A) and 10-day-old seedlings, including shoot and root (B–I). GUS activity was also typically detected in vascular tissue of mature plants (J), the trichome base (indicated by arrows) (J, K), hydathodes (J), silique (L), developing seed chalaza (M) and different parts of the flower (N), including the style, filament, receptacle and pedicel (indicated by arrows). Experiments were repeated at least twice with similar results.
expression in germinating seeds revealed GUS activity in embryo cotyledons and shoot apical meristems during testa rupture and radical emergence (Fig. 3B), confirming a role for PLC3 in seed germination.

Since the delayed seed germination in the plc3 mutants could have consequences for the observed difference in primary root growth, we analyzed the relative root growth of mutants and the WT at various days after germination. From these analyses, it became clear that the primary roots of plc3 mutants grew with similar speed to the WT. The differences in lateral root densities remained, however. (Supplementary Fig. S4).

Loss of PLC3 function results in decreased sensitivity to ABA-inhibited seed germination

To test whether the difference in seed germination was due to ABA sensitivity, germination experiments were repeated on 1/2 MS plates ± ABA. Without ABA, plc3 mutants germinated more slowly than the WT, as was described above (Fig. 3A, C). Addition of ABA inhibited seed germination; however, in this case, the plc3 mutants were clearly found to be less sensitive to ABA inhibition (Fig. 3C). For example, after 40 h of 1 µM ABA, 12.5% of plc3-2 and 10.5% of plc3-3 seeds had germinated, but only 2.5% of the WT seeds had germinated. These differences remained or even increased over time (Fig. 3C, left panel). Using 2 µM ABA, seed germination was more strongly inhibited, but again revealed higher germination rates for both plc3 mutants than for the WT (Fig. 3C, right panel).

Loss of PLC3 function results in decreased sensitivity to ABA-induced stomatal closure

Since ABA also plays a key role in guard cell signaling (Munemasa et al. 2015), and since antisense PLC expression had been shown to reduce the stomatal closure response to ABA in Nicotiana tabacum (Hunt et al. 2003), we investigated the potential involvement of PLC3 in Arabidopsis guard cell ABA responses.

First, we determined whether PLC3 was expressed in guard cells since this was not obvious from previous histochemical analyses (Fig. 2), or from the eFP browser (Winter et al. 2007). Performing histochemical GUS analyses on epidermal leaf peels clearly indicated PLC3 expression in guard cells (Fig. 4A). Next, the stomatal closure response was tested in leaf peels of the WT and plc3 mutants after treatment 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 the WT and plc3 mutants were found (Fig. 4B). However, with increasing concentrations of ABA, both plc3-2 and plc3-3 clearly exhibited reduced stomatal closure responses (Fig. 4B). These results indicate that PLC3 plays a role in ABA sensitivity, both in seed germination and in stomatal closure. We also tested the effect of ABA on the root architecture of WT and plc3 seedlings, but found no significant difference (Supplementary Fig. S5).

ABA triggers PIP2 synthesis in germinating seeds, seedlings and guard cells

Next, we analyzed the phospholipid levels in response to ABA in different tissues and genotypes using in vivo 32P labeling. First, germinating seeds were analyzed. As shown in Fig. 5, the 32P-labeled PIP2 levels were significantly higher in plc3 mutants than in WT seeds, i.e. 23% and 22% for plc3-2 and plc3-3,
Values are means ± the SE of at least three independent experiments (n ≥ 100).

respectively (Figs. 5A, B). No significant differences in PIP or PA levels were found, or in the structural phospholipids (Fig. 5A, B; data not shown). Upon ABA treatment, a significant increase in PIP$_2$ (27%) in WT seeds was found, which was absent from the plc3 mutants (Fig. 5B). No significant changes in any of the other phospholipids were found for either genotype (Fig. 5A, B).

To check the lipid signaling responses in guard cells, epidermal leaf peels from WT and mutant leaves were used. In these peels, most of the mesophyll cells are dead, while guard cells remain alive, which was checked by fluorescein diacetate/propi- dium iodide staining (not shown). In this case, the $^{32}$P pre-labeling was restricted to only 3 h as longer incubation times negatively affected the viability of the guard cells. Similar to what we observed in germinating seeds, plc3 mutants contained slightly higher PIP$_2$ levels in guard cells, while the levels of PIP, PA and the structural phospholipids were the same as in the WT (Fig. 5B; not shown). Upon ABA treatment (15 min), a significant increase of PIP$_2$ was found for the WT, which was not seen in the plc3 mutants (Fig. 6). No significant differences for PIP, PA or any of the structural phospholipids were found (P < 0.05).

Seedlings also revealed an increase in PIP$_2$ upon ABA treatment, but this only became apparent after 30–60 min (Supplementary Fig. S6A–D). However, in this case, no differences between WT and plc3 seedlings were observed (Supplementary Fig. S6E, F). No effect on the other phospholipids or differences between genotypes were found, (Supplementary Fig. S6B–D, G, H).

Overexpression of PLC3 increases drought tolerance

To test whether the reduced ABA responsiveness of guard cells in leaf peels of plc3 mutants was detectable in planta, the drought tolerance of WT and plc3 mutant plants was investigated. Well-watered plants were grown on soil for 4 weeks, after which watering was stopped and subsequent survival rates were scored. However, no significant differences between the
mutants were 32Pi labeled for 3 h and then treated in buffer (i.e. hydrolysis of PIP2) was responsible for the phenotype re-cycling of the chosen PLC or whether the canonical PLC function has been shown to increase their drought tolerance (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011). Whether this was formed. Data shown are the means ± SD (n = 3) from one representative experiment. Data were analyzed by two-way ANOVA. Statistically significant differences between genotypes are indicated by letters (P < 0.05, Dunn’s method).

WT and plc3 mutants could be observed in three independent experiments (Supplementary Fig. S7).

In maize, canola and tobacco, overexpression of PLC has been shown to increase their drought tolerance (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011). Whether this was caused by a specific, maybe as yet unknown, substrate specificity of the chosen PLC or whether the canonical PLC function (i.e. hydrolysis of PIP2) was responsible for the phenotype remains unknown. To test whether PLC3 overexpression could increase the drought tolerance of Arabidopsis, we generated transgenic plants ectopically expressing PLC3 using the UBQ10 promoter. Independent transgenic plants were selected from T0 to T1 generation plants, and multiple homozygous lines were obtained. Two homozygous lines, PLC3-OE9 and PLC3-OE16 were selected for further analyses, which overexpressed PLC3 by approximately 48-fold and approximately 20-fold, respectively (Fig. 7A). When growing these overexpression lines next to WT plants on soil or agar plates, no phenotype was observed. However, when their drought tolerance was analyzed on 4-week-old soil-grown plants, the PLC3-OE lines were found to perform better than the WT (Fig. 7B, C). Upon drought, the fresh weight of the WT shoot decreased by approximately 21%, which occurred less in PLC3-OE9 or PLC3-OE16 (17% and 12%, respectively, Fig. 7D). The dry weight was also higher in PLC3-OE lines and this was independent of the drought treatment (Fig. 7E). When the loss of water in detached rosettes of 4-week-old WT and PLC3-OE plants was compared, the latter lost significantly less water (Fig. 7F), which was also reflected in increased values of relative water content (RWC %) of PLC3-OE rosettes under drought conditions (Supplementary Fig. S8).

Guard cell responsiveness in leaf peels revealed that the stomatal aperture of PLC3-OE plants was strongly reduced (~30%; Fig. 7G). Upon 0.1 μM ABA treatment, stomatal closure was initiated in each genotype, but the aperture of the PLC3-OE lines was still significantly smaller than that of the WTs. Above 1 μM ABA, these differences were lost (Fig. 7G).

**PLC3-overexpressing plants respond to osmotic stress with stronger PIP2 responses**

To determine whether overexpression of PLC3 caused any changes in the level of the signaling lipids, 32P-labeled seedlings were treated or not with sorbitol to mimic drought stress. As shown in Fig. 8, no major differences between WT and PLC3-OE lines were found under control conditions. However, upon sorbitol treatment, a much stronger PIP2 response was observed in the PLC3-OE lines. In the WT, the PIP2 levels increased by about 300%, whereas the OE lines revealed an approximately 600% increase (Fig. 8B). The osmotic stress-induced decrease in PIP and increase in PA appeared to be similar between mutant and WT seedlings (Fig. 8B). These results suggest that PLC3-OE lines exhibit a more potent PIP2 response under osmotic stress.

**Discussion**

In this study, new roles for PLC in stress signaling and development have been identified. Using Arabidopsis loss-of-function mutants, we provide genetic evidence that AtPLC3 is involved in seed germination, root development, stomatal movement and ABA signaling. The phenotypes are subtle, but were consistent in two independent mutants, plc3-2 and plc3-3, and in different seed batches over 10 years. Redundancy of any of the other eight PLC genes, of which several exhibit overlapping expression profiles with PLC3 (Tasma et al. 2008), may be responsible for this. Overexpression of PLC3 had no effect on the root architecture or seed germination, but did increase the plant’s tolerance to drought, as has been observed when overexpressing PLC in maize, tobacco and canola (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011, Nokhrina et al. 2014). That overexpression of a single gene can increase the plant’s tolerance to drought is interesting, and may have
agricultural applications. Currently, we are investigating the underlying molecular mechanism of this phenotype and whether other Arabidopsis PLC genes can achieve this too, or whether it is PLC3 specific.

While the above findings underline the importance of PLC in signaling plant stress and development, we still know very little about how this is achieved at the molecular level. Theoretically, there are several possibilities. Activation of PLC would produce DAG and IP3 or IP2 (depending on whether PIP or PIP2 is used as substrate), and while plants lack the classical targets of the mammalian paradigm (i.e. the IP3 receptor, PKC), it is likely that the phosphorylated products, i.e. PA and higher IPPs (including PP-IPPs) fulfill the second messenger function in various biological processes have been linked to these molecules, and several protein targets involved in signal transduction and metabolism have also been identified (see below). In guard cells, for example, ABA has been shown to induce the formation of IP6 and to release Ca2+ from an intracellular store (Lemtiri-Chlieh et al. 2000, Lemtiri-Chlieh et al. 2003), so the plant PLC system could potentially achieve this. We found no changes in the basal levels of PPIs, PA or IPP in the plc3 mutants, which is likely to be due to genetic and/or biochemical redundancy (Supplementary Fig. S6). Upon ABA treatment, however, plc3 mutants revealed enhanced PIP2 responses, probably the result of reduced PIP2 hydrolysis in WT cells expressing PLC3. Promoter–GUS analyses revealed that this may be very
local, i.e. in guard cells and vascular tissue, in particular in the phloem companion cells (Fig. 2). This means that, independent of PLC redundancy, its very local expression may cause the differences in PLC’s substrate and/or product levels between plc3 and the WT to be difficult to identify.

It is possible that PLC hydrolyzes PIP rather than PIP2 (Munnik 2014). Concentrations of the latter are extremely low in the plasma membranes of plants, whereas PIP4P levels are comparable with those found in mammalian cells, or even more abundant (van Leeuwen et al. 2007, Vermeer et al. 2009, Laha et al. 2015, Laha et al. 2016). However, plants also use IPPs to store high amounts of IP6 during their development as a potential source of phosphate (e.g. for DNA, ATP, membranes and PP-IIPs are also emerging as signaling molecules in plants (York 2006, Michell 2008, Burton et al. 2009, Desai et al. 2015). In order to make IP6 out of IP2 or IP3, the same two inositol polyphosphate kinases (IPKs) are involved. IPK2 is an inositol multiphosphate kinase that can phosphorylate the 3-, 5- and 6-position of the inositol ring to produce IP6, while IPK1 specifically phosphorylates IP5 at the 2-position to produce IP6. VIH2 is a recently discovered IPK that is responsible for the production of the PP-IIPs, i.e. IP6. Like in animals and fungi, IPPs and PP-IIPs are also emerging as signaling molecules in plants (York 2006, Michell 2008, Burton et al. 2009, Desai et al. 2014, Laha et al. 2015). However, plants also use IPPs to store phosphate as phytate and, even though this probably occurs in distinct cells, tissues and even organelles, it makes studying their levels extremely complex (Stevenson-Paulik et al. 2005, Munnik and Vermeer 2010, Desai et al. 2014, Kuo et al. 2014, Laha et al. 2015, Laha et al. 2016).

Another function of PLC could be to attenuate PIP2 signaling. Even though the concentration of this lipid in plants is extremely low under control conditions, during certain aspects of plant growth and development, and upon certain stresses, PIP2 is readily produced and suggested to fulfill a second messenger function itself (Ischebeck et al. 2010, Gillaspy 2013, Rodriguez-Villalon et al. 2015, Heilmann 2015, Heilmann 2016, Gerth et al. 2017, Zarza et al. 2017). Potential targets include proteins involved in ion transport (e.g. K+ channels), membrane trafficking (endo/exocytosis via clathrin and Exo70) and the cytoskeleton (e.g. small G-protein, Rop) (Ischebeck et al. 2010, Gillaspy 2013, Munnik 2014, Heilmann 2016). Similarly, PLC could function as an attenuator of PI4P signaling (Balla 2013, Munnik 2014). As far as we know, PLCs are not able to hydrolyze D3-phosphorylated inositol lipids [i.e. PI3P and PI(3,5)P2] or PI5P (Balla 2013, Munnik 2014). Whether the newly linked PLC3 functions observed here reflect PLC’s role as second messenger producer or attenuator (or both), remains to be established. Below, a broader perspective of our results is given and some potential molecular mechanisms are discussed.

Role for PLC3 in seed germination

The delayed germination phenotype of plc3 mutants (Fig. 3A) together with the promoter–GUS activity in germinating seeds (Fig. 3B) indicate a role for PLC3 in seed germination. Since ABA is known to inhibit seed germination (Nambara et al. 2010, Nakashima and Yamaguchi-Shinozaki 2013), we investigated whether the delayed germination of plc3 seeds was caused by hypersensitivity to ABA. Surprisingly, plc3 mutants were found to be less sensitive to ABA (Fig. 3C). The latter results are in agreement with Sanchez and Chua (2001), who found that the ABA sensitivity of seed germination and downstream gene expression was lost when silencing PLC1 in Arabidopsis. Interestingly, we also found that guard cells of plc3 mutants were less sensitive to ABA, so this could point to a more general role for PLC3 in ABA signaling (see below). The above results indicate, at least, that the delayed germination rate in plc3 mutants is not linked to ABA hypersensitivity.

PIP2 levels were significantly higher in germinating plc3 seeds (Fig. 5), which would be consistent with a reduction of PLC3 activity 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 they are extremely difficult to label with [3H]inositol (Stevenson-Paulik et al. 2005). Seeds hardly take up the label, and the high endogenous phytate concentration may also be the reason why young seedlings require relatively long labeling times (i.e. 4–11 d) to incorporate some label. Seeds typically store high amounts of IP6 during their development as a source of phosphate (e.g. for DNA, ATP, membranes and...
sugars) and inositol (for IPPs, PPIs and cell wall sugars), which is required for germination and the growth and development of the seedling (Munnik and Nielsen 2011). This ‘storage’ IP₆ is difficult—if not impossible—to distinguish from the IP₆ that has a ‘signaling’ function, even though they may be differentially localized in cells and tissues (Munnik and Vermeer 2010, Gillaspy 2011, Gillaspy 2013). During seed germination, IP₆ is rapidly broken down to IP₃ (Luo et al. 2012), which could be an alternative explanation for what has been assumed to be PLC-generated IP₆ (Munnik and Vermeer 2010). Raffinose family oligosaccharides (RFOs) are another group of molecules that are linked to inositol. RFOs serve as transport sugars in phloem, as storage sugars in various tissues and as desiccation protectants in seeds (van den Ende 2013, Sengupta et al. 2015). In Arabidopsis, RFOs are required for the rapid germination of seeds in the dark (Gangl and Tenhaken 2016). RFOs are sucrose derivatives to which a galactosyl unit is attached via galactitol. The latter is produced via UDP-galactose and myo-inositol by the enzyme galactitol synthase. To make RFOs, free myo-inositol is required and this is predominantly formed through cyclization of glycolytic glucose 6-phosphate into inositol-3-phosphate, and subsequent dephosphorylation by inositol monophosphatase. Theoretically, however, inositol could be produced via dephosphorylation of PLC-generated IPPs (Munnik and Vermeer 2010). We therefore analyzed the soluble carbohydrate composition in seeds but found no significant differences between the WT and plc3 mutants (Supplementary Fig. S9A). Of course, changes could be local and small, so it is possible differences remain unobserved.

Promoter–GUS analyses of developing seeds revealed PLC3 expression at the chalaza (Fig. 2M), the non-micropylar end of the seed, which is probably the chalaza endosperm and/or seed coat. 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 transport. Alternatively, PLC could be involved in the production of IP₆ for 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 a distinct cation (Mg, Mn, Zn, K and Ca) composition per compartment (Otegui et al. 2002). As such, this could be another mechanism by which loss of PLC3 redundantly affects seed germination and root development.

**Role for PLC3 in lateral root formation and auxin signaling?**

Loss-of-function PLC3 mutants displayed reduced lateral root densities (Fig. 1). This was due to fewer initiation sites, not development (data not shown). Promoter–GUS analyses revealed a typical, segmented PLC3 expression pattern at the emerging site of the lateral root, whereby lateral roots always emerged from a segment but not every segment resulted in a lateral root. Normally, our agar plates are grown vertically in an angle of 80°, which typically gave two or three segments per lateral root. By tilting the agar plate more horizontally (45°), roots start to undulate more and a lateral root emerges at every bend (Ditengou et al. 2008). Using the latter set-up drastically reduced the number of segments and revealed almost a 1 : 1 correlation between lateral root emergence and PLC3 expression. These results indicate that PLC3 is required before lateral root initiation, and that in the 80° set-up the primary root is less sure as to where and when it will produce a lateral root than in the 45° set-up where the decision is forced at the bending sites. That the phenotype in plc3 mutants is quite mild may indicate redundancy of other PLC genes. Using the eFP browser, we found three other PLC genes, i.e. PLC2, PLC5 and PLC7, also being expressed in the phloem and/or companion cells (not shown; Winter et al. 2007). Auxin plays an important role in lateral root formation, and the mechanism by which auxin is perceived is well characterized (Péret et al. 2009a, Péret et al. 2009b, Benkó and Bielach 2010). The auxin receptor TIR1 is an F-box protein in complex with SCF (ubiquitin protein ligase), which promotes ubiquitin-dependent proteolysis of the transcriptional repressor Aux/IAAs. Interestingly, IP₆ was found in the crystal structure of TIR1, where it is suggested to play a role in auxin binding and TIR1 function (Tan et al. 2007, Munnik, 2014). Where this IP₆ comes from is unknown, but potentially this could be generated via PLC3 at the ‘segments’. As such, less PLC3-generated IP₆ in the plc3 mutants may explain the reduced auxin responsiveness (see Fig. 9A). That the effect is not very strong may well be due to redundant PLC genes.

In seedlings, no difference in PPI or PA levels between WT and plc3 seedlings was found (Supplementary Figs. S4, S11A, B). Since PLC3 is only expressed in a limited number of cells (mainly phloem companion cells), it is possible that potential differences are in fact diluted out in whole seedlings. We also did not observe major differences in the IPPs (Supplementary Fig. S10C–F), though slightly lower levels of IP₇ or IP₈ were found in plc3 mutants, depending on the seedling age and labeling procedure (Supplementary Fig. 11D, F). PP-IPPs are emerging as novel signaling molecules (Desai et al. 2014, Lahaye et al. 2015, Lahaye et al. 2016) for which there is already plenty of evidence in yeast and animal cells (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, other IPPs could be locally generated via this PLC, for example in guard cells, where IP₆ may be responsible for the release of intracellular Ca²⁺ (Lemtiri-Chlieh et al. 2000, Lemtiri-Chlieh et al. 2003). For auxin, this type of Ca²⁺ signaling could also be relevant (Zhang et al. 2011). The main bottleneck still after the first discoveries >25 years ago (Blatt et al. 1990, Gilroy et al. 1990, Allen and Sanders 1994) is the identification of a genuine IP₆ (or other IPP-) gated channel (Munnik 2014).

Several low phytic acid (LPA) mutants have been identified with reduced IP₆ levels in Arabidopsis. These include besides IPK1 and IPK2, inositol tris/tetrakisphosphate kinases (ITPKs), PI syntases (PI5s) and AtMRP5 (Murphy et al. 2008, Nagy et al. 2009, Gonzalez et al. 2010, Kim and Tai 2011, Desai et al. 2014,
Kuo et al. 2014). The latter is not involved in IP₆ synthesis but in IP₆ transport, and shown to affect guard cell signaling, phytate storage and root architecture (Gaedeke et al. 2001, Nagy et al. 2009).

Since RFOs are important for carbohydrate transport and storage, they could be involved in loading sucrose to sink organs (van den Ende, 2013, Sengupta et al. 2015, Gangl and Tenhaken 2016) and hence affect lateral root density as sugars are stored in lateral roots. Since sugar transport occurs through the phloem where PLC3 is specifically expressed, we analyzed the sugar composition of the phloem sap of the WT and plc3 mutants. Interestingly, increased amounts of sucrose were found in the plc3 mutants (Supplementary Fig. S9B). If sucrose is not properly transported to, or into, lateral roots via a tentative PLC-dependent RFO pathway, then sucrose levels could indeed be higher in the phloem, and theoretically affect root...
growth and lateral root formation. It will be worth following up on this hypothesis using other PLC loss-of-function mutants that are also expressed in the phloem.

**Role for PLC3 in stomatal closure and ABA signaling**

Both plc3 mutants revealed reduced ABA responses, i.e. inhibition of seed germination and induction of stomatal closure (Figs. 3C, 4B). These results confirm earlier observations when PLC was silenced in tobacco (Hunt et al. 2003, Mills et al. 2004). We found that ABA triggered significant increases in PIP2 in three different tissues, i.e. germinating seeds, guard cell-enriched leaf peels and seedlings, and this response was reduced in plc3 mutants or even lost in the germinating seeds and leaf peels, but not in the seedlings (Figs. 4, 6; Supplementary Fig. S4). ABA can activate PLC3, causing an increase in PIP2 hydrolysis, but also in its subsequent replenishment by PIP kinase (PIP5K). Due to the nature of our 32P-labeling experiments, synthesis of [32P]PIP2 may dominate the breakdown, and hence be witnessed as an increase in PIP2 (Munnik et al. 1998a, Munnik et al. 1998b).

Earlier, PIP2 was implicated in stomatal opening (Lee et al. 2007). Exogenously administered PIP2, induced swelling of guard cell protoplasts and triggered stomatal opening. Moreover, a mutant of Arabidopsis PIP5K4 exhibited a reduced stomatal opening phenotype, which could be rescued by complementation with the full-length PIP5K4 or exogenous PIP2 application (Lee et al. 2007). Electrophysiological experiments suggest that PIP2 inhibits anion channel activity, probably the slow anion channel 1 (SLAC1) (Lee et al. 2007), as well as K+ efflux (Lee et al. 2007, Ma et al. 2009). A potential model summarizing how PLC, PIP5K and PIP2 could regulate stomatal opening is shown in Fig. 9B. In the light, the H+-ATPase pump is active, causing hyperpolarization of the plasma membrane and 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, causing stomata to open. ABA-INSENSITIVE 1 (ABI1), a type 2C protein phosphatase (PP2C), inhibits SNF1-Related kinase 2 (SnRK2, i.e. OST1), preventing it from activating SLAC1 (Munemasa et al. 2015, Assmann and Jegla 2016). The PIP5K4-generated PIP2 inhibits SLAC1 (Lee et al. 2007) and K+ efflux (Ma et al. 2009), co-facilitating the low water potential. Upon ABA treatment, the PYR/PYL receptor dimer dissociates and forms PYR–ABA or PYL–ABA complexes that bind PP2C so that it can then no longer inhibit SnRK2/OST1. As a consequence, SnRK2/OST1 autophosphorylates itself and activates SLAC1, which results in a decrease of intracellular Cl− (Munemasa et al. 2015, Assmann and Jegla 2016). In our model, ABA also activates PLC, which causes PIP2 to be hydrolyzed, thereby releasing the inhibition of SLAC1 and K+ efflux, and allowing IPPs to be produced. IP3 releases Ca2+ from internal stores (Lemtiri-Chlieh et al. 2000, Lembrit-Chlieh et al. 2003, Munnik, 2014), which in turn inhibits the K+ influx channel (Lemtiri-Chlieh et al. 2000) and co-activates SLAC1 (Siegel et al. 2009). Together, these activities drive the net efflux of K+ and Cl−, causing water to leave the guard cells and stomatal pores to close. We propose that PLC3 is one of the PLCs involved in this process since the response is significantly reduced in plc3 mutants. How PLC is activated by ABA remains elusive.

**Overexpression of PLC3 enhances drought tolerance**

Plants use different strategies to cope with drought stress (Mickelbart et al. 2015, Zhu 2016), and lipid signaling has been implicated as one of the factors in various plant systems (Munnik and Meijer 2001, Meijer and Munnik 2003, Munnik and Vermeer 2010, Hou et al. 2016). Moreover, overexpression of a PLC in maize, tobacco and canola has been shown to improve drought tolerance (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011). We showed here that overexpression of PLC3 using the UB10 promoter improves drought tolerance in Arabidopsis. Under non-stressed conditions, PLC3-OE plants looked like WT plants but upon drought stress they clearly performed better (Fig. 7B, C). The molecular mechanism behind this may well reflect what is discussed above (Fig. 9B), but could also involve other factors, e.g. compatible solutes, gene expression, etc.

In an attempt to mimic drought in 32P-pre-labeled seedlings using sorbitol, increased PIP2 and PA levels were found, and responses were stronger in the PLC3-OE lines (Fig. 8). Again, this may reflect the increased turnover of PIP2 and phosphor-ylation of DAG that is readily picked up by our method, although the increase in PA could also result from PLD activation (Munnik and Vermeer 2010, Hou et al. 2016).

Since the UBQ10 promoter is constitutively expressed, the accumulation of PA and PIP2, is likely to occur in numerous cells and tissues, which is totally different from the local, endogenous PLC3 expression in the vasculature and guard cells. Both lipids have been implicated as second messengers, playing roles in important cellular events, including regulation of the cytoskeleton, vesicular trafficking and ion transport, and probably play a role in the plant’s response to water stress (Munnik and Vermeer 2010, Testerink and Munnik 2011, Heilmann and Ischenbeck 2016, Heilmann 2016, Gerth et al. 2017, Rodriguez-Villalon et al. 2015, Gujas et al. 2017). Further unraveling of the molecular mechanisms requires identification and characterization of some main targets of these lipid second messengers. For PA, this has already started (Testerink et al. 2004, Arisz et al. 2009, McLoughlin et al. 2013, Testerink and McLoughlin 2013, Julkowska, et al. 2015), but, for PIP2, this is still an untouched area.

For many years, the PLC/IP3/Ca2+ system has been claimed to regulate the gravitropism response (Perera et al. 1999, Stevenson et al. 2000, Perera et al. 2006, Boss et al. 2010). We tested this in our plc3 mutants by rotating the agar plate by 90° and following the root growth direction (Supplementary Fig. S11A, B), but we found no significant differences in bending between the WT and plc3 mutants (Supplementary Fig. S11C). This could be due to PLC redundancy. Most evidence for PLC/IP3/Ca2+ enrollment in gravitropism is based on IP3 measurements using an unreliable IP3 displacement kit (Munnik 2014). Since auxin is an important factor regulating gravitropism, an alternative hypothesis is that it reflects an IP3--
Materials and Methods

Plant material

Arabidopsis thaliana (Columbia-0, Col-0) T-DNA insertion mutants plc-2 (SALK_037453) and plc-3 (SALK_054406) were obtained from the SALK collection (signal.salk.edu). Homozygous plants were identified by PCR in the T2 generation using gene-specific primers in combination with the left border primer, Lba (Supplementary Table S1).

RNA extraction and RT–PCR

Expression in the T2-DNA insertion mutants was confirmed by RT–PCR. Total RNA was extracted with Trizol reagent (Invitrogen). RNA (5 μg) was converted to cDNA using oligo(dT)12 primers, dNTPs and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. PLC3 and TiBLIN7 were PCR amplified for 40 or 30 cycles, respectively, with gene-specific primers (Supplementary Table S1).

Cloning and plant transformation

To generate the reporter construct, PLC3pro::GUS-YFP, a 2,437 bp PLC3 promoter region was amplified from genomic DNA using PLC3prom HindIIIw 5′-CCCAAGCTTCAAGTCGCCGAACGAGACATC-3′ and PLC3prom NheIrev 5′-CTGCTCTTCTTCTTCTTACTTGTTAG-3′ and cloned into HindIII and NheI sites of pGreen0179 using NotI.

For the PLC3 overexpressor, the MultiSite Gateway Three-Fragment Vector Construction Kit (www.lifetechnologies.com) was used to generate UBQ10pro-PLC3 by cloning PLC3 cDNA into the pGreen0125 expression vector according to the supplier’s protocol (www.thermofisher.com). Constructs were transformed into Agrobacterium tumefaciens strain GV3101, which was used to transform WT A. thaliana Col-0 by floral dip (Clough and Bent 1998). Homozygous lines were selected in the T1 generation and used for further experiments.

Q-RT-PCR

The primer pairs used for confirmation of the PLC3 (At4g38530) expression level were: 5′-TCCGATTCTTCTGCAAGTATTGGGA-3′ (forward) and 5′-TATA GGAATCCTAGTGGACAGAC-3′ (reverse). For cDNA synthesis, 1 μg of total RNA from 10-day-old seedlings was used. Q-PCR analyses was performed with an ABI 7500 Real-Time PCR System (Applied Biosystem). The relative gene expression was determined by the comparative threshold cycle value. Transcript levels were normalized by the level of TUBULIN expression was determined by the comparative threshold cycle value.

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 PLC3prom::GUS-YFP were grown for the indicated times and specific tissues were incubated in X-Gluc reaction solution containing 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) in 50 mM phosphate buffer (pH 7.0) and 0.1% Triton X-100. Materials were incubated overnight at 37 °C. 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 PLC3prom::GUS-YFP seedlings were grown for 5 d and then transferred to object slides containing a fixed cover slide that was separated by a spacer of approximately 0.32 mm (Munnik and Zarzsa 2013). This allowed seedlings to grow in liquid medium (1/2 MS with 1% sucrose, pH 5.8) for 1–2 d so that they could be used directly for microscopy without damage to the root hairs. Microscopy was performed using a Zeiss LSM 510 CLSM (confocal laser scanning microscope) (Carl-Zeiss), implemented on an inverted microscope (Axiovert 100, Carl-Zeiss). For YFP imaging, confocal configurations were used as described before (Vermeer et al. 2006).

Seed germination

Mature seeds were harvested and stored at room temperature. Seeds were surface sterilized with chlorine gas in a desiccator by using 20 ml of bleach (5% NaClO) and 1 ml of 37% HCl for 3 h, then sown on square Petri dishes containing 30 ml of 1/2 MS medium, 0.5% sucrose, pH 5.8, and 1.2% Daishin agar ± ABA (0, 1 or 2 μM), and kept in the dark for 2 d at 4 °C, after which the plates were transferred to long-day conditions (i.e. 22 °C, 16 h of light and 8 h of darkness). Germination was scored as radical emergence at the indicated time points using a binocular microscope (Leica MZFLIII).

Root growth

Seeds were sterilized and stratified as described above. Plates were transferred to long-day conditions and placed vertically under an angle of 80°. Four-day-old seedlings with comparable size were then transferred to a 1/2 MS agar plate. Plates were imaged 8 d later (12 d after germination) using an Epson Perfection V700 scanner. Primary root length and lateral root number for each genotype were quantified using ImageJ analysis software (NIH). Lateral root density was expressed as the lateral number per primary root length (LR number/PR length). For gravitropic responses, seedlings were grown on 1/2 MS plates with 0.5% sucrose for 4 d. Plates were then rotated 90° and scanned 2 d later. Bending was expressed as curvature angle, which was quantified by ImageJ analysis software (NIH) (Perera et al. 2006).

Stomatal aperture

Stomatal apertures were measured according to Distefano et al. (2012) with some minor changes. Treatments were performed on epidermal leaf strips excised from the abaxial side of fully expanded Arabidopsis leaves from 3-week-old plants grown at 22 °C for 16 h with light and 8 h of darkness. Strips were floated onto ‘opening buffer’ containing 5 mM MES-KOH, pH 6.1, and 50 mM KCl for 3 h. Strips were maintained in the same opening buffer and exposed to different ABA concentrations. After 90 min, stomata were digitzed using a Nikon DS-Fi 1 camera coupled to a Nikon Eclipse Ti microscope. The stomatal aperture width was measured using ImageJ (NIH).

32P-phospholipid labeling, extraction and analysis

For the determination of PPI and PA levels, different tissues were 32P labeled. Germinating seeds. Seeds were sterilized and stratified on 1/2 MS with 0.5% sucrose (pH 5.8) and germinated under long-day conditions for around 20 h when the testa ruptured. Germinating seeds were then transferred to 200 μl of labeling buffer (2.5 mM MES, pH 5.8, 1 mM KCl) containing 5–10 μCi of 32PO4⁻ (100 Ci/mmol) (carrier free, Perkin-Elmer) in 2 ml of Eppendorf SafeLock microcentrifuge tubes for 24 h. Samples were treated with 200 μl of buffer ± 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 darkness were stripped and immediately floated on 100 μl of opening buffer (10 mM MES, pH 6.1, and 50 mM KCl) containing 32P (5–10 μCi) in a 48-well cell culture plate (Greiner bio-one) for 3 h. Samples were treated with 400 μl of buffer (10 mM MES-KOH, pH 6.1, 2.5 μM CaCl2) supplemented or not with ABA for the times and concentrations indicated.

Seedlings. Five-day-old seedlings were transferred to 200 μl of 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 (~16 h) or for 3 h. Samples were treated the next day by adding 200 μl of labeling buffer, supplemented or not with ABA or sorbitol for the times and concentrations indicated.

All treatments were stopped by adding perchloric acid to a final concentration of 5% (v/v) for 5–10 min, after which the supernatant was removed (2 min at 13,000 × g) and the lipids extracted with 400 μl of CHCl3/MeOH/HCl.
the total [32P]lipid fraction.

Healthcare). Individual phospholipid levels were expressed as the percentage of autoradiography and quantified by phosphoimaging (Typhoon FLA 7000, GE Healthcare). Individual phospholipid levels were expressed as the percentage of the total [32P]lipid fraction.

Inositol polyphosphates: [3H]inositol labeling, extraction and HPLC analyses

For the measurement of IPPs, two labeling procedures were used. The first was based on the method described by Laha et al. (2015). Seedlings were grown under short days (22°C, 12 h light/12 h darkness) in 1/2 MS containing 2% sucrose, pH 5.7, and 0.6% phytagel for 11 d, after which 10 seedlings were transferred to 2 ml of liquid medium (1/4 MS, pH 5.7, 0.3% phytagel) containing [3H]-inositol (80 μCi, Biortoc, ART-0261-5). After 7 d, seedlings were washed twice with water before harvesting, and snap-frozen in liquid N2. IPPs were subsequently extracted (Azevedo and Saiardi 2006) and resolved by strong anion exchange HPLC (using the Partisphere SAX 4.6 x 125 mm column; Whatman) at a flow rate of 0.5 ml min⁻¹, using a shallow gradient formed by buffer A (1 mM EDTA) and buffer B (1.3 M ammonium phosphate (pH 3.8, H₃PO₄), 1 mM EDTA). Fractions were collected every minute and radioactivity quantified by liquid scintillation counting. Results are expressed as a percentage of the total. The latter was determined by counting [3H] in all fractions from 13 min to the end of the run.

Alternatively, IPPs were determined as described by Desai et al. (2014). In this case, seedlings were grown in 1/2 MS with 0.8% agar under long-day conditions (16 h light and 8 h darkness) for 4 d, after which seedlings were incubated in 50 μl of medium (1× MS, 1% sucrose, pH 5.7) supplemented with 100 μl of aqueous myo-[2-3H(N)]inositol (100 μCi; American Radiolabeled Chemicals Cat. #ART 0116A; specific activity 20 Ci mmol⁻¹). Samples were incubated with supplemental light for 4 d. IPPs were extracted as described by Azevedo and Saiardi (2006), by vortexing the tissue with glass beads in extraction buffer (25 mM EDTA, 10 mM NaCl, 10 mM K₂CO₃, and dried to a volume of 70 μl). Samples were neutralized to approximately pH 6–8 with 250 mM EDTA and 1 M K₂CO₃ and dried to a volume of 70 μl. Samples were prepared using a binary HPLC pump (Beckman Coulter) equipped with a Partisphere-SAX (4.6 x 125 mm) column and 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⁻¹. An on-line IN/US radiation detector was used to generate chromatograms. A 4 ml aliquot of Ultima-Fluor scintillation cocktail (Perkin Elmer) was added to each 1 ml of the eluted fraction post-detector to quantify the radioactivity of the eluted fractions using the [3H] window of a Beckman Coulter LS6000 Scintillation Counter. The [3H]-inositol c.p.m. incorporated into total IPPs was calculated by taking the sum of the c.p.m. of all fractions and subtracting the peak of free [3H]inositol c.p.m. The amount of each IPP was calculated as follows: [(Σ c.p.m. in peak)/(total IPP)]×100.

Drought tolerance

Determination of survival rates, fresh and dry weights under water deficit conditions and water loss were performed as described in the literature (Hua et al. 2012; Oubakar et al., 2013), with some minor modifications. Seeds were stratified in the dark for two nights at 4°C and directly sown on soil (45 cm x 45 cm x 7.5 cm), with each pot containing nine plants and 80 g of soil. Plants were grown under short-day conditions (22°C with 12 h light/12 h darkness) for 4 weeks and then subjected to drought stress by withholding water for 2 weeks, while control plants were watered normally. Plants were photographed, re-watered for another week and again photographed. Surviving green plants were counted and the survival rate determined by the percentage of green plants compared with the total amount of plants. Each experiment used 36 plants per genotype, and experiments were repeated at least three times.

To determine the fresh and dry weights under dehydration stress, plants were grown under short-day conditions for 4 weeks as described above, and exposed to drought for 1 week by withholding water, while control plants were watered normally. Rosette fresh weights were scored immediately after detachment by weighing. After complete drying, dry weights were determined. Eighteen plants from each genotype were measured, and the experiment was repeated three times.

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

**Supplementary Data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

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