Knock-Down of Arabidopsis PLC5 Reduces Primary Root Growth and Secondary Root Formation While Overexpression Improves Drought Tolerance and Causes Stunted Root Hair Growth

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Phospholipase C (PLC) is a well-known signaling enzyme in metazoaans that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol 1,4,5-trisphosphate and diacylglycerol as second messengers involved in multiple processes. Plants contain PLC too, but relatively little is known about its function there. The model system Arabidopsis thaliana contains nine PLC genes. Reversed genetics have implicated several roles for PLCs in plant development and stress signaling. Here, PLCs is functionally addressed. Promoter–β-glucuronidase (GUS) analyses revealed expression in roots, leaves and flowers, predominantly in vascular tissue, most probably phloem companion cells, but also in guard cells, trichomes and root apical meristem. Only one plc5-1 knock-down mutant was obtained, which developed normally but grew more slowly and exhibited reduced primary root growth and decreased lateral root numbers. These phenotypes could be complemented by expressing the wild-type gene behind its own promoter. Overexpression of PLC5 (PLC5-OE) using the UBQ10 promoter resulted in reduced primary and secondary root growth, stunted root hairs, decreased stomatal aperture and improved drought tolerance. PLC5-OE lines exhibited strongly reduced phosphatidylinositol 4-monophosphate (PIP) and PIP2 levels and increased amounts of phosphatic acid, indicating enhanced PLC activity in vivo. Reduced PIP2 levels and stunted root hair growth of PLC5-OE seedlings could be recovered by inducible overexpression of a root hair-specific PIP5-kinase, PIP5K3. Our results show that PLC5 is involved in primary and secondary root growth and that its overexpression improves drought tolerance. Independently, we provide new evidence that PIP2 is essential for the polar tip growth of root hairs.

Abbreviations: DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphatase; GUS, β-glucuronidase; IP3, inositol 1,4,5-trisphosphate; IP6, inositol hexakisphosphate; IPP, inositol polyphosphate; KD, knock-down; MS, Murashige and Skoog; OE, overexpressing; PA, phosphatidic acid; PIP, phosphatidylinositol 4-monophosphate; PIP4, phosphatidylinositol 4,5-bisphosphate; PIP5K3, PIP5-kinase; PKC, protein kinase K; PLC, phospholipase C; PP-IPP, pyrophosphorylated inositol polyphosphate; Q-PCR, quantitative PCR; TRP, transient receptor potential.

Introduction

Phospholipase C (PLC) signaling is best known from animal systems where it generates the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) by catalyzing the hydrolysis of the minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2). The water-soluble IP3 diffuses into the cytosol where it triggers the release of Ca2+ from the endoplasmic reticulum (ER) via a ligand-gated Ca2+ channel, while the lipid DAG remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family or stimulates TRP- (transient receptor potential) type ion channels. The PLC signaling system is activated by hundreds of receptors and regulates numerous cellular events and physiological processes (Balla 2013, Nakamura and Fukami 2017).

Plants contain PLCs too, but the signaling system is probably different from that of animals since they lack the primary targets for IP3 and DAG, i.e. IP3 receptors, PKCs and TRP channels (Zonia and Munnik 2006, Wheeler and Brownlee 2008, Munnik and Testerink 2009, Munnik 2014). Also different is that the plasma membrane of flowering plants hardly contains any PIP2, which is the presumed substrate of PLC (Munnik et al. 1994, van...
Leeuwen et al. 2007, Simon et al. 2014). In vitro, PLCs can also hydrolyze its precursor, i.e. phosphatidylinositol 4-monophosphate (PIP). The latter is relatively abundant in plant plasma membranes (Vermeer et al. 2009, Simon et al. 2014, 2016), but it remains unknown what the typical substrate for PLC is in vivo (see Munnik 2014).

Initially, IP$_3$ could be linked to the release of intracellular Ca$^{2+}$ in plants (Blatt et al. 1990, Gilroy et al. 1990, Allen and Sanders 1994, Hunt and Gray 2001), but later evidence indicated that this response was caused by its phosphorylation into IP$_6$ (inositol hexakisphosphate) (Lemtiri-Chlieh et al. 2000, 2003, Sanders 1994, Hunt and Gray 2001), but later evidence indicated that this response was caused by its phosphorylation into IP$_6$ (inositol hexakisphosphate) (Lemtiri-Chlieh et al. 2000, 2003, Sanders 1994, Hunt and Gray 2001). Similarly, not DAG but its phosphorylated product, phosphatidic acid (PA), has emerged as the plant lipid second messenger (Munnik 2001, Testerink and Munnik 2005, Arisz et al. 2009, Testerink and Munnik 2011, Munnik 2014, Pokotylo et al. 2014, Heilmann 2016a, Heilmann 2016b, Hong et al. 2016, Hou et al. 2016). PA can be further phosphorylated into diacylglycerol pyrophosphate (DGPP) by PA kinase, an enzyme lacking in animals but present in fungi, oomycetes and trypanosommes (Munnik et al. 1996, van Schooten et al. 2006). Whether DGPP’s function is to attenuate PA signaling or to form a new signal (it is normally absent) remains unknown. Meanwhile, various inositol polyphosphates (IP$_p$) other than IP$_6$ have been emerging as signaling molecules. In fungi and animals, IPPs have been shown to regulate ion channels, mRNA transport and gene transcription (Tsu and York 2010, Gillaspy 2011, Gillaspy 2013, Williams et al. 2015). In plants, IP$_4$ has been implicated in regulating a chloride channel (Zonia et al. 2002), while IP$_5$ and IP$_6$ were discovered in the crystal structures of TIR1 and CO1 receptors for auxin and jasmonate signaling, respectively (Tan et al. 2007, Sheard et al. 2010). Similarly, Gle1, an mRNA export factor, is an IP$_6$-binding protein and key activator of the ATPase/RNA helicase, LOS4 (low expression of osmotically responsive genes 4), similar to the yeast paradigm, Glec1-IP$_6$-Dbp5 (a LOS4 homolog) (Lee et al. 2015). IP$_6$ is also required for basal resistance to plant pathogens (Murphy et al. 2008) and binds SPX domains that are typically present on proteins involved in phosphate homeostasis (Puga et al. 2014, Wild et al. 2016). IPPs are clearly involved in phosphate signaling (Stevenson-Paulik et al. 2005, Kuo et al. 2014, Kuo et al. 2018) and some are pyrophosphorylated to IP$_7$ and IP$_8$ [pyrophosphorylated inositol polyphosphate (PP-IPPs)], which have been implicated in jasmonic acid and plant defense signaling (Mosblech et al. 2011, Laha et al. 2015, Williams et al. 2015, Laha et al. 2016). Besides a lipid/PLC-generated pathway, IPPs and PP-IPPs can also be synthesized de novo, via the conversion of glucose 6-phosphate into inositol 3-phosphate and subsequently inositol, which is then stepwise phosphorylated by various IPP kinases (Munnik and Vermeer 2010, Gillaspy 2011, Gillaspy 2014, Kuo et al. 2014, Laha et al. 2015, Williams et al. 2015, Williams et al. 2016, Kuo et al. 2018).


Genetic evidence that PLC is involved in stress responses is also emerging. In Arabidopsis and tomato, PLC has been linked to plant defense signaling, in particular to the production of reactive oxygen species (Vossen et al. 2010, Goronzy et al. 2016, D’Ambrosio et al. 2017). For Arabidopsis, this was recently shown to involve PLC2 and RbohD (D’Ambrosio et al. 2017). PLC2 has also been implicated in the ER stress response pathway (Kanekara et al. 2015). Arabidopsis PLC3 and PLC9 have been implicated in thermotolerance (Zheng et al. 2012, Gao et al. 2014), PLC3 in ABA signaling (Zhang et al. 2018) and PLC4 was recently shown to regulate salt tolerance negatively (Xia et al. 2017).

PLC signaling has also been implicated in plant growth and development. PLC is important for pollen tube growth in petunia and tobacco (Dowd et al. 2006, Helling et al. 2006) and affects male and female gametophyte development in Arabidopsis and Torenia (Song et al. 2008, Li et al. 2015, Di Fino et al. 2017). In Physcomitrella, PLC is involved in cytokinin and gravity responses (Repp et al. 2004). Historically, IP$_3$ responses have been correlated to gravitropism and Ca$^{2+}$ signaling (Perera et al. 1999, Stevenson et al. 2000, Perera et al. 2006, Boss et al. 2010), but recent advances with respect to IP$_6$–TIR1 and auxin signaling, and the role of PLC therein, remain unknown. Loss-of-AtPLC3 seedlings exhibited normal gravitropism responses (Zhang et al. 2018). The latter study did reveal, however, novel phenotypes for PLC, including reduced lateral
root formation, reduced seed germination and reduced sensitivity to ABA with respect to induction of stomatal closure and inhibition of seed germination (Zhang et al. 2018). PLC3 overexpression increases the plant’s tolerance to drought stress (Zhang et al. 2018), consistent with earlier studies in canola, maize and tomato (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011).

Arabidopsis contains nine PLC genes (Hunt et al. 2004, Tasma et al. 2008, Munnik 2014, Pokotylo et al. 2014). Since AtPLC3 was mainly expressed in the vasculature, in particular in the phloem and companion cells (Zhang et al. 2018), and since knock-down (KD) mutants exhibited a lateral root phenotype, we searched for other PLC genes being specifically expressed in the phloem. This resulted in the identification of AtPLCS5, which belongs to a clade of the PLC family different from AtPLC3 (Hunt et al. 2004, Tasma et al. 2008, Munnik 2014). In this study we provide evidence that AtPLC3 plays a role in lateral root development too and that its overexpression increases drought tolerance. PLC5 overexpression also led to stunted root hair growth for which we provide genetic and biochemical evidence that this is caused by the increased hydrolysis of PIP2 at the tip of the root hair that is essential for its tip growth.

Results

Knock-down of PLC5 affects root development

Earlier, we found that Arabidopsis KD mutants of PLC3 were affected in lateral root development, and that this was linked to its specific expression in the root phloem/companion cells (Zhang et al. 2018). Via the eFP browser, we found that Arabidopsis PLC5 was also predicted to be expressed in the phloem/companion cells (eFPBrowser Ar5g58690) (Winter et al. 2007). To characterize PLC5 functionally, we tried obtaining homozygous T-DNA insertion mutants. Eventually, we uncovered only one homozygous line, plc5-1 (SALK_144469; Fig. 1a), which turned out to be a KD mutant (Fig. 1b).

Seedlings of plc5-1 contained shorter primary roots (~10%) and fewer (~20%) lateral roots than the wild type (Fig. 1c–f), a phenotype we found earlier for plc3 mutants (Zhang et al. 2018). The phenotype of plc5-1 could be rescued by complementation with wild-type PLC5 driven by its own promoter (Fig. 1c–f), confirming PLC5’s role in root development.

Analysis of the different lateral root stages (Supplementary Fig. S1) indicated that the plc5-1 phenotype concerned lateral root initiation rather than emergence. To investigate the potential redundancy of PLC3, plc3plc5 double mutants were created by crossing plc3-2 (Zhang et al. 2018) with plc5-1. In homozygous T2 lines, the lateral root phenotype was only marginally enhanced (22% fewer lateral roots compared with the wild type; Supplementary Fig. S2), indicating additional redundancy. Further analysis on the eFP browser (Winter et al. 2007) revealed that PLC2 and PLC7 were also expressed in the phloem. Unfortunately, however, plc3plc5plc7 triple and plc2 single mutants were found to be homozygous lethal (Di Fino et al. 2017, Munnik lab unpublished).

Expression of PLC5 during growth and development

Quantitative PCR (Q-PCR) analysis has revealed some variation in PLC5 expression among the various organs (Tasma et al. 2008). To investigate this topologically in more detail, we analyzed a PLC5 promoter–β-glucuronidase (GUS) reporter line, kindly provided by Dr. Julie Gray (Hunt et al. 2004). As shown in Fig. 2a, pPLC5-GUS expression was already apparent during germination (28 h after vernalization/transfer to 22°C) in the cotyledons, hypocotyl and root of the embryo. Upon further development, expression was mainly observed in the vasculature throughout all tissues, i.e. root, cotyledons, leaves (including hydathodes), hypocotyl and flower, including the stamen, style, receptacle and pedicel (Fig. 2b–l). Intact trichomes also revealed expression (Fig. 2i), which is different from that of PLC3, which was only expressed at the base of a trichome, as a ring (Zhang et al. 2018). Expression in the root was not homogenous; it was ‘segmented’ at the distal side of the root maturation zone, continuous in the apical root maturation zone, was lacking near the transition zone, but was expressed at the root tip (Fig. 2d–g; Supplementary Fig. S3). This pattern was very similar to the expression of PLC3, except for the root tip (Zhang et al. 2018). Noticeably, lateral roots always emerged from a ‘segment’, even though not every segment led to a lateral root, like PLC3 (Fig. 2c; Supplementary Fig. S3; Zhang et al. 2018). Both PLC5 segmentation and root tip expression were also observed during tertiary root formation (Fig. 2e).

Overexpression of PLC5 increases drought tolerance

In maize, canola and tobacco, overexpression of PLC increased the plant’s tolerance to drought (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011). We confirmed this phenotype when overexpressing PLC3 in Arabidopsis (Zhang et al. 2018). It is not known whether specific PLC isoenzymes are required for this or whether any PLC could achieve this. To investigate the
potency of PLC5, transgenic overexpressing plants were generated using the ubiquitin (UBI10) promoter. Ten transgenic plants were selected from T0 to T3, and two independent homozygous lines, PLC5-OE2 and PLC5-OE3, overexpressing PLC5 about 300- and 100-fold, respectively (Fig. 4a), were selected for further studies.

Overexpression of PLC5 caused a slight reduction in growth (Supplementary Fig. S4), something we did not observe when overexpressing PLC3 (Zhang et al. 2018). Nonetheless, PLC5-OE lines were consistently (more than six independent experiments) more drought tolerant than the wild type (Fig. 4b) and also lost less water (Fig. 4c). The drought tolerance of plc5-1 plants was similar to that of the wild type (Supplementary Fig. S5).

To analyze the stomatal aperture and ABA sensitivity, leaf peels were isolated (Zhang et al. 2018). Under control conditions, stomatal opening in both PLC5-OE lines was significantly reduced compared with the wild type (Fig. 4d). Upon ABA
Fig. 2  pPLC5::GUS expression analyses in seedlings and mature tissues of Arabidopsis. (a) GUS activity was present in embryo cotyledons and roots during seed germination, from testa rupture until radical emergence (28 h after transfer from 4°C to 22°C), in the vasculature of 2-day-old (b) and 10-day-old seedlings, including leaf (c) and roots (d–g), in vascular tissue of mature 3-week-old plants (h), trichomes (i), hydatodes (indicated by arrows) (j), guard cells (m), siliques (l) and in different parts of the flower (k), including the style, filament, receptacle and pedicel.

Fig. 3  PPI and PA levels in wild-type and plc5-1 seedlings. Five-day-old seedlings were labeled with $^{32}$PO$_4$$^{3-}$ overnight and the next day their lipids were extracted and separated by thin-layer chromatography (TLC). (a) Autoradiograph of a typical labeling experiment, each lane representing one-fifth of the extract of three seedlings. (b) Quantification of $^{32}$P-labeled PIP$_2$, PIP and PA levels in the wild type and plc5-1. Values are calculated as the percentage of total $^{32}$P-labeled phospholipids and are represented as means ± SD (n = 3). The experiment was repeated twice with similar results.
treatment, stomatal closure was induced for all genotypes; however, the amplitude of the response between 0 and 10 μM ABA was significantly lower in the OE lines than in the wild type (Fig. 4d).

**Overexpression of PLC5 triggers enhanced PPI and PA responses**

To analyze whether PLC5 overexpression caused any changes in PPI and/or PA levels, $^{32}$P labeling experiments on seedlings (3 h labeling) were performed and the effect of sorbitol tested to mimic water stress. Under control conditions, PLC5-OE lines exhibited a clear reduction in PIP$_2$ (~80%) and PIP (~20%), and an increase in PA (~30%) (Fig. 5), confirming a constitutively higher PLC activity in vivo. Overnight $^{32}$P labeling conditions gave similar percentages (Supplementary Fig. S5e). Upon sorbitol treatment, a much stronger increase in PIP$_2$ was observed in the OE lines. While PIP$_2$ levels increased about 4-fold in the wild type, in the OE lines a massive 12-fold increase was witnessed, even though the absolute levels of PIP$_2$ and PIP remained below wild-type levels. PA and PIP responses in wild-type and OE lines were similar (Fig. 5b).

**Overexpression of PLC5 causes stunted root hair growth**

PLC5 overexpression did not increase the lateral root numbers but, instead, led to smaller roots and reduced lateral root densities (Supplementary Fig. 5e–h). Analyzing the roots in more detail led to the discovery that the root hairs of the PLC5-OE lines were stunted (Fig. 6a, b). Measuring individual root hairs revealed a strong reduction in root hair length for both PLC5-OE (~90%) and PLC5-OE3 (~80%) (Fig. 6c).

PIP$_2$ has been implicated in root hair elongation by the results of several studies. It specifically accumulates at the tip of growing root hairs and has been shown to involve PIP 5-kinase (PIP5K3) (van Leeuwen et al. 2007, Kusano et al. 2008, Stenzel et al. 2009, Grierson et al. 2014). Since the PLC5-OE lines exhibited strongly reduced PIP$_2$ levels (i.e. 83% in PLC5-OE2 and 77% in PLC5-OE3), which correlated with the severity of the root hair phenotype (Fig. 6), and phenocopied the reduced root hair length in pip5k3 KD mutants (Kusano et al. 2008), we hypothesized that the root hair phenotype in PLC5-OE lines could be caused by the increased hydrolysis of PIP$_2$ at the root hair tip that is essential for tip growth. To analyze this, we crossed...
PLC5-OE2 with an estradiol-inducible overexpressor of PIP5K3 (ER8-PIP5K3) that is known to produce PIP2 at the root hair tip and to induce root hair formation (Kusano et al. 2008). T3 transgensics of PLC5-OE2 × ER8-PIP5K3 were selected and grown together with the wild type and both individual mutant lines, PLC5-OE2 and ER8-PIP5K3, for 4 d on half-strength Murashige and Skoog (1/2 MS) plates and then transferred to 1/2 MS plates with and without 10 μM estradiol for another 3 d. Without estradiol, the ER8-PIP5K3 lines showed similar root hair growth to the wild type, while PLC5-OE2 × ER8-PIP5K3 clearly exhibited the reduced root hair phenotype described above (Fig. 7a, b). After estradiol induction, however, the root hair length significantly increased in both ER8-PIP5K3 and PLC5-OE2 × ER8-PIP5K3 lines, but did not change in the wild type or
PLC5-OE2 (Fig. 7a, b). Determining the PIP2 levels in these lines revealed that without estradiol, PLC5-OE2 and PLC5-OE2 ER8-PIP5K3 lines contained PIP2 levels that were significantly lower than the wild type and ER8-PIP5K3. However, upon induction by estradiol, PIP2 levels in both ER8-PIP5K3 and PLC5-OE2 ER8-PIP5K3 increased sharply, while they remained the same for the wild type and PLC5-OE2 (Fig. 7c, d). Thus, increasing PIP2 levels by estradiol-induced overexpression of PIP5K3 clearly recovered the stunted root hair growth that resulted from PLC5 overexpression.

Discussion

Earlier, we found that loss-of-function mutants of the phloem/companion cell-expressed Arabidopsis PLC3 were affected in seed germination, lateral root development and ABA sensitivity, and that ectopic overexpression improved drought tolerance (Zhang et al. 2018). Here, we show that PLC5, which belongs to a different subclade of the Arabidopsis PLC family (Tasma et al. 2008), is also predominantly expressed in phloem companion cells, and that a KD mutant is negatively affected in primary root growth and lateral root development, while no effects on germination or ABA sensitivity were found. Overexpression of PLC5 led again to an increase in drought tolerance, a phenotype that is shared with PLC-overexpressing rapeseed, maize or tobacco (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011). In the case of PLC5 overexpression, retarded root and shoot growth were found, and a novel phenotype, i.e. a strong retarded root hair growth.

It is still not clear how plant PLCs achieve this molecularly, but several scenarios are possible. As flowering plants lack the metazoan targets for IP3 and DAG (i.e. ligand-gated Ca2+, TRP channels and PKC), it is not very likely that these molecules themselves will fulfill a second messenger role in plants, even though we cannot completely rule out the possibility that plants may have evolved distinct targets. In contrast, there is accumulating evidence that the phosphorylated products of IP3...

Overexpression of PLC5 resulted in decreased levels of PIP and PIP₃, and increased levels of PA, but not of DGPP. While DGPP responses are relatively abundant in algae and cell suspensions (van Schooten et al. 2006, Meijer et al. 2017), in Arabidopsis seedlings this lipid is hardly detectable, even under stress (Arisz et al. 2013).

While PIP₃ is the authentic PLC substrate in animal systems, its concentration in plants is relatively low and hardly detectable in plasma membranes where most of the PLC activity resides (Munnik et al. 1998a, Meijer and Munnik 2003, van Leeuwen et al. 2007, Munnik 2014). This is in contrast to PI4P, which is hydrolyzed equally well in vitro (Munnik et al. 1998a, Munnik 2014), is 20–30 times more abundant than PIP₂ and is highly enriched in plasma membranes (Munnik et al. 1994, Vermeer et al. 2009, Vermeer and Munnik 2013, Simon et al. 2014, Simon et al. 2016, Vermeer et al. 2017). Hence, in vivo, PI4P may be an important plant PLC substrate. Obviously, this could be different under stress conditions where PIP₂ levels in vivo, PI4P may be an important plant PLC substrate. Obviously, this could be different under stress conditions where PIP₂ levels are raised, e.g. in response to ABA, hyperosmotic stress, salinity or heat (Pical et al. 1999, DeWald et al. 2001, Takahashi et al. 2001, van Leeuwen et al. 2007, Darwish et al. 2009, Mishkind et al. 2009, Zhang et al. 2018). PI4P levels have been reported to drop in response to, for example, salt and cold stress (Cho et al. 1993, Pical et al. 1999, DeWald et al. 2001, Ruelland et al. 2002, Vermeer et al. 2009, Arisz et al. 2013), but it remains to be shown whether this reflected the hydrolysis by a PLC or a phosphatase, or is a consequence of a PIPK activation. Nevertheless, PLC hydrolysis of PI4P would still generate DAG and IP₂, which can be converted into PA and IPPs through DGK and IPks, respectively, and into PP-IPPs via VIH2 (Laha et al. 2015, 2016) to activate signaling responses.

While basal levels of PIP₃ in flowering plants are extremely low, the lipid itself is clearly emerging as a second messenger too, involving various stress and developmental responses, including tip growth in pollen tubes and root hairs, vascular differentiation, salt and heat stress responses, the organization of the cytoskeleton and membrane trafficking (endo- and exocytosis). Potential targets include small G-proteins, K⁺ channels, clathrin adaptor proteins and EXO70 (Ischebeck et al. 2010, Munnik and Nielsen 2011, Gillaspy 2013, Rodriguez-Villalon et al. 2015, Heilmann 2016a, Heilmann 2016b, Heilmann and Ischebeck 2016, Gerth et al. 2017, Gjus et al. 2017, Kalmbach et al. 2017, Noack and Jallais 2017, Wu et al. 2017). Similarly, PI4P itself has been emerging as a lipid second messenger (Stevenson et al. 2000, Vermeer et al. 2009, Munnik and Nielsen 2011, Heilmann 2016a, Heilmann 2016b, Simon et al. 2016, Noack and Jallais 2017). In such cases, PLC could then act as an attenuator of PPI signaling. Whether PLC attenuates second messengers or produces them (or both) will require more research. This will not be easy, considering the high degree of redundancy in PPI, PA and IPP pathways, e.g. Arabidopsis has nine PLC, 11 PIPK, 12 PIK, seven DGK and 12 PLD genes, and various IPKs (Munnik and Testerink 2009, Kuo et al. 2018). Events may also be very local and small in nature, as here, in the phloem companion cells.

A role for PLC5 in root development and auxin signaling?

Knock-down of PLC5 led to shorter primary roots and reduced lateral root numbers, while promoter–GUS analyses suggest that PLC5 is predominantly expressed in the root vasculature (Fig. 2), which is confirmed by eFP data (Winter et al. 2007; Supplementary Fig. S3). GUS expression was not homogenous but showed some degree of segmentation. Moreover, lateral roots always emerged from a segment, even though not every segment resulted in a lateral root, similar to what we found for PLC3 (Zhang et al. 2018). Lateral roots revealed a similar segmentation pattern, indicating that tertiary roots initiate from PLC segments too (Fig. 2d, e). That the lateral root phenotype is relatively mild in either plc3 or plc5 KD mutants may point to redundancy, especially since plc3 plc5 double mutants displayed a similar reduction in primary root length and lateral root number (Supplementary Fig. S2). In the eFP browser, two additional PLC genes were found in the phloem vasculature: PLC2 and PLC7. Unfortunately, single plc2 mutants and triple plc3 plc5 plc7 mutants were found to be homozygous lethal (Di Fino et al. 2017, Munnik lab unpublished). Inducible silencing may offer new perspectives to investigate their role in lateral root formation.

Auxin plays a key role in root system architecture (Benková and Bielech 2010). It regulates massive changes in gene expression by promoting the degradation of the transcriptional Aux/IAA repressors through binding and activation of the auxin receptor, TIR1. The latter is an F-box protein that forms an SCF complex that functions as a multiprotein E3 ubiquitin ligase complex, which catalyzes the ubiquitination of Aux/IAAs destined for proteosomal degradation (Kepinski and Leyser 2005). Interestingly, the crystal structure of TIR1 contains IP₆ that is anticipated to regulate auxin binding and TIR1 activity (Tan et al. 2007, Hao and Yang 2010, Calderón Villalobos et al. 2012, Zhang et al. 2018). While it is unclear from where the IP₆ originates, we proposed that it could be formed though PLC, generating IP₃/IP₅ with subsequent phosphorylation into IP₆, occurring at the PLC3 segments from which lateral roots emerge (Zhang et al. 2018). Our data here imply a similar, yet redundant role for PLC5 and possibly for other PLCs, i.e. PLC2 and PLC7. Alternatively, chemical redundancy could play a role, e.g. by synthesizing IP₆ de novo from inositol (Munnik and Vermeer 2010, Gillaspy 2011, Gillaspy 2013, Kuo et al. 2018). To couple IP₆ with TIR1 functionally, it will be important to determine the amount of IP₆ bound to TIR1 in wild-type and plc3 plc5 mutants. For the latter, we would need induced silencing lines in combination with KD and knock-out mutants.

An alternative explanation for the root phenotype in plc5 could be related to the metabolism of inositol-based raffinose family oligosaccharides (RFOs) that are important for carbohydrate transport and storage into sink organs such as lateral roots (van den Ende 2013, Sengupta et al. 2015). We checked...
myo-inositol and sugar levels in seedlings (leaf and root) and phloem sap of the wild type and plc5-1 but found no significant differences (Supplementary Figs. S7, S8; Zhang et al. 2018).

**Overexpression of PLC5 enhances drought tolerance**

Overexpression of PLC has earlier been shown to improve drought tolerance in maize, tobacco and canola (Wang et al. 2008, Georges et al. 2009, Tripathy et al. 2011), and we confirmed this for Arabidopsis when overexpressing PLC3 (Zhang et al. 2018) and again here with PLC5. Even though PLC3 and PLC5 are phloem-expressed PLCs, we do not know whether this is a requirement or whether any PLC could achieve this. For the overexpression in maize, tobacco and canola, such information is not available. Overexpression of a non-phloem-specific AtPLC would shed light on this.

Under control conditions, PLC5-OE plants grew slightly more slowly, and eventually produced less biomass (Supplementary Fig. S4). Upon drought stress, however, plants performed much better than the wild type (Fig. 4b, c), even better than the PLC3-OE lines (Zhang et al. 2018). The latter revealed no growth penalty, nor did the crop plants mentioned above, so it is indeed the overexpression of PLC that causes the increase in drought tolerance. The molecular mechanism of this drought tolerance, however, remains unknown. PLC5-OE plants lost significantly less water than the wild type, which could be related to the fact that the stomata are less open than in the wild type (Fig. 4c, d), since there was no difference in the number of stomata (not shown). The reduction in apertures could also be a reason why PLC5-OE plants are smaller, since the capacity to convert CO2 into sugars could be more limited; however, glucose, fructose and sucrose levels in shoots and roots of wild-type and PLC5-OE seedlings revealed no difference (Supplementary Fig. S8), nor could we find a difference in PII activity in photosynthesis (Supplementary Fig. S9).

PLC5-OE lines displayed reduced levels of PIP and PIP2, and increased levels of PA, which is in agreement with an increase in PLC activity. PIP2 has been shown to be important for stomatal opening by lowering the water potential through inhibition of SLAC1 (Lee et al. 2007) and K+ efflux channels (Ma et al. 2009), which would favor the influx of water and open the stomata (Zhang et al. 2018). As such, the closed-stomata phenotype of PLC5-OE lines could also be due to the increased PIP2 hydrolysis. Subsequent increases in IP6 would facilitate the stomatal closure by triggering the release of intracellular Ca2+ (Lemtiri-Cliéh et al. 2000, Lemtiri-Cliéh et al. 2003, Zhang et al. 2018).

To mimic drought stress and analyze the effect on PA and PPI levels, we treated seedlings with sorbitol. Interestingly, PIP2 levels dramatically increased upon osmotic stress and the accumulation was much stronger in the PLC5-OE lines (Fig. 5). The latter may reflect an increased basal turnover of PIP2, due to enhanced PLC5 hydrolysis, which would readily be picked up by these types of 32P labeling experiments (Munnik et al. 1998b, Arisz and Munnik 2013). Since the UBQ10 promoter drives the expression of PLC5 in many more cells than its normal endogenous, vascular expression, increased PLC-derived signals (e.g. PA and IPPs) or PPI attenuation may affect distinct cells, tissues and processes relevant to osmotic stress (Munnik and Vermeer 2010).

PLC5-OE lines also exhibited stunted root hairs (Fig. 6), which could be another explanation for their smaller plant size, as nutrient and mineral uptake could be affected (Leitner et al. 2009). Strikingly, PLC3-OE lines did not reveal this root hair phenotype, nor did they exhibit changes in the basal levels of PPIs or PA (Zhang et al. 2018). From the amino acid sequence, it is not clear why PLC5 would be more active than PLC3. All AtPLCs contain the same domain structure, including two EF-hands, a catalytic XY-domain and a CalB/C2 domain (Tasma et al. 2008, Munnik 2014). Nonetheless, PLC3 and PLC5 belong to different PLC subclades, so there might be subtle changes in enzymatic properties, Ca2+ sensitivity or interaction with other proteins that explains their difference. In vitro characterization of their activities and analyses of the proteins they interact with, will shed light on this.

**PIP2 generation is essential for root hair tip growth**

Overexpression of PLC5 resulted in plants with shortened, stunted root hairs (Fig. 6), which is very probably due to the reduction of PIP2, that is required for tip growth (Fig. 5b; Supplementary Fig. S5). Evidence for this came from the induced overexpression of PIP5K3 in the PLC5-OE background that restored PIP2 levels and rescued the root hair phenotype (Fig. 7). T-DNA insertion mutants of PIP5K3 exhibit similar stunted root hairs, and this lipid kinase has been shown to be responsible for generating PIP2 at the plasma membrane of the growing tip (van Leeuwen et al. 2007, Kusano et al. 2008, Stenzel et al. 2008). Similar results have been found in pollen tubes (Kost et al. 1999, Helling et al. 2006, Ischebeck et al. 2008, Kost 2008, Ischebeck et al. 2010, Ischebeck et al. 2011, Hempel et al. 2017), whose tip growth resembles that of root hairs (Ovečka et al. 2005, Zonia and Munnik 2008, Ischebeck et al. 2010, Griersson et al. 2014). PLC is important there too since silencing of PLC1 in petunia pollen tubes led to arrested and depolarized growth, which was accompanied by a disorganization of the actin cytoskeleton (Dowd et al. 2006). Overexpression of NtPLC3 in tobacco pollen tubes shortened their length (Helling et al. 2006). Moreover, fluorescent protein-tagged NtPLC3 was shown to localize at the flanks of the growing tip, while PIP2, visualized by a biosensor, accumulated at the apex (Helling et al. 2006), similar to what was found in growing root hairs (van Leeuwen et al. 2007, Kusano et al. 2008). In the plc5-1 mutant, no aberrant root hair morphology was observed, but normally PLC5 is not expressed there either (Fig. 2; Supplementary Fig S3).

How PIP2 drives polar tip growth is still unclear, but is likely to involve a complex signaling network between membrane trafficking and cytoskeletal dynamics (Ovečka et al. 2005, Kost 2008, Ischebeck et al. 2010, Griersson et al. 2014, Heilmann 2016a, Heilmann 2016b, Tejos et al. 2014, Grebnev et al. 2017, Hempel et al. 2017, Noack and Jaillais 2017). Tip growth is sustained by exocytosis of vesicles containing growth materials, such as polysaccharides and proteins, for the growing cell wall and membrane (Griersson et al. 2014). In animal cells,
PIP2 is involved in priming exocytosis and vesicle fusion by binding EXO70, a subunit from the exocyst complex (Aikawa and Martin 2003, Munson and Novick 2006, Liu et al. 2007). Arabidopsis contains 23 EXO70s, and the exocyst complex is essential for pollen tube germination and growth, but also in cell division (Synnek et al. 2006, Hálá et al. 2008, Gujas et al. 2017, Kalmbach et al. 2017, Wu et al. 2017). The local accumulation of PIP2 has also been shown to correlate with vesicle secretion (Kalmbach et al. 2017, Wu et al. 2017). The local accumulation of PIP2 has also been shown to correlate with vesicle secretion (Ischebeck et al. 2008). Similarly, PIP2 has been implicated in clathrin-mediated endocytosis (CME) by recruiting clathrin adaptor proteins (König et al. 2008, Zhao et al. 2010, Baisa et al. 2013, Ischebeck et al. 2013). To deliver and return vesicles to and from expanding cell areas, the actin cytoskeleton plays a crucial role too, and PIP2 has been suggested to participate there as well (Logan and Mandated 2006, Balla 2013). Rho GTPases, in plants called Rop (Rho of plants), are crucial regulators of tip growth (Kost 2008), controlling the actin cytoskeleton and membrane trafficking (Lee et al. 2008), and there is evidence that PIP2 is involved in its regulation (Kost et al. 1999, Klahre et al. 2006, Kost 2008, Ischebeck et al. 2011).

Clearly, further studies are required to decipher what the precise role of PLC is and what the downstream targets are for individual PPIs, IPPs and PA. Generating additional knock-out, KD and OE mutants and characterizing their phenotypes will provide useful tools, which may generate new answers as well as new questions.

### Materials and Methods

#### Plant material

The Arabidopsis thaliana (Columbia-0) T-DNA insertion mutant plc5-1 (SALK_144469) was obtained from SALK (signal.salk.edu). Homozygous plants were identified by PCR in F2 and F3 generations using gene-specific primers (forward primer 5'-TGGAAACCTGGGATATGGTC-3'; reverse primer 5'-TTGCCGTCTTTGATATTACGGG-3') and by the combination between reverse primer and left border primer LbI1,3 (5'-ATTITGGCGATTTTTCGGAAC-3'). A double mutant, plc3plc5 (plc3,5) was created by crossing plc3-2 (SALK_037453) and plc5-1 (SALK_144469) and selecting homozygous lines in F2 and F3 generations. The pPLC5-GUS line was kindly provided by Dr. Julie E. Gray (University of Sheffield; Hunt et al. 2004).

#### Root growth

Seeds were surface sterilized in a desiccator using 20 ml of bleach and 1 ml of 37% HCl for 3 h, and then sown on square Petri dishes containing 30 ml of 1/2 MS medium (pH 5.8), supplemented with 0.5% sucrose, and 1.2% Daishin agar (Duchefa Biochemie). Plates were stratified at 4°C in the dark for two nights, and then transferred to long-day conditions (22°C, 16 h of light, 8 h of dark), placed vertically at an angle of 70°. Four-day-old seedlings of comparable size were transferred to 1/2 MS-agar plates without sucrose and scanned after 4–6 d (Epson Perfection V700 scanner). Primary root length, lateral root number and average lateral root length were quantified for each genotype using ImageJ analysis software (National Institutes of Health). For root hair visualization, seedlings were grown on 1/2 MS medium with 0.5% sucrose for 7 d, viewed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera). More than 200 root hairs per seedling were quantified and about 10 seedlings for each genotype were used for the measurement. For indolucine expression, seedlings were grown on 1/2 MS medium with 0.5% sucrose for 4 d and then transferred to agar medium supplemented with 10 µM β-estradiol for another 3 d.

### Cloning and plant transformation

A Multisite Gateway Three-Fragment Vector Construction Kit (www.life-technologies.com) was used to generate pPLCS::PLCS. Oligonucleotide primers (5'-GGGACACACTTGTAGAAAGTTGTATATAGTAAAGAAGACTTGCATATC-3' and 5'-GGGGACTGCTTTTTTTGTACAACTTGCATTTGCCTTCGAAAAGTTGTTCCACATTAG3'), including attB1 and attB2 sites, were used to PCR amplify a region of approximately 770 bp upstream of the predicted PICS ATG start codon. The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX1. Oligonucleotide primers (5'-GGGACACAGTTGTACAAAAAGACGGCTATGAAAGATGGGAGTAGTAC3' and 5'-GGGGACACATTGTAGAAAGTTGTATATAGTAAAGAAGACTTGCATATC-3'), including attB1 and attB2 sites, were used to PCR amplify the PLC5 coding sequence (CDS). The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX2. BOX3 was the pGEM-T-NOS entry clone containing attB2 and attL2 sites. The three entry clones and a destination vector (pGreen0125) were used in a MultiSite Gateway LR recombination reaction to create an expression clone (Multi gateway protocol).

To generate PLC5-OS insert, pUBQ10::PLCS was constructed. The PLC5 CDS was amplified from cDNA using the following primers: AtPLC5-fw (5'-GA GCTTGAACATAAGAGATAGGTC-3') and AtPLC5-T-BamHI (5'-CGGGACTGCTTTTTTTGTACAACTTGCATTTGCCTTCGAAAAGTTGTTCCACATTAG3'). The PCR product was transformed into pET1.2, sequenced and digested with BsrGI and BamHI. After gel extraction, the BsrGI-A1-PLCS-BamHI fragment was cloned into the BsrGI/BamHI-digested pGreen0129V-PUBQ10 MCS vector.

All constructs were transformed into Agrobacterium tumefaciens strain GV3101, which was used to transform either Arabidopsis (Col-0) wild-type plants or the plc5-1 mutant background by floral dip (Clough and Bent 1998). Homozygous lines were selected in the T1 generation and used for further experiments.

### RNA extraction and Q-PCR

The primer pair to measure PLC5 (At5g58690) expression levels was: 5'-CCTCTCAACATGCAGCGGCTATGGAAGG-3' and 5'-GAGAATTATTGTTCATAAAGTCCACGGATACAGGCAAG-3'. The relative gene expression was normalized by comparative threshold cycle value. Transcript levels were normalized by the level of SAND (At2g28390; forward primer: 5'-AAC TCT ATG CAG CAT TTG ATC CAT C-3', reverse primer: 5'-TGA TTG CAT ATC TTT ATC GCC ATC-3'). Three biological replicates and two technical replicates were used for the values of means and SDs (Han et al. 2013).

### Histological analyses for GUS activity

The pPLCS::GUS line was generated by Hunt et al. (2004) and kindly provided by Dr. Julie E. Gray (University of Sheffield). Plants and seedlings were grown for the times indicated (Fig. 2; Supplementary Fig. S3) and incubated overnight at 37°C in X-Glc solution, containing 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM phosphate buffer pH 7.0 and 0.1% Triton X-100 (Zhang et al. 2018). Next day, solutions were replaced by 70% ethanol to de-stain the tissue, after which the material was analyzed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera).

### Stomatal aperture

Stomatal aperture measurements were performed according to Di Stefano et al. (2012) with minor changes. Epidermal strips were excised from the abaxial side of fully expanded Arabidopsis leaves of 3-week-old plants grown at 22°C under 16 h of light and 8 h of dark, and immediately floated in opening buffer (5 mM MES-KOH, pH 6.1, and 50 mM KCl) for 3 h. Strips were then treated with ABA (0–10 µM). Stomata were digitized using a Nikon DS-Fi 1 camera, coupled to a Nikon Eclipse Ti microscope. The stomatal aperture width was subsequently measured using ImageJ software (National Institute of Health).
32P, phospholipid labeling, extraction and analysis

Two different tissues were used, whole seedlings and epidermal leaf peels. For seedlings, 5-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). Samples were treated next day by adding 200 μl of labeling buffer with or without sorbitol for the times and concentrations indicated. For epidermal leaf peels, leaves of 3-week-old plants grown at 22°C under short-day conditions at 22°C were detached and the fresh weight was determined by weighing them every hour. Water content was calculated as a loss, rosettes from 4-week-old plants were detached and the fresh weight was determined by weighing them every hour. Water content was calculated as a loss. For epidermal leaf peels, leaves of 3-week-old plants grown at 22°C under short-day conditions at 22°C were stratified for 2 d at 4°C in a 48-well cell culture plate (Greiner bio-one) for 3 h. Treatments were stopped by adding perchloric acid at a final concentration of 5% (v/v), and the lipids were extracted and separated as described previously (Munnik et al. 1994, Munnik and Zarra 2013). Radioactive lipids were visualized by autoradiography and quantified by phosphoimaging (Typhoon FLA 7000, GE Healthcare). Individual phospholipid levels are expressed as the percentage of the total [32P]lipid fraction.

Drought tolerance

Drought assays were performed as described earlier (Zhang et al. 2018). Seeds were stratified for 2 d at 4°C in the dark, and sown on soil. Each square pot (4.5 x 4.5 x 7.5 cm) contained 80 g of soil and nine plants, which were grown under short-day conditions at 22°C with 12 h light/12 h dark for 4 weeks and then subjected to dehydration by withholding water for 2 weeks while control plants were watered normally. Each experiment (n = 3) used 36 plants per genotype, and experiments were repeated at least four times. To assay water loss, rosettes from 4-week-old plants were detached and the fresh weight was determined by weighing them every hour. Water content was calculated as a percentage from the initial fresh weight. Twenty plants were used for each experiment, and each 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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Q. Zhang et al. | Functional characterization of Arabidopsis PLCs


