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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 metazoans that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate and diacylglycerol as second messengers involved in mutiple 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, *PLC5* 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 PIP_2 levels and increased amounts of phosphatidic acid, indicating enhanced PLC activity in vivo. Reduced PIP_2 levels and stunted root hair growth of *PLC5-OE* seedlings could be recovered by inducible overexpression of a root hair-specific PIP 5-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 PIP_2 is essential for the polar tip growth of root hairs.

Keywords: Drought tolerance • Lateral root formation • PIP_2 • Root hair tip growth • Stomatal aperture.

Abbreviations: DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; GUS, β-glucuronidase; IP_3, inositol 1,4,5-trisphosphate; IP_6, inositol hexakisphosphate; IPP, inositol polyphosphate; KD, knock-down; MS, Murashige and Skoog; OE, overexpressing; PA, phosphatidic acid; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP5K3, PIP 5-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 (IP_3) and diacylglycerol (DAG) by catalyzing the hydrolysis of the minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). The water-soluble IP_3 diffuses into the cytosol where it triggers the release of Ca^{2+} from the endoplasmic reticulum (ER) via a ligand-gated Ca^{2+} channel, while the lipid DAG remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family or stimulates TRP- (transient receptor potential) type ion channels. 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 IP_3 and DAG, i.e. IP_3 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 PIP_2, 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, reviewed in Munnik and Vermeer 2010). 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, Heilman 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 trypanosomes (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 polyphosphate (IPP) species 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 (Tsui 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 COI1, 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, Gle1-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).

Plant PLC signaling has been implicated in various biotic and abiotic stress responses. Many *PLC* genes are up-regulated in response to abiotic stress, including salt, drought, heat and cold (Hirayama et al. 1995, Hunt et al. 2004, Kim et al. 2004, Lin et al. 2004, Mills et al. 2004, Das et al. 2005, Vergnolle et al. 2005, Liu et al. 2006, Tasma et al. 2008, Sui et al. 2008, Li et al. 2017), but also to pathogens (Vossen et al. 2010, Gonorazky et al. 2014, Abd-El-Haliem et al. 2016, Gonorazky et al. 2016, D'Ambrosio et al. 2017). Some of these stresses have been correlated with changes in IP_3 using a commercial binding assay (DeWald et al. 2001, Takahashi et al. 2001, Ruelland et al. 2002, Zheng et al. 2012, Gao et al. 2014), that is under debate in the plant field (Munnik and Vermeer 2010, Munnik 2014). Increases in PA have also been reported, and some of these were, at least in part, generated by diacylglycerol kinase (DGK) rather than phospholipase D (PLD), which is another important PA generator (Wang et al. 2006, Arisz et al. 2009, Arisz and Munnik 2013, Arisz et al. 2013, Hong et al. 2016, Hou et al. 2016). As such, PLC–PA responses have been associated with plant defense (van der Luit et al. 2000, Hartog et al. 2003, De Jong et al. 2004, Laxalt et al. 2007, Vossen et al. 2010, Lanteri et al. 2011, Raho et al. 2011, Gonorazky et al. 2014, Gonorazky et al. 2016, Cacas et al. 2017, D'Ambrosio et al. 2018), as well as to cold, heat, salt osmotic and ABA responses (Munnik et al. 2000, DeWald et al. 2001, Munnik and Meijer 2001, Ruelland et al. 2002, Zonia and Munnik 2004, van Leeuwen et al. 2007, Darwish et al. 2009, Mishkind et al. 2009, Arisz et al. 2013, Simon et al. 2014). In response to salt, heat and ABA, an increase in PIP_2 is observed (Pical et al. 1999, DeWald et al. 2001, Zonia and Munnik 2004, van Leeuwen et al. 2007, König et al. 2008, Darwish et al. 2009, Mishkind et al. 2009, Horvath et al. 2012, Simon et al. 2014, Li et al. 2017, Zhang et al. 2018). Whether this reflects a mechanism to increase PLC's substrate or a role for PIP_2 as a second messenger itself remains unknown (Munnik and Vermeer 2010, Munnik 2014, Heilmann and Heilmann 2015, Heilmann 2016a, Heilmann 2016b, Heilmann and Ischebeck 2016, Gerth et al. 2017).

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, Gonorazky 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 (Kanehara 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-At*PLC3* 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 *AtPLC5*, 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 *AtPLC5* 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 PIP_2 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 At5g58690) (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 T_3 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 22ºC) 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 hydatodes), 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 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**). pPLC5-GUS also showed strong expression in guard cells (**Fig. 2m**).

Together, our results confirm that *PLC5* is expressed throughout the plant (Hunt et al. 2004, Tasma et al. 2008), but also reveal that its expression is predominantly restricted to the vasculature, and in lower amounts to the trichomes and guard cells. The latter led us to look into guard cells and trichome, but no aberrations in structure or numbers were apparent (not shown).

Analysis of PPI and PA levels in *plc5-1* mutants

To determine whether the knock-down of *PLC5* caused any changes in the levels of PLC's substrates (i.e. PIP and PIP_2) or product (conversion of DAG into PA) (Munnik et al. 1998b, Ruelland et al. 2002, Arisz et al. 2009, 2013), seedlings were $^{32}P_i$ labeled overnight and their lipids were extracted and analyzed. However, no significant differences in PIP_2, PIP and PA were found between wild-type and *plc5-1* seedlings (**Fig. 3**). This may be due to redundancy or reflect the low percentage of cells expressing PLC5.

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

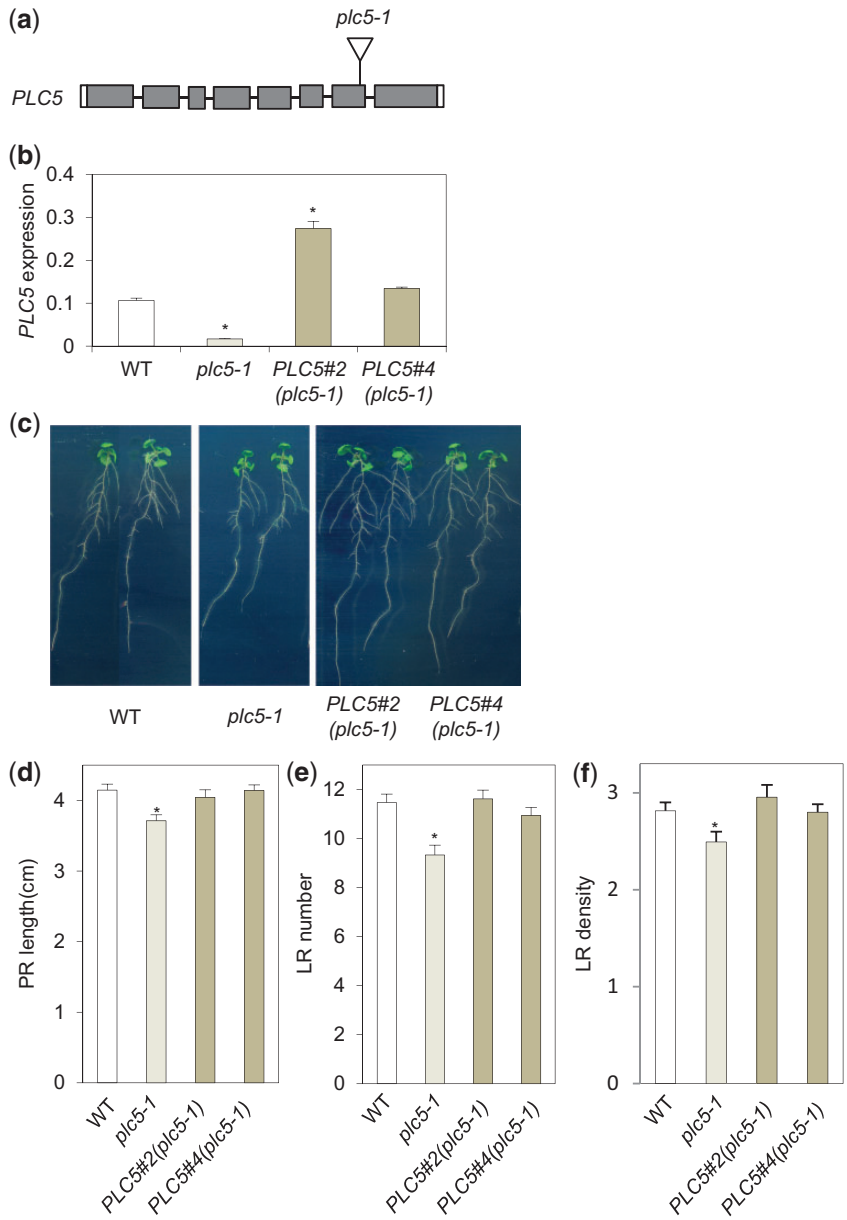

Fig. 1 Phenotypic analysis of *plc5-1* seedlings and complementation by the wild-type *PLC5* gene. (a) Representation of the *PLC5* gene and T-DNA insertion position of *plc5-1*. Filled boxes and lines represent exons and introns, respectively. Open boxes and the triangle represent untranslated regions and T-DNA insertion, respectively. (b) Q-PCR analysis of the *PLC5* expression level in the wild type, *plc5-1* and two complementation lines, *PLC5#2* and *#4* (in the *plc5-1* background using *SAND* as a reference gene. Values are means ± SD (n = 3). (c) Seedling morphology of the wild-type, *plc5-1* and complementation lines. Seeds were germinated on 1/2 MS plates with 0.5% sucrose for 4 d, then transferred to 1/2 MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d) Primary root (PR) length and lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n >20). An asterisk (*) indicates significance at P <0.05 compared with the wild type, based on Student's t-test.

potency of PLC5, transgenic overexpressing plants were generated using the ubiquitin (UBI10) promoter. Ten transgenic plants were selected from T_0 to T_3, 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

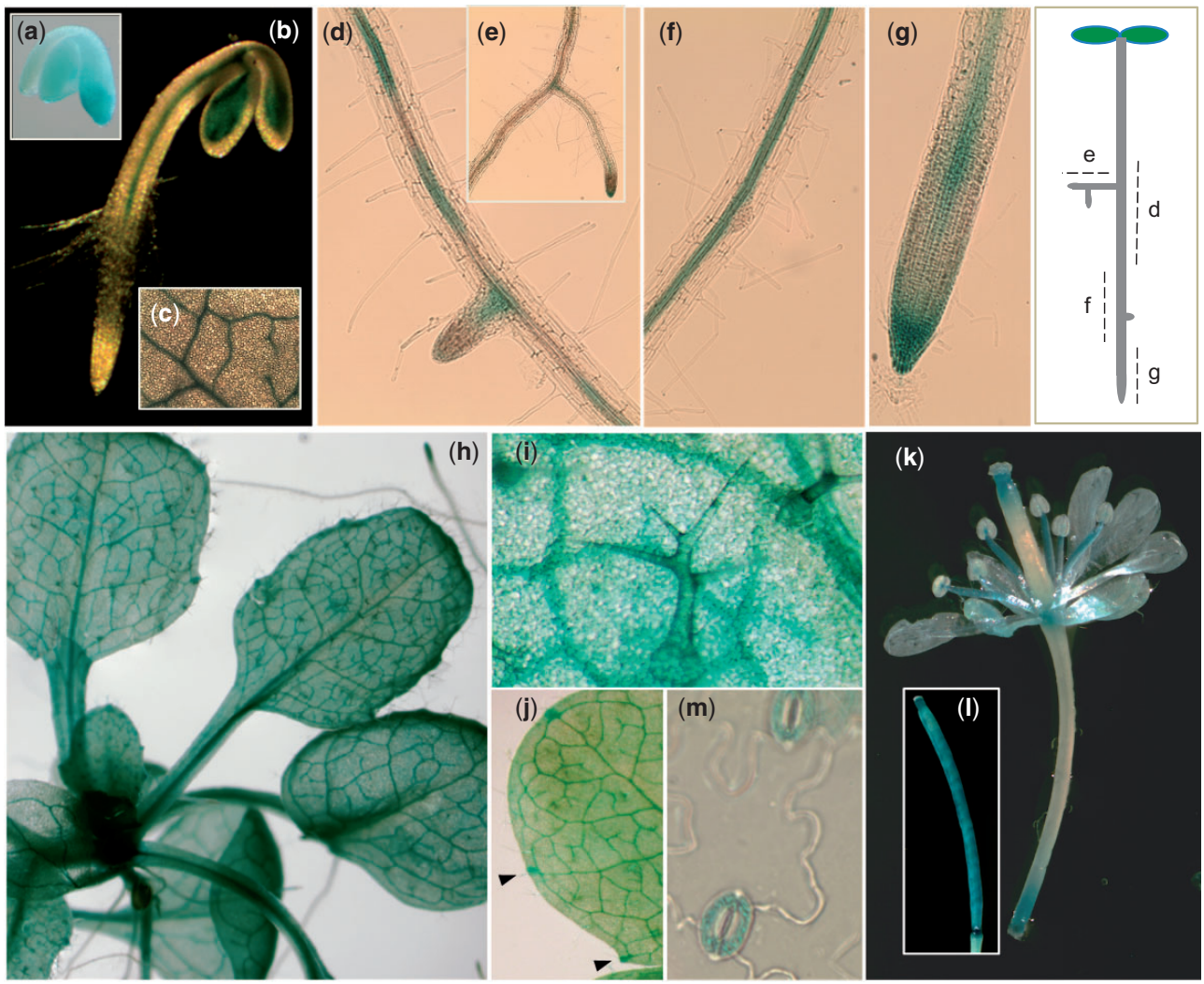

Fig. 2 *pPLC5::GUS* expression analyses in seedlings and mature tissues of Arabidopsis. (a) GUS activity was present in embryo cotyledons and roots during seed germination, from testa rupture until radical emergence (28 h after transfer from 4°C to 22°C), in the vasculature of 2-day-old (b) and 10-day-old 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.

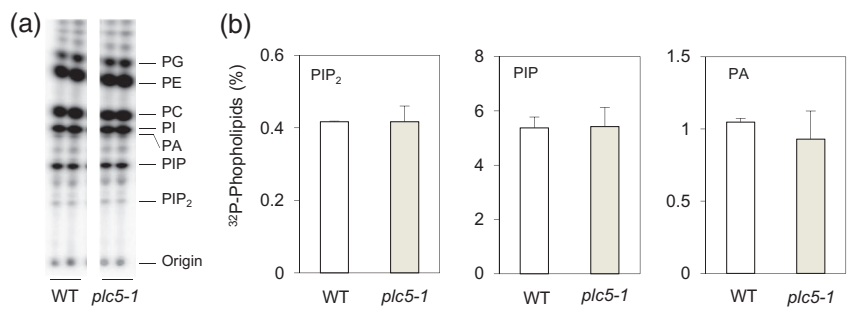

Fig. 3 PPI and PA levels in wild-type and *plc5-1* seedlings. Five-day-old seedlings were labeled with $^{32}PO_4^{3-}$ overnight and the next day their lipids were extracted and separated by thin-layer chromatography (TLC). (a) Autoradiograph of a typical labeling experiment, each lane representing 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.

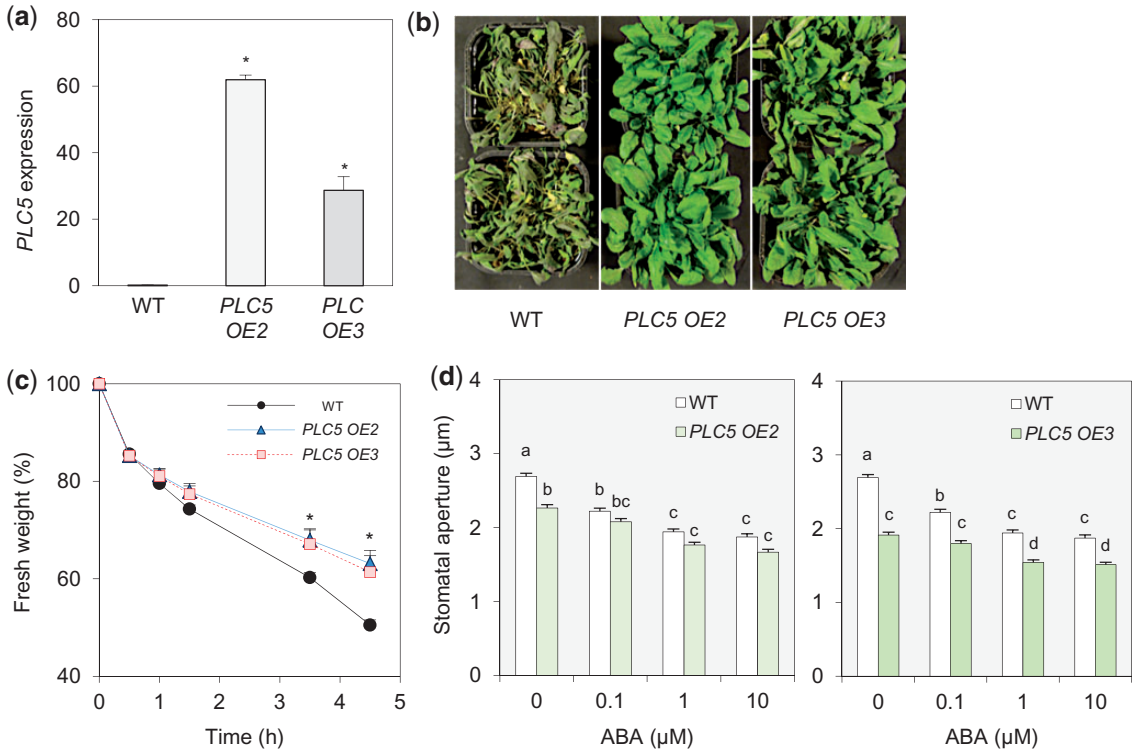

Fig. 4 *PLC5* overexpression increases drought tolerance. Overexpression lines were generated and the expression level of *PLC5* determined by Q-PCR, relative to the expression of *SAND*. Values are means ± SD ($n = 3$) for one representative experiment. The experiment was independently repeated three times with similar results. (b) Phenotype of 4-week-old wild-type and *PLC5-OE* plants, grown on soil and exposed to drought by withholding water for 2 weeks. (c) Water loss of detached rosettes. Water loss was measured at the indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment ($n = 36$). Experiments were repeated at least three times and all gave similar results. (d) ABA-induced stomatal closure in wild-type, *PLC5-OE2* (left), *PLC5-OE3* (right) plants. Leaves from 3-week-old plants were stripped and incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentrations of ABA for 90 min, after which stomata were digitalized and the aperture width measured. Data were analyzed by one-way ANOVA. Statistically significant differences between genotypes are indicated by letters ($P < 0.05$, Dunn's method). Values are means ± SE of at least three independent experiments ($n \geq 100$).

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. S6). 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. S4e–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-OE2* (~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. 2008, 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

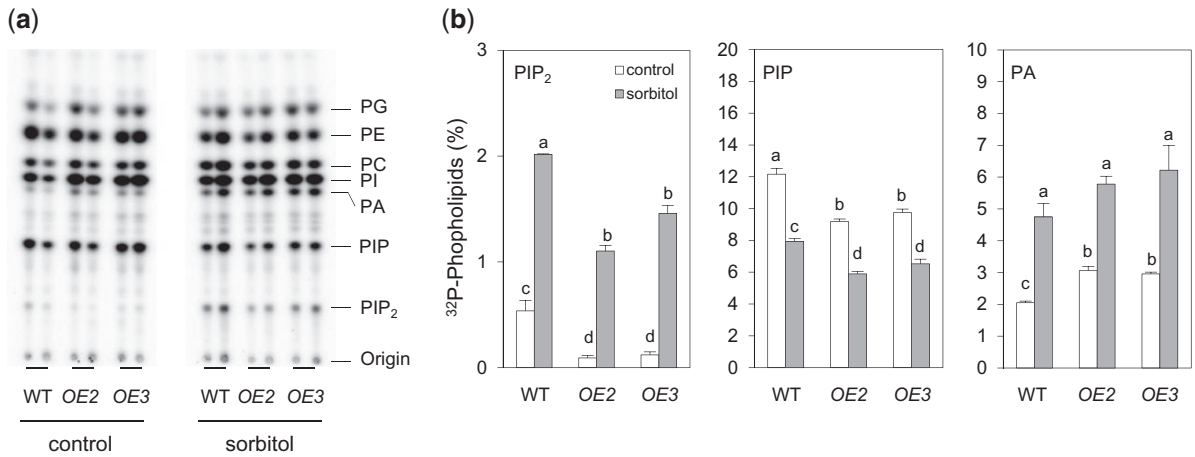

Fig. 5 PIP, PIP_2 and PA levels in *PLC5-OE* lines with and without osmotic stress. Six-day-old seedlings were $^{32}P_i$ labeled for 3 h and then treated with buffer ± 600 mM sorbitol for 30 min. Lipids were extracted, analyzed by thin-layer chromatography (TLC) and quantified by phosphoimaging. (a) Typical TLC profile with each lane containing one-fifth of the lipid extract of three seedlings. (b) ^{32}P levels of PIP_2, PIP and PA of the wild type and *PLC5-OE* lines #2 and #3 under control conditions and with sorbitol. Data shown are the means ± SE (n = 3) from three independent experiments. Data were analyzed by two-way ANOVA. Statistically significant differences between genotypes are indicated by letters ($P < 0.05$).

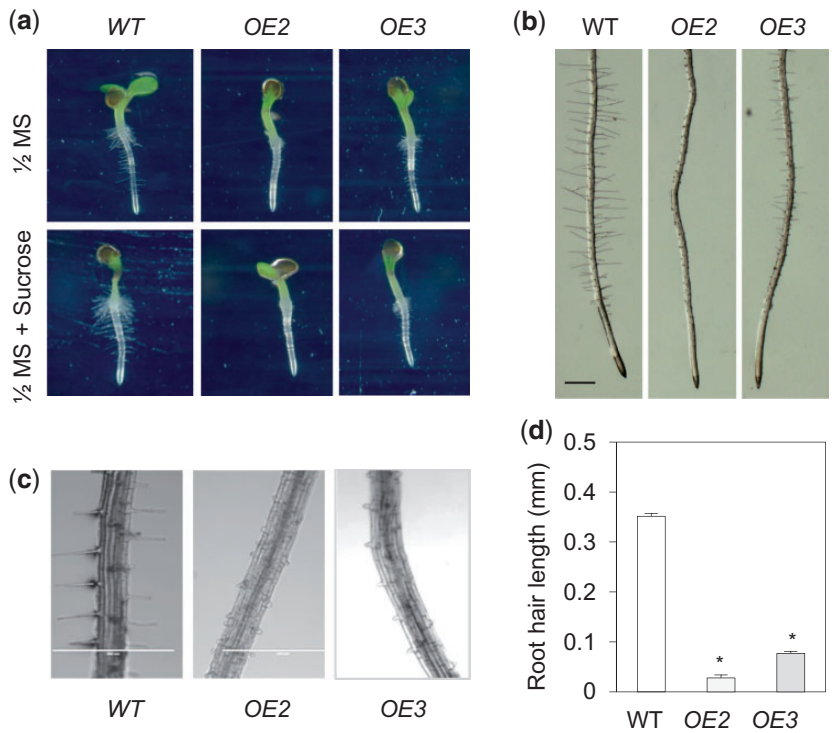

Fig. 6 Overexpression of *PLC5* affects root hair growth. (a–c) Root hair phenotypes of the wild type and *PLC5-OE* lines #2 and #4 of (a) 2-day-old seedlings on 1/2 MS plates ± sucrose to check sugar dependency, (b) 6-day-old seedlings and (c) details of their root hairs (4 d old). (d) Root hair length measurements of wild-type and *PLC5-OE* 6-day-old seedlings. Values are means ± SE of three independent experiments (n >200). An asterisk (*) indicates significance at $P < 0.05$ compared with the wild type, based on Student's t-test. Scale bar = 0.5 mm.

PLC5-OE2 with an estradiol-inducible overexpressor of PIP5K3 (*ER8-PIP5K3*) that is known to produce PIP_2 at the root hair tip and to induce root hair formation (Kusano et al. 2008). T_3 transgenics 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

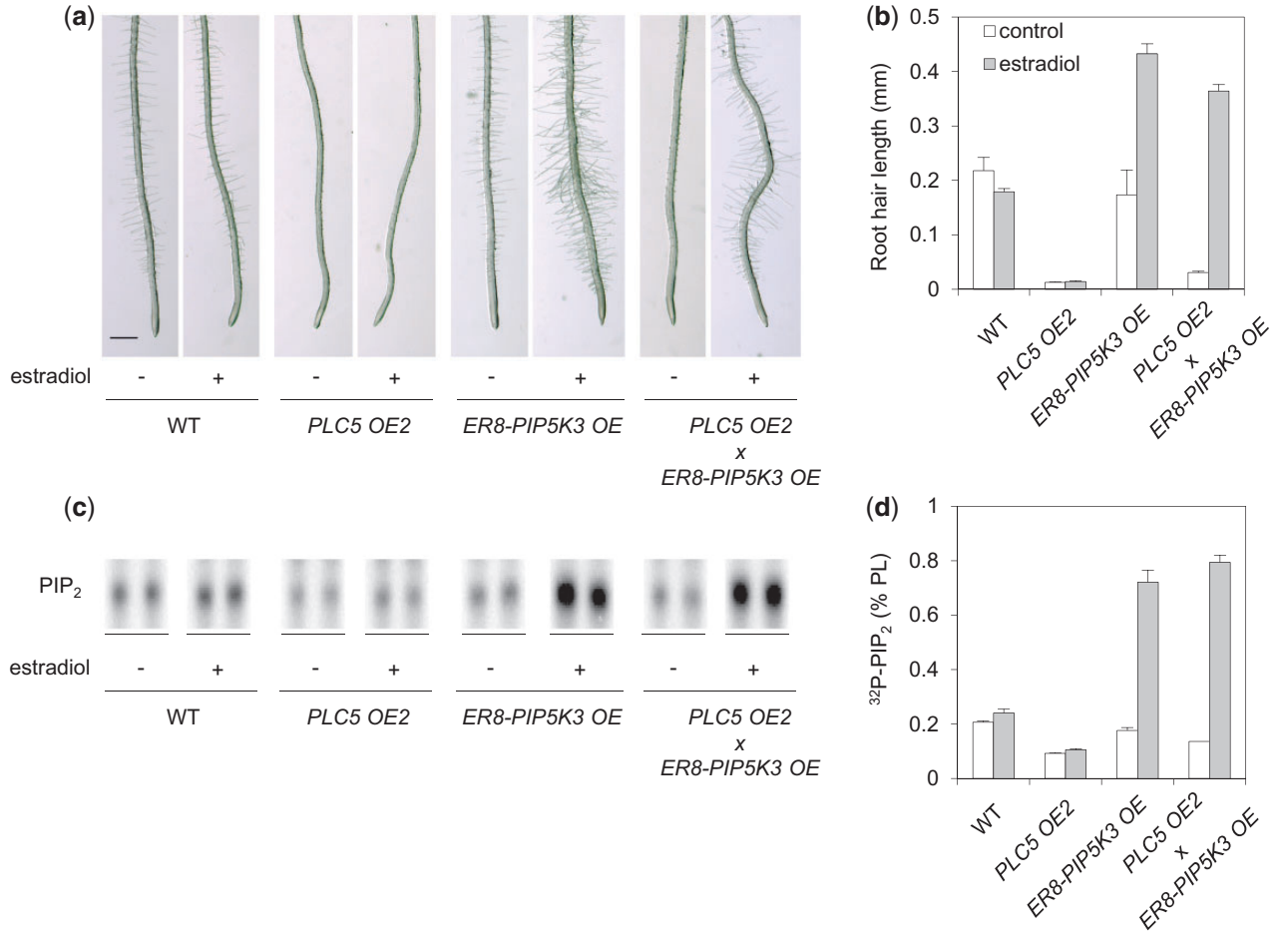

Fig. 7 Root hair phenotype of *PLC5-OE* lines is rescued by inducible expression of *PIP5K3*. (a) Root hair phenotypes of the wild type, *PLC5-OE2*, *ER8-PIP5K3* and *PLC5-OE2*×*ER8-PIP5K3* ± estradiol induction. *ER8-PIP5K3* is an estradiol-inducible overexpression line (Kusano etal. 2008). Seeds were first germinated on 1/2 MS plates supplemented with 0.5% sucrose for 4 d, and then transferred to plates ± 10 μM estradiol. Seedlings were scanned 3 d after transferring. Scale bar = 0.5 mm. (b) Quantification of root hair length after estradiol induction. (c) PIP_2 levels after estradiol induction. For the latter, seedlings were grown on 1/2 MS plates with 0.5% sucrose for 4 d and then transferred to plates ± 10 μM estradiol for 3 d, after which they were labeled overnight with $^{32}P_i$ to measure the changes in PIP_2 (d). Values are calculated as the percentage of total ^{32}P-labeled phospholipids and represented as means ± SD (n = 3). The experiment was repeated twice with similar results.

PLC5-OE2 (**Fig. 7a, b**). Determining the PIP_2 levels in these lines revealed that without estradiol, *PLC5-OE2* and *PLC5-OE2 ER8-PIP5K3* lines contained PIP_2 levels that were significantly lower than the wild type and *ER8-PIP5K3*. However, upon induction by estradiol, PIP_2 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 PIP_2 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 IP_3 and DAG (i.e. ligand-gated Ca^{2+}, 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 IP_3

and DAG, i.e. IPPs and PP-IPPs, and the lipids PA and DGPP, act as plant signaling molecules (Wang et al. 2006, Arisz et al. 2009, Munnik and Vermeer 2010, Testerink and Munnik 2011, Gillaspy 2013, Hou et al. 2016, Gerth et al. 2017, Noack and Jallais 2017).

Overexpression of *PLC5* resulted in decreased levels of PIP and PIP_2, 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 PIP_2 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 PIP_2 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 PIP_2 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 IP_2, 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 PIP_2 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 organiztion 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, Gujas et al. 2017, Kalmbach et al. 2017, Noack and Jaillais 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 Jaillais 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 promotor–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 Bielach 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 proteasomal degradation (Kepinski and Leyser 2005). Interestingly, the crystal structure of TIR1 contains IP_6 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 IP_6 originates, we proposed that it could be formed though PLC, generating IP_2/IP_3 with subsequent phosphorylation into IP_6, 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 IP_6 de novo from inositol (Munnik and Vermeer 2010, Gillaspy 2011, Gillaspy 2013, Kuo et al. 2018). To couple IP_6 with TIR1 functionally, it will be important to determine the amount of IP_6 bound to TIR1 in wild-type and *plc* mutant backgrounds. For the latter, we would need induced *PLC* 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 aperture could also be a reason why *PLC5-OE* plants are smaller, since the capacity to convert CO_2 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 PSII activity in photosynthesis (Supplementary Fig. S9).

PLC5-OE lines displayed reduced levels of PIP and PIP_2, and increased levels of PA, which is in agreement with an increase in PLC activity. PIP_2 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 PIP_2 hydrolysis. Subsequent increases in IP_6 would facilitate the stomatal closure by triggering the release of intracellular Ca^{2+} (Lemtiri-Clieh et al. 2000, Lemtiri-Clieh 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, PIP_2 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 PIP_2 due to enhanced PLC5 hydrolysis, which would readily be picked up by these types of ^{32}P 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, Ca^{2+} 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.

PIP_2 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 PIP_2 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 PIP_2 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 PIP_2 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, Grierson 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 Nt*PLC3* 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 PIP_2, visualized by a biosensor, accumulated at the apex (Helling et al. 2006), similar to what was found in growing root hairs (van Leeuwen et al. 2007, Kusano et al. 2008). 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 PIP_2 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, Grierson 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 (Grierson et al. 2014). In animal cells,

PIP_2 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 (Synek et al. 2006, Hála et al. 2008, Gujas et al. 2017, Kalmbach et al. 2017, Wu et al. 2017). The local accumulation of PIP_2 has also been shown to correlate with vesicle secretion (Ischebeck et al. 2008). Similarly, PIP_2 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 PIP_2 has been suggested to participate there as well (Logan and Mandato 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 PIP_2 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 F_2 and F_3 generations using gene-specific primers (forward primer 5′-TGGAAACTCGCAGGATATGTC-3′; reverse primer 5′-TTGCGTCTTTGATATTCAGGG-3′) and by the combination between reverse primer and left border primer LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-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 F_2 and F_3 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 through 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 inducible 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.lifetechnologies.com) was used to generate *pPLC5::PLC5*. Oligonucleotide primers (5′-GGGGACAACTTTGTATAGAAAAGTTGCTTTATAATAGATTAAGAAGCT TCATATC-3′ and 5′-GGGGACTGCTTTTTTGTACAAACTTGCTCTTCAAAAA GTTCCTGCAATTTAG-3′), including attB4 and attB1r sites, were used to PCR amplify a region of approximately 770 bp upstream of the predicted *PLC5* ATG start codon. The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX1. Oligonucleotide primers (5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGAGAGATATGGG GAGTTAC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGAA AGTGAAACCGCATGAGAA-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-TNOS entry clone containing attR2 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*-OE lines, *pUBQ10::PLC5* was constructed. The *PLC5* CDS was amplified from cDNA using the following primers: AtPLC5-*Bsr*GI-fw (5′-GA GCTGTACAATGAAGAGAGATATGGGG-3′) and AtPLC5-T-*Bam*HI (5′-CGGG ATCCTTAAAGAAAGTGAAACCGCATGAG-3′). The PCR product was transformed into pJET1.2, sequenced and digested with *Bsr*GI and *Bam*HI. After gel extraction, the *Bsr*G1-AtPLC5-*Bam*HI fragment was cloned into the *Bsr*GI/*Bam*HI-digested pGreenII0029JV-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 T_3 generation and used for further experiments.

RNA extraction and Q-PCR

The primer pair to measure *PLC5* (*At5g58690*) expression levels was: 5′-CTTTCA ACATGCAGGGCTATGGAAG-3′ and 5′-GAGATTATTGTTCATCATAAAGTCC GG-3′. Total RNA was extracted with Trizol reagent (Invitrogen). RNA (1.5 μg) from 10-day-old seedlings was converted to cDNA using oligo$(dT)_{18}$ primers, dNTPs and SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Q-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystem). The relative gene expression was determined by comparative threshold cycle value. Transcript levels were normalized by the level of *SAND* (At2g28390; forward primer: 5′-AAC TCT ATG CAG CAT TTG ATC CAC T-3′, reverse primer: 5′-TGA TTG CAT ATC TTT ATC GCC ATC-3′). Three biological replicates and two technical replicates were used for the values of means and SDs (Han et al. 2013).

Histochemical analyses for GUS activity

The *pPLC5::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-Gluc reaction 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 destain 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 Distéfano 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).

$^{32}P_i$ 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 $^{32}P_i$ (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 16 h of light and 8 h of dark were stripped and immediately floated on 100 µl of opening buffer (10 mM MES, pH 6.1 and 50 mM KCl), containing $^{32}P_i$ (5–10 µCi) 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 Zarza 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 [^{32}P]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 (4.5×4.5×7.5 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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