Novel roles for phospholipase C in plant stress signalling and development

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Chapter 3

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

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ABSTRACT

In animal cells, phospholipase C (PLC) is known to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which release intracellular Ca^{2+} and activate protein kinase C (PKC), respectively, orchestrating a wide range of cellular- and physiological processes. Plants contain PLCs too but lack IP_3 receptors and PKC, and it still remains largely unclear what its physiological role is in plants and what molecular targets it has. The genome of *Arabidopsis thaliana* encodes 9 *PLC* genes. Earlier work on *PLC2*, *PLC3* and *PLC9* revealed roles for PLC in gametogenesis, ABA signalling, lateral root formation and heat stress tolerance. Here, we functionally characterised the role of *PLC5*. Promoter-GUS analyses revealed that this gene is predominantly expressed in vascular tissue, most likely the phloem, including roots, leaves and flowers, but expression was also detected in the root-apical meristem, in guard cells and in trichomes. We only managed to find one homozygous T-DNA insertion line, *plc5-1,* which turned-out to be a knock-down mutant, suggesting that a KO mutant is probably lethal. Growth of *plc5-1* plants on agar plates consistently exhibited a ~20% reduction in their lateral root formation. The latter was caused by a decrease in initiation rather than emergence of the lateral roots. PLC3 was found to be required for lateral root formation earlier (Chapter 2), but a double *plc3plc5* mutant did not intensify the phenotype, indicating the involvement of possible additional redundant PLCs. Complementation of *plc5-1* with the PLC5-wt gene, expressed behind its own promoter, restored growth and rescued the lateral root phenotype. UBQ10-Overexpression of *PLC5* did not affect lateral root development, but was found to stunt root hair growth, to decrease the stomatal aperture and to increase their tolerance to drought stress. *In vivo* $^{32}P_i$-labeling analyses of PLC's substrate/product lipids revealed no differences in *plc5-1* seedlings, however, *PLC5*-overexpression lines clearly exhibited reduced levels of PIP- and PIP_2 and increased levels of phosphatidic acid (PA), the latter likely produced through phosphorylation of PLC-generated DAG. Inducible overexpression of *PIP5K3* in *PLC5* overexpressor lines recovered the stunted root-hair growth and restored PIP_2 level. These results provide independent evidence for PIP_2's role in polar tip growth of root hairs, confirm PLC's involvement in lateral root formation, and that overexpression of *PLC* seems to increase drought tolerance in general.

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

INTROUDCTION

Phospholipase C (PLC) signaling is implicated in various cellular events and plays crucial roles in all eukaryotic cells. The pathway is best known from animal systems where it generates the second messengers, inositol 1,4,5- trisphosphate (IP_3) and diacylglycerol (DAG), which are formed though PLC-catalyzed hydrolysis of the minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). The water-soluble IP_3 diffuses into the cytosol where it triggers the release of Ca^{2+} from the ER via a ligand gated-Ca^{2+} channel, while the lipid DAG, remains in the plasma membrane where it recruits and activates members of the protein kinase C (PKC) family or stimulates TRP (transient receptor potential) type- ion channels. Stimulation of the PLC pathway activates multiple downstream signaling cascades, regulating multiple cellular processes (Irvine, 2006; Michell, 2008; Balla, 2013).

Plants also contain PLCs, but the signaling pathway is likely different from animal systems, as plants lacks the primary targets for both IP_3 and DAG, i.e., the IP_3 receptor, PKC and TRP channels (Zonia & Munnik, 2006; Wheeler and Brownlee, 2008; Munnik, 2014). Also different, is that its potential substrate, PIP_2 is hardly present in plant plasma membranes, while its precursor, phosphatidylinositol 4-monophosphate (PIP) is relative abundant (Munnik and Testerink, 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011; Munnik, 2014; Heilmann, 2016). *In vitro,* plant PLCs do hydrolyze both PIP and PIP_2 but *in vivo* it is still unknown what the normal substrate is (Munnik, 2014). Biochemical subfractionation analyses showed that most (>90%) PLC activity is present in the plasma membrane fraction (Munnik et al., 1998a).

IP_3 was initially linked to the release of intracellular Ca^{2+} (Gilroy et al., 1990; Blatt et al., 1990; Allen and Sanders, 1994; Hunt and Gray, 2001), but later this was shown to be caused by IP_6 that was produced by phosphorylating IP_3 (Lemtiri-Chlieh et al., 2000, 2003; Munnik and Vermeer, 2010). Similarly, not DAG, but its phosphorylated product, phosphatidic acid (PA) has been emerging as the plant lipid-second messenger (Munnik, 2001; Testerink and Munnik, 2005; Arisz et al., 2009; Pokotylo et al., 2014; Munnik, 2014; Heilmann 2016). In plants, PA can be further phosphorylated into diacylglycerolpyrophosphate (DGPP) by PA kinase, an enzyme that is lacking from animals but is present in fungi, oomycetes and trypanosomes (van Schooten *et al.*, 2006a). Whether the formation of DGPP reflects an attenuation of the PA signal or the formation of a new signal (this lipid is normally not there either), remains unknown. Meanwhile, various other inositolpolyphosphates (IPPs) than IP_6 are emerging as signaling molecules, also in fungi and animals, where they have been implicated in ion channel binding, phosphate sensing, transcription and embryonic development etc. In plants, IP_4 has been proposed to regulate a chloride channel (Zonia *et al.*, 2002), while IP_5 and IP_6 were discovered in the crystal structure of TIR1 and COI1, which are receptors for auxin- and jasmonate signaling, respectively(Tan *et al.*, 2007; Sheard *et al.*, 2010). Gle1, an mRNA export factor, is an IP_6-binding protein that has recently been identified as a key activator of the ATPase/RNA helicase, LOS4 (low expression of osmotically responsive genes 4), similar to the Gle1-IP_6-Dbp5 (a LOS4 homolog)

paradigm in yeast (Lee *et al.*, 2015). Recently, SPX-domain containing proteins were also identified as interactors with IP_6 and many of these proteins are involved in phosphate homeostasis(Kuo *et al.*, 2014; Puga *et al.*, 2014; Wild *et al.*, 2016). Besides Ca^{2+} release in guard cells upon ABA stimulation (Lemtiri-Chlieh *et al.*, 2003), IP_6 production has also been linked to disease resistance (Murphy *et al.*, 2008). Recently, it has been discovered that IP_6 can also be pyrophosphorylated to IP_7 and IP_8, which have also been implicated in signaling, including JA and plant defense (Laha *et al.*, 2015, 2016; Williams *et al.*, 2015). Besides the lipid-generated pathway via PLC, IPPs can also be synthesized by the conversion of glucose 6-phosphate into inositol 3-phosphate, which is then stepwise phosphorylated by various IPP kinases (Munnik and Vermeer, 2010).

Plant PLCs have been implicated in various abiotic stress responses. *PLC* gene expression is typically induced by various stresses, including salt, drought, heat, and cold stress (Hirayama et al., 1995; Hunt et al., 2004; Lin et al., 2004; Das et al., 2005; Vergnolle et al., 2005; Zhai et al., 2005; Skinner et al., 2005; Liu et al., 2006a; Tasma et al., 2008; Sui et al., 2008). Some of these have been correlated with changes in IP_3 (DeWald *et al.*, 2001; Takahashi *et al.*, 2001; Ruelland *et al.*, 2002; Zheng *et al.*, 2012; Gao *et al.*, 2014), but we currently know that these measurements are strongly subject to artifacts and to changes in the flux of IP_6, the most abundant IPP (for discussion, see Munnik and Vermeer, 2010; Munnik 2014). Increases in PA have also been reported and some of these were indeed generated (or at least partly) by DAG kinase (DGK) rather than phospholipase D (PLD), which is another important PA generator (Arisz *et al.*, 2009, 2013; Arisz and Munnik, 2013).

Increases in PIP_2 upon salt- or heat stress have been reported (DeWald *et al.*, 2001; van Leeuwen *et al.*, 2007; Mishkind *et al.*, 2009; Simon *et al.*, 2014), and these are mainly due to activation of a PI4P 5-kinase (PIPK) rather than inhibition of a PLC (Mishkind *et al.*, 2009; Zarza, 2017; Munnik Lab, unpublished). Decreased PIP levels have been reported in response to osmotic- and temperature stresses (Cho *et al.*, 1993; Pical *et al.*, 1999; DeWald *et al.*, 2001; Zonia and Munnik, 2004; van Leeuwen *et al.*, 2007; Darwish *et al.*, 2009; Mishkind *et al.*, 2009; Vermeer *et al.*, 2009; Horvath *et al.*, 2012; Munnik, 2014; Zarza *et al.*, 2016). While many of these responses have been correlated to PLC signaling, none of them have ever been functionally linked.

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

PLC has also been associated to biotic-stress responses (Luit et al., 2000; Hartog et al., 2003; De Jong et al., 2004; Vossen et al., 2010; Gonorazky et al., 2014, 2016), some in relation to nitric oxide (NO) signaling (Laxalt *et al.*, 2007; Lanteri *et al.*, 2011; Raho *et al.*, 2011). Genetic evidence for PLC's involvement in disease resistance has been obtained for tomato (Vossen *et al.*, 2010; Gonorazky *et al.*, 2014, 2016; Abd-El-Haliem *et al.*, 2016) and recently for Arabidopsis *PLC2* (D'Ambrosio *et al.*, 2017). In both cases, PLC seems to be involved in the production of reactive oxygen species (ROS); for AtPLC2 this involved RBOHD (D'Ambrosio *et al.*, 2017) .

While plant-PLC signaling is often linked to stress, there is also evidence for its involvement in growth- and developmental responses. For example, PLC is important for pollen tube growth in petunia and tobacco (Dowd *et al.*, 2006; Helling *et al.*, 2006) and affects male- and female gametophyte development in *Arabidopsis* and *Torenia fournieri* (Song et al., 2008; Li et al., 2015; Di Fino *et al*., 2017). In *Physcomitrella,* PLC is involved in cytokinin- and gravity responses (Repp *et al.*, 2004). In higher plants, IP_3 changes have been correlated to gravitropism and Ca^{2+} signaling too (Perera *et al.*, 1999, 2006; Stevenson *et al.*, 2000; Boss *et al.*, 2010) but it is unclear whether this involves a PLC. Loss-of-*PLC3* in Arabidopsis did not alter gravitropism response (chapter2; Zhang et al., 2017). The latter did, however, reveal reduced lateral root formation, reduced seed germination, and reduced sensitivity to ABA with respect to stomatal aperture and the inhibition of seed germination (chapter2; Zhang et al., 2017). Arabidopsis *plc3*- and *plc9* mutants also exhibit reduced thermotolerance responses (Zheng *et al.*, 2012; Gao *et al.*, 2014).

The Arabidopsis genome encodes 9 *PLC* genes (Hunt et al., 2004; Tasma et al., 2008; Munnik, 2014; Pokotylo et al., 2014). While *AtPLC3* is mainly expressed in the vasculature, most likely in the phloem and companion cells (Chapter 2; Zhang et al., 2017), we searched for other PLC genes specifically expressed in the phloem, and this resulted in the identification of *AtPLC5,* which belongs to a different subfamily than *AtPLC3* (Hunt et al., 2004; Tasma et al. 2008). Here, we provide evidence that *AtPLC5* plays a role in lateral root development and that its overexpression increases the plant's tolerance to drought. Interestingly, this overexpression led to a stunted root-hair phenotype, which is likely caused by the increased hydrolysis of PIP_2 at the tip of the root hair, required for its growth.

RESULTS

Knockdown of *PLC5* affects root development

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

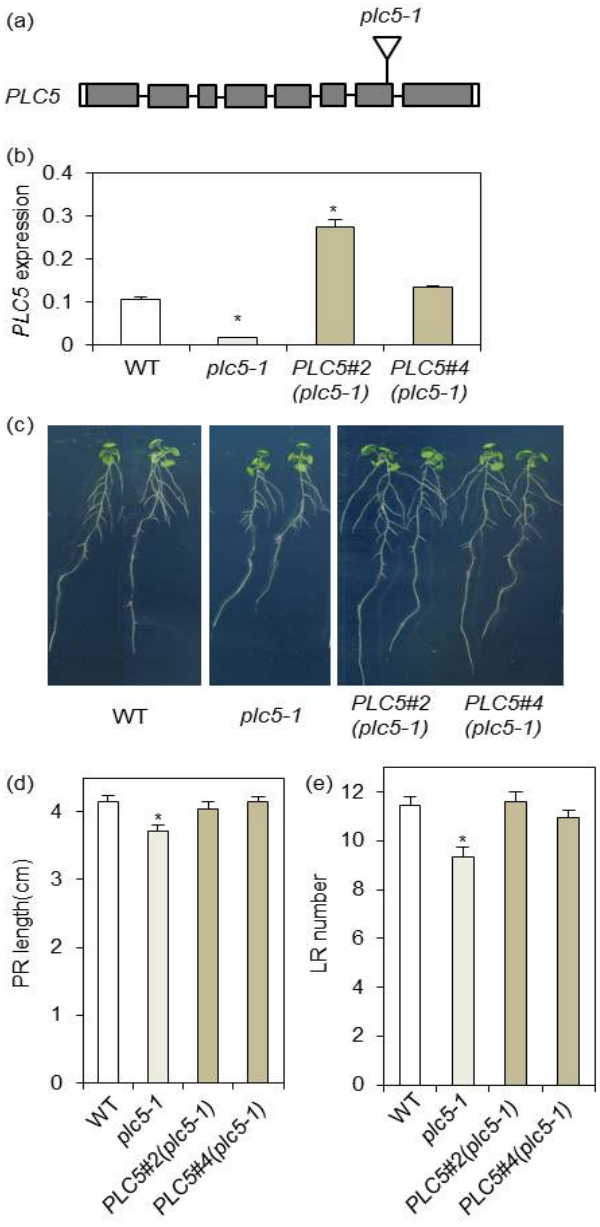

Figure 1. Phenotypic analysis of *plc5-1* seedlings and complementation by wild-type *PLC5* gene.
(a) Representation of the *PLC5* gene and T-DNA insertion position of *plc5-1*. Filled boxes and lines represent exons and introns, respectively. Open boxes and triangle represent untranslated regions and T-DNA insertion, respectively. (b) Q-PCR analysis of *PLC5* expression level in wild type, *plc5-1* and two complementation lines, *PLC5#2* and *#4* (in *plc5-1* background using *SAND* as a reference gene. Values are means ± SD (n=3). (c) Seedling morphology of wild type, *plc5-1* and complementation lines. Seeds were germinated on ½MS with 0.5% sucrose for 4 days, and then transferred to ½MS plates without sucrose. Photographs were taken 12 days after germination (DAG). (d) Primary root (PR) length and lateral root (LR) number at 12 DAG. Values are means ± SE of three independent experiments (n>20). Asterisk (*) indicate significance at P<0.05 compared to wild type, based on Student's *t* test.

Analyzing *plc5-1* mutant seedlings revealed shorter primary roots (10 %) and contained less (19 %) lateral roots than wild type (Fig. 1c-e), a phenotype that was also found for *plc3* mutants (Chapter 2; Zhang et al., 2017). Expression and phenotype could, however, be complemented by expressing the *PLC5* gene driven by its own promoter in the *plc5-1* mutant background in independent T3 lines (Fig. 1c-e), confirming PLC5's role in root development.

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

Expression of *PLC5* during plant development

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

Together, these results confirm that *PLC5* is expressed throughout the plant (Hunt *et al.*, 2004; Tasma *et al.*, 2008), but that expression is clearly restricted to the vasculature, trichomes and guard cells.

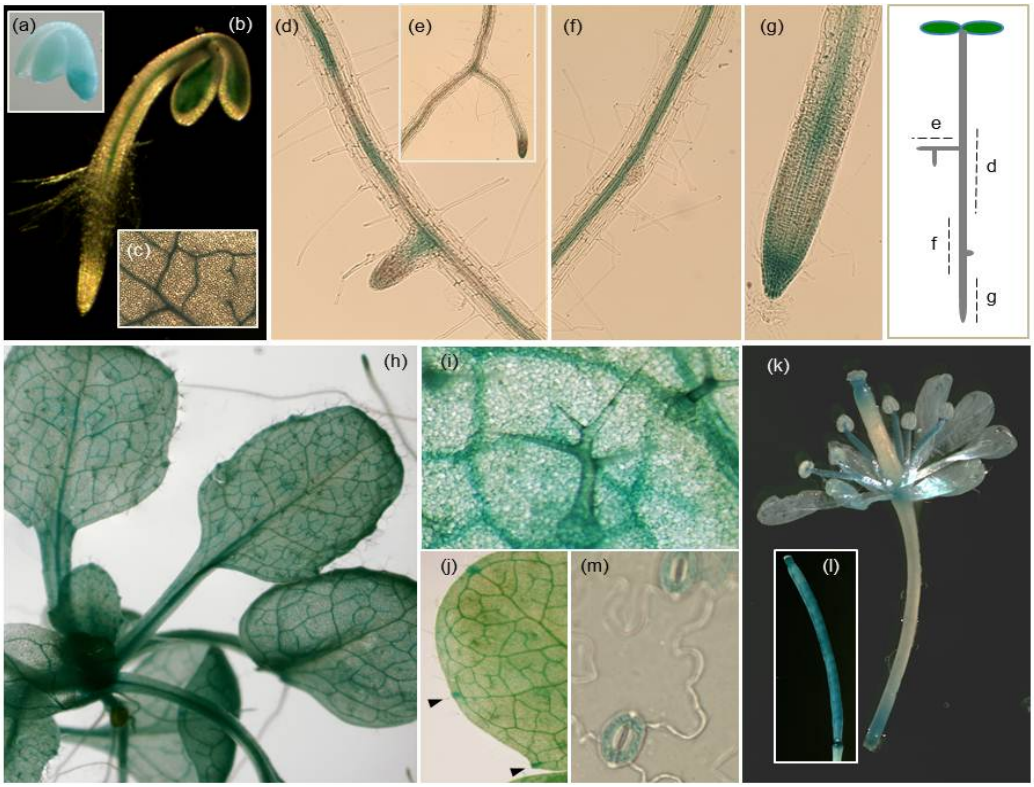

Figure 2. *pPLC5::GUS* expression analyses in Arabidopsis seedlings and mature tissues.
(a) GUS activity was present in embryo cotyledons and roots during seed germination, from testa rupture until radical emergence (28 hrs after transfer from 4°C to 22°C). GUS staining was observed in the vasculature of 2-d old- (b) and 10-d old seedlings, including leaf (c) and roots (d-g). GUS activity was also found in vascular tissue of mature 3 weeks old plants (h), trichomes (i), hydathodes (indicated by arrows) (j), guard cells (m), siliques (l) and in different parts of the flower (k), including style, filament, receptacle and pedicel.

Analysis of PPI- and PA levels in *plc5-1* mutant Arabidopsis seedlings

To determine whether the knockdown-of-*PLC5* caused any changes in the levels of PLC's substrates (i.e. PIP and PIP_2) or -product (i.e. the conversion of DAG into PA) (Munnik et al., 1998b; Ruelland et al., 2002; Arisz et al., 2009, 2013), seedlings were $^{32}P_i$- labeled O/N and their lipids extracted and analyzed. As shown in Figures 3a and 3b, no significant differences in PIP_2, PIP and PA levels were found between wt and *plc5-1* seedlings.

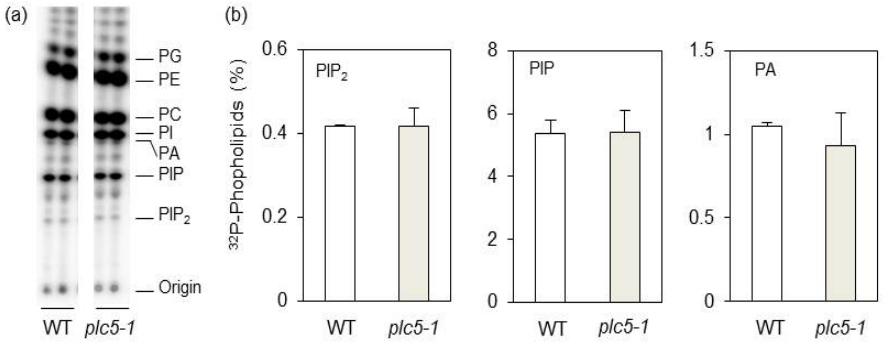

Figure 3. PPI- and PA levels in wild type- and *plc5-1* seedlings.
Five-day old seedlings were labelled with $^{32}PO_4^{3-}$ overnight and the next day their lipids extracted and separated by TLC. (a) Autoradiograph of a typical experiment, each lane representing 1/5th of the extract of three seedlings. (b) Quantification of ^{32}P-labelled PIP_2-, PIP- and PA levels in wild type and *plc5-1*. Values are calculated as the percentage of total ^{32}P-labelled phospholipids and are represented as means ± SD (n=3). The experiment was repeated twice with similar results.

Overexpression of *PLC5* increases tolerance to drought

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

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

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

To check the stomatal response in *PLC5-OE* lines, leaf peels were isolated as before and the ABA sensitivity analyzed. As shown in Figure 4d, stomatal opening in both *PLC5-OE* lines was significantly reduced compared to wt under control conditions, like *PLC3-OE* lines (Chapter 2, Fig 5). However, upon ABA treatment (0.1 μM), the stomates closed rapidly for all genotypes, but the aperture of the two *PLC5-OE* lines was significantly smaller than in wild type. Above 1 μM, this difference was maintained in *PLC5-OE3*, but was lost in *PLC5-OE2* (Fig. 4d).

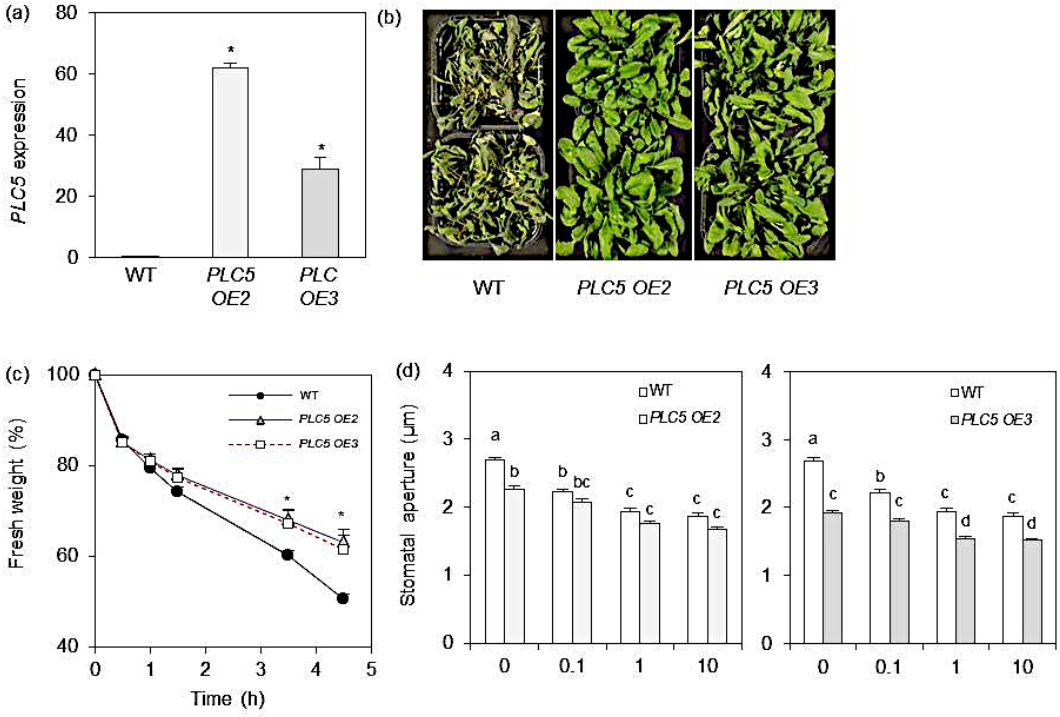

Figure 4. Overexpression of *PLC5* increases tolerance to drought.
Overexpression lines were generated and the expression level of *PLC5* determined by Q-PCR, relative to the expression of *SAND*. Values are means ± SD (n = 3) for one representative experiment. At least three experiments have been repeated with similar result. (b) Phenotype of 4-weeks old wild type- and *PLC5 OE* plants, grown on soil and exposed to drought by withholding water for 2 weeks. (c) Water loss of detached rosette. Water loss was measured at indicated time points and expressed as a percentage of the initial fresh weight. Values are means ± SD for one representative experiment (n=36). At least three experiments has been repeated with similar result. (d) ABA-induced stomatal closure in wild type, *PLC5 OE2* (left), *PLC5 OE3* (right) plants. Leaves from 3-weeks old plants were striped and incubated in opening buffer with light for 3 h until stomata were fully open. Peels were then treated with different concentration of ABA for 90 mins, after which stomata were digitized and the aperture width measured. Values are means ± SE of at least three independent experiments (n > 100). Asterisk (*) marks that *PLC5-OE* levels are significantly different from wild-type, based on Student's t-test (P< 0.05).

PLC5-overexpressing plants exhibit increased PPI responses

To determine whether overexpression of *PLC5* caused any changes in PPI- and/or PA levels, ^{32}P-labeling experiments were performed on seedlings (3h labeling) and the effect of sorbitol tested to mimic water stress. Interestingly, under control conditions, *PLC5-OE* lines exhibited a clear reduction in PIP_2- and PIP levels, by about 80- and 20% respectively, and an increase in PA by 30% (Fig. 5), indicating a constitutively higher PLC activity *in vivo*. O/N ^{32}P-labeling conditions gave similar results (Supplemental Fig. S4). Upon sorbitol treatment, however, a much stronger relative increase in PIP_2 was observed in the OE lines. While PIP_2 levels increased about 4-fold in wt, in the OE lines a massive, 12-fold increase was witnessed, although the absolute levels of both PIP_2 and PIP remained below that of wild type. The relative increase in PA and PIP was similar, however, for both wt and OE lines (Fig. 5b).

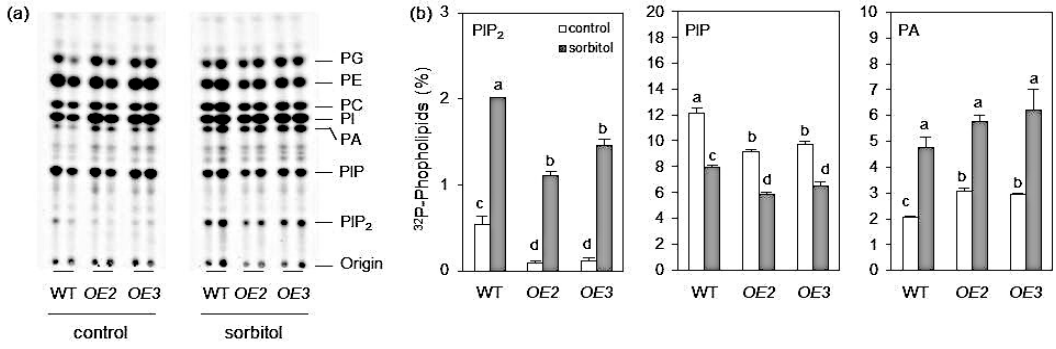

Figure 5. *PLC5 OE* lines have altered PIP-, PIP_2- and PA responses, both in control conditions and upon osmotic stress.
Six-day-old seedlings were $^{32}P_i$-labeled for 3h and then treated with buffer ± 600 mM sorbitol for 30 min. Lipids were extracted, analyzed by TLC and quantified by phosphoimaging. (a) Typical TLC profile with each lane representing 1/5th of the extract of 3 seedlings. (b) ^{32}P-levels of PIP_2, PIP and PA of wild-type and *PLC5 OE* lines #2 and #3 under control conditions and with sorbitol. Data shown are the means ± SE (n=3) from three independent experiments. Data was analyzed by 2-way ANOVA. Statistical significant differences between genotypes are indicated by letters (P<0.05).

Overexpression of *PLC5* stunts root hair growth by hydrolyzing essential PIP_2 at the tip

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

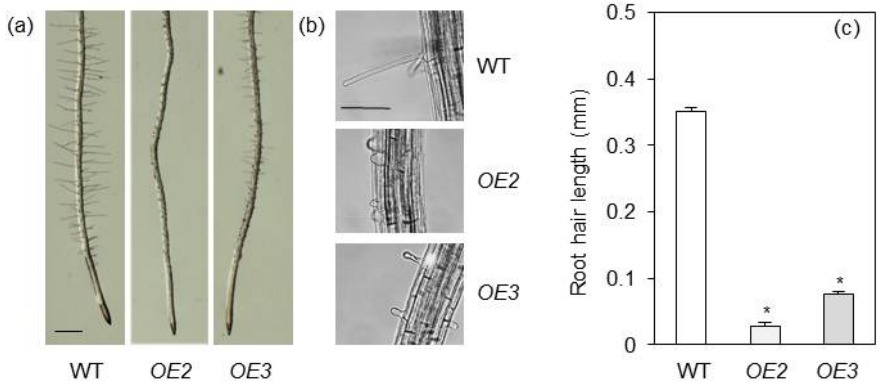

Figure 6. Overexpression of *PLC5* affects the growth of root hairs.
(a, b) Root hair phenotypes of wild type and *PLC5-OE* lines #2 and #4 of 6 days old seedlings (c) Root hair-length measurements of wild-type and *PLC5 OE* lines. Values are means ± SE of three independent experiments (n>200). Asterisk (*) indicates significance at P<0.05 compared to wild type, based on Student's *t* test. Bar = 0.5 mm.

Earlier, PIP_2 was found to promote root hair elongation at the tip of growing root hairs, and that PIP5K3 was involved in its generation(van Leeuwen *et al.*, 2007; Kusano *et al.*, 2008; Stenzel *et al.*, 2008; Grierson *et al.*, 2014). Since the *PLC5-OE* lines had reduced PIP_2 levels, i.e 83% in *PLC5 OE2* and 77% in *PLC5 OE3*, which corresponded to the severity of the root hair phenotype (Fig. 6a) and phenocopied the reduced root hair length of four independent T-DNA insertion *pip5k3* mutants, we

hypothesized that the root hair phenotype in *PLC5-OE* lines was caused by the continuous removal of PIP_2 from the tip that is essential for its growth. To confirm that PIP_2 is a key regulator for root hair elongation, we crossed *PLC5-OE2* with an estradiol-inducible over-expressor of PIP5K3 (*ER8-PIP5K3*) that is known to increase PIP_2 and to induce massive root hair formation (Kusano et al., 2008). T3 transgenic of *PLC5-OE2* x *ER8-PIP5K3* were selected and grown together with wild type, and the individual mutant lines, *PLC5-OE2* and *ER8-PIP5K3,* for four days on ½MS plates and then transferred to ½MS plates with and without 10 μM estradiol for another three days. Without estradiol, the *ER8-PIP5K3* lines showed similar root hair growth as wild type, while, the crossed line, *PLC5-OE2* x *ER8-PIP5K3* clearly exhibited the reduced root hair phenotype described above (Figs. 7a and 7b). After estradiol induction, the root hair length significantly increased in both *ER8-PIP5K3* and *PLC5-OE2* x *ER8-PIP5K3* lines, but did not change in wild type or *PLC5-OE2* (Figs. 7a and 7b). Determining the PIP_2 levels in all above lines, revealed that without estradiol *PLC5-OE2* and *PLC5-OE2 ER8-PIP5K3* lines showed PIP_2 levels that were significantly lower than wild type and *ER8-PIP5K3* line. However, upon induction by estradiol, PIP_2 levels in both *ER8-PIP5K3* and *PLC5-OE2 ER8-PIP5K3* went up sharply, while remained the same for wild type and *PLC5-OE2* (Fig. 7c and 7d). Increasing PIP_2 levels by estradiol-induced overexpression of PIPK3 clearly recovered root hair growth in the *PLC5-OE2* background.

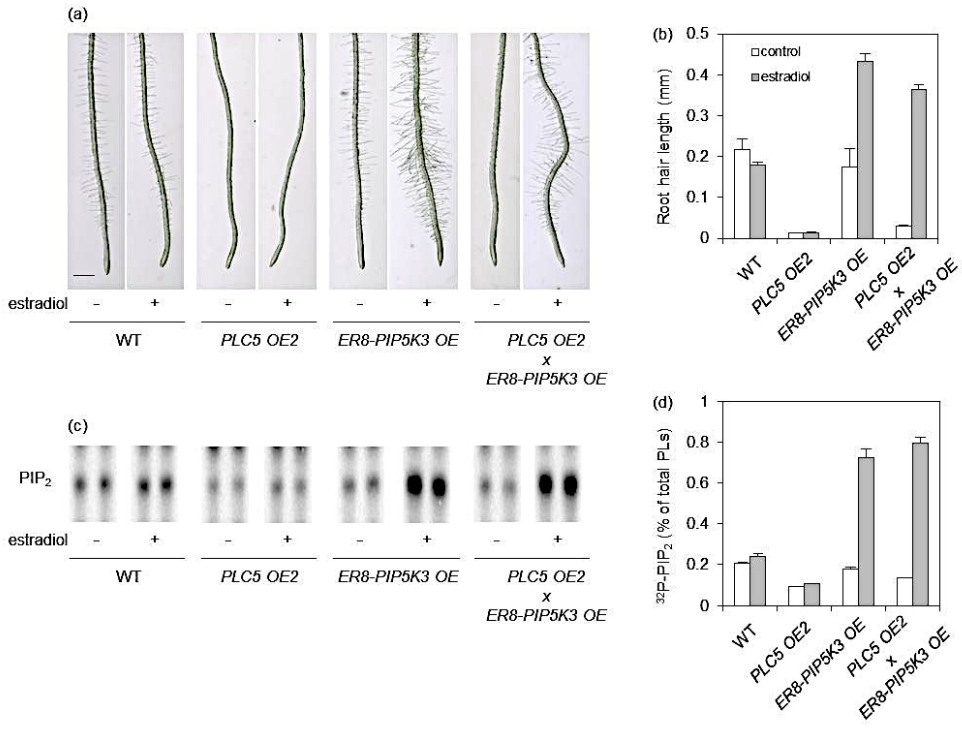

Figure 7. Root hair phenotype in *PLC5-OE* lines is rescued by inducible overexpression of *PIP5K3*.
(a) Root hair phenotypes of wild type, *PLC5-OE2*, *ER8-PIP5K3* and *PLC5-OE2* x *ER8-PIP5K3* after estradiol induction. *ER8-PIP5K3* is an estradiol-inducible overexpression line (ref). Seeds were first germinated on ½MS plates supplemented with 0.5% sucrose for 4 days, and then transferred to plates containing ± 10 μM estradiol. Seedlings were scanned three days after transferring. Bar = 0.5 mm. (b) Quantification of root hair length after estradiol induction. (c) PIP_2 levels after estradiol induction. For the latter, seedlings were grown on ½MS plates with 0.5% sucrose for 4 days and then transferred to the plates containing ± 10 μM estradiol for three days, after which they were labelled overnight with $^{32}P_i$ 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.

DISCUSSION

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

How PLC achieves this, is still largely unknown, but there are several possibilities. Plants lack the primary targets for IP_3 and DAG, so these molecules are unlikely to fulfill a second messenger role, even though we cannot completely rule this out, as plants may have evolved distinct targets than animals. Nonetheless, there is accumulating evidence that the phosphorylated products of IP_3 and DAG, which includes various IPPs and PP-IPPs and the lipids, PA and DGPP, are acting act the plant signaling molecules (van Schooten *et al.*, 2006b; Arisz *et al.*, 2009; Munnik and Vermeer, 2010; Testerink and Munnik, 2011; Gillaspy, 2013; Hou *et al.*, 2016). The reason for this remains unknown, but it is striking since seedlings are quite active in PA signaling (Wang *et al.*, 2006; Testerink and Munnik, 2011; Hou *et al.*, 2016).

Overexpression of *PLC5* resulted in decreased PIP- and PIP_2 levels and increased levels of PA, however not of DGPP. While DGPP responses are relatively abundant in algae and cell suspensions (Wissing *et al.*, 1994, 1995, 1992, 1993; Wissing and Behrbohm, 1993; Munnik *et al.*, 1996, 2000; Pical *et al.*, 1999; van der Luit *et al.*, 2000; Munnik and Meijer, 2001; Meijer and Munnik, 2003; Meijer *et al.*, 2017), in Arabidopsis seedlings this lipid is hardly detectable, even under stress (Arisz *et al.*, 2013).

While PIP_2 is the authentic PLC substrate in animal systems, in plants, PIP_2 concentrations are extremely low and hardly detectable in plasma membranes where most of the PLC activity is believed to reside(Munnik et al., 1998a, 1998b; Meijer and Munnik, 2003; van Leeuwen et al., 2007; Munnik, 2014; Simon et al., 2014; Tejos et al., 2014). In contrast, PI4P is 30-100 times more abundant, highly enriched in plasma membranes (Munnik *et al.*, 1994; Vermeer *et al.*, 2009; Vermeer and Munnik, 2013; Simon *et al.*, 2014, 2016), and is hydrolyzed equally *in vitro* (Munnik et al., 1998a; Munnik, 2014). Hence, *in vivo*, PI4P may actually be the common PLC substrate for plants. Obviously, this may be very different in stressed cells, where PIP_2 levels do go up, *e.g.* in response to ABA, salt stress or heat (Takahashi et al., 2001; DeWald et al., 2001; van Leeuwen et al., 2007; Mishkind et al., 2009; Darwish et al., 2009; Zhang et al., 2017) or during events where local PIP_2 turnover is high, but levels too low to be visualized and measured. Indeed, PI4P levels have been reported to drop in response to stimuli, e.g. upon salt and cold stress (Cho et al., 1993; Pical et al., 1999; DeWald et al., 2001; Ruelland et al.,

2002;Vermeer et al., 2009; Zarza et al., 2016), even though it still remains to be shown whether this reflects hydrolysis by PLC or PI4P phosphatase or an activation of PIPK, for example. Nonetheless, PLC hydrolysis of PI4P would still generate DAG and IP_2 that can be converted into PA and IPPs through DGK, IPK2 and IPK1, respectively (Munnik and Vermeer, 2010), and into PP-IPPs via VIP2 (Laha *et al.*, 2015, 2016), to activate downstream-signaling responses.

While basal levels of PIP_2 in plant cells are extremely low, this lipid is clearly emerging as a second messenger itself, involving various stress and developmental responses, including tip growth in pollen and root hairs, vascular differentiation, salt- and heat stress, ion-channel regulation, cytoskeletal organization, and membrane trafficking, including endo- and exocytosis (Ischebeck *et al.*, 2010; Gillaspy, 2013; Rodriguez-Villalon *et al.*, 2015; Heilmann, 2016) . Potential targets include small G-proteins like, K^+ channels, clathrin-adaptor proteins, and Exo70 (Ischebeck et al., 2010; Munnik and Nielsen, 2011; Gillaspy, 2013; Munnik, 2014; Heilmann, 2016). Similarly, PI4P has been emerging as a lipid second messenger too under certain conditions, and in particular cells (Stevenson *et al.*, 2000; Meijer and Munnik, 2003; Vermeer *et al.*, 2009; Munnik and Nielsen, 2011; Heilmann, 2016; Simon *et al.*, 2016) . In such cases, PLC could also function as an attenuator of signaling. However, whether PLC attenuates second messengers or produces them (or likely both!), clearly requires more research and will be difficult to address anyway because of the huge redundancy of both PPI/PA pathways (e.g. Arabidopsis has 9 *PLC*, 11 *PIPK*, 12 *PIK*, 7 *DGK* and 12 *PLD* genes), but also due to the highly localized events, such as within the vascular system (phloem, companion cells) in this case.

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

Knock-down of *PLC5* led to shorter primary roots and fewer lateral roots, which was functionally complemented by expressing *PLC5* behind its own promotor. Promotor-GUS analyses indicated that *PLC5* is predominantly expressed in the root vasculature, which was not homogenous but revealed some form of segmentation. Moreover, lateral roots always emerged from a segment, even though not every segment gave a lateral root, which was similar to what we found for *PLC3* (Chapter 2; Zhang et al., 2017). Interestingly, the emergence of tertiary roots revealed the same segmentation pattern, indicating that tertiary roots initiate from *PLC* segments in lateral roots (Figs. 2d,e). That the lateral root phenotype is quite mild in either *plc3* (knockout) or *plc5* (knockdown) mutants pointed to gene redundancy. We therefore created *plc3plc5*-double mutants, but they displayed a similar reduction in primary-root length and lateral-root number as the single mutants (Supplemental Fig. S2). These results indicate that more *PLCs* are involved in this phenotype. Re-analyzing the eFP browser further, we found two other *PLCs* expressed in the phloem vasculature, i.e., *PLC2* and *PLC7* (Chapter 4; Di Fino et al., 2017; Van Wijk, Laxalt and Munnik, unpublished). Unfortunately, *plc3plc5plc7*-triple mutants or *plc2*-single mutants were found to be homozygous lethal (Di Fino et al., 2017; Chapter 4; Munnik lab, unpublished). Inducible-silencing should offer new perspectives here.

Root growth and -branching are important events in root development and the phytohormone, auxin plays a key role in this (Péret, De Rybel, *et al.*, 2009; Péret, Larrieu, *et al.*, 2009; Benková and Bielach, 2010). Auxin regulates massive changes in gene expression by promoting the degradation of the transcriptional repressor, Aux/IAA through its receptor, TIR1. The latter is an F-box protein that forms an SCF complex that functions as a multi-protein E3 ubiquitin-ligase complex, which catalyzes the ubiquitination of Aux/IAAs destined for proteasomal degradation (Kepinski and Leyser, 2005). Interestingly, the crystal structure of TIR1 was found to contain IP_6, which is anticipated to regulate auxin binding and TIR1 activity (Tan et al., 2007; Chapter 3; Zhang et al., 2017). So far, it has remained unknown where this IP_6 would be coming from, but earlier we proposed that it could be formed though a PLC3-generated IP_2/IP_3 response, with subsequent phosphorylation into IP_6 at the PLC segments from which the lateral roots emerge (Chapter 3; Zhang et al., 2017). Our data here, indicates a similar role for PLC5. Double-*plc3plc5* mutants were expected to produce less PLC-generated IP_6 and hence, exhibit reduced auxin responsiveness and root development, but likely redundant PLCs, such as *PLC2* and *PLC7*, take over, even though it should be noted that *plc5-1* is a knock-down mutant. Alternatively, chemical redundancy could play a role, *e.g.* by making IP_6 via *de novo* synthesis (Munnik and Vermeer, 2010; Gillaspy, 2011; 2013). To functionally couple IP_6 with TIR1, it will be important to determine the amount of IP_6 in TIR1 in both wt and *plc*-mutant backgrounds. For the latter, we may require induced *PLC*-silencing lines in combination with KD- and KO mutants.

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

Overexpression of *PLC5* enhances drought tolerance

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

Under control conditions, *PLC5-O*E plants grew slightly slower, and eventually developed smaller in size and produced less biomass (Supplemental Fig. S4). However, upon drought stress, they performed much better (Figs. 4b,c), also better than *PLC3-OE* lines (not shown). *PLC5-O*E plants loose significantly less water than wild type, which is likely related to the increased number of closed stomata that we found under normal growth conditions (Fig. 4d), as there was no difference in the number of stomata (not shown). The reduction in stomatal aperture could also be the reason why *PLC5-OE* plants tend to be smaller, as CO_2 uptake and sugar production could be affected (Cornic and Massacci, 1996). Similarly, *PLC5-OE* lines led to a stunted root-hair growth (Fig. 6) (see discussion below), which could be another explanation for their smaller size, as nutrient uptake could be affected (Leitner *et al.*, 2009). To validate this, we measured glucose-, fructose- and sucrose levels in shoots and roots of 10-day old seedlings but found that *PLC5-OE* lines even contained slightly higher carbohydrate concentrations (Supplemental Fig. S6). Whether this is related to their drought tolerance or a consequence of their lower water content remains unclear.

PLC5-OE lines displayed reduced PIP- and PIP_2 levels and increased PA levels, likely reflecting increased PLC activity. Earlier, PIP_2 has been proposed to be important for stomatal opening by lowering the water potential through inhibition of SLAC1 (Lee *et al.*, 2007) and K^+-efflux channels (Ma *et al.*, 2009), which would favor the influx of water and open the stomata. Hence, the closed-stomata phenotype of *PLC5-OE* lines could be due to the strongly reduced PIP_2 levels.

In an attempt to mimic drought stress in seedlings and to analyze the effect on the PA- and PPI levels, we used sorbitol. Interestingly, PIP_2 levels dramatically increased upon this osmotic stress, and the accumulation was much stronger in the *PLC5-OE* lines (Fig. 5). The latter may reflect the increased turnover of PIP_2 due to enhanced PLC5 hydrolysis that would readily be picked-up by these ^{32}P-labeling experiments. Since UBQ10 drives *PLC5* expression in all cells, which is totally different from the endogenous, limited *PLC5* expression in the vasculature, enhanced PLC-signal formation (PA and IPPs) or PIP_2 attenuation may affect many cells, tissues and processes relevant to control responses to osmotic stress (Munnik and Vermeer, 2010).

Similarly, *PLC5-OE* lines led to a stunted root-hair growth (Fig. 6) (see discussion below), and this could be another explanation for their smaller size, as nutrient uptake could be affected (Leitner *et al.*, 2009). Strikingly, these lipid changes were not observed in the *PLC3-OE* lines, which did not reveal a root-hair phenotype either. It is not clear from the amino-acid sequence why PLC5 would be more active than PLC3. All AtPLCs contain the same domain structure, i.e. two EF-hands, a catalytic and CalB/C2 domain. So there must be subtle changes in the enzymatic properties, caused by Ca^{2+} sensitivity, K_m or V_{max} values, for example, or by interacting other proteins.

PLC-dependent PIP_2 generation is essential for root hair tip growth

Overexpression of *PLC5* also resulted in a very-short-root hair phenotype (Fig. 6), which is very likely due to the low amounts of basal PIP_2 required for tip growth (Fig. 5b; supplemental Fig. 6). Interestingly, these lipid changes and root-hair phenotype were not observed in the *PLC3-OE* lines, indicating differences in PLC3/5 activity or regulation. Evidence that this phenotype was coupled to PIP_2 came from the induced-overexpression of *PIP5K3* in *PLC5-OE2* line, which restored PIP_2 levels and rescued the root hair phenotype (Fig. 7). T-DNA insertion mutants of *PIP5K3* exhibit a similar short-root hair phenotype as *PLC5-OE* lines, and this lipid kinase has been shown to be responsible for generating the PIP_2 at the plasma membrane of the growing tip (van Leeuwen et al., 2007; Kusano et al., 2008; Stenzel et al., 2008; Ischebeck et al., 2008).

Similar results have been found in tobacco pollen tubes (Ischebeck *et al.*, 2008), whose tip growth resembles that of root hairs(Ovečka et al., 2005; Zonia and Munnik, 2008;Ischebeck et al., 2010; Grierson et al., 2014). Reduced PLC1 activity in petunia pollen tubes led to arrested- and depolarized pollen tubes, which was accompanied by a disorganization of the actin cytoskeleton (Dowd *et al.*, 2006). On the other hand, overexpression of Nt*PLC3* was shown to reduce pollen tube length (Helling *et al.*, 2006). FP-tagged NtPLC3 was localized at the flanks of the growing tip, while the PIP_2, visualized by a biosensor, accumulated at the apex (Helling *et al.*, 2006), similar to what was found in growing root hairs (van Leeuwen *et al.*, 2007; Kusano *et al.*, 2008). We found no aberrant root hair morphology in the *plc5-1* mutant, which could be due to the fact that this is not a KO mutant and/or due to *PLC* redundancy.

How PIP_2 is exactly involved in this polar tip growth remains unknown, but is likely to involve a complex signaling network between membrane trafficking and cytoskeletal dynamics (Ovečka et al., 2005; Ischebeck et al., 2010; Munnik and Nielsen, 2011; Grierson et al., 2014). Tip growth is sustained by exocytosis of vesicles containing growth materials, like polysaccharides and proteins, for growing cell wall and membrane (Grierson *et al.*, 2014). In animal cells, PIP_2 is involved in priming exocytosis and vesicle fusion by binding EXO70, a subunit form the exocyst complex (Aikawa and Martin, 2003; Munson and Novick, 2006; Liu *et al.*, 2007). Arabidopsis contains 23 EXO70s, and the exocyst complex is essential for pollen tube germination and growth (Synek *et al.*, 2006; Hála *et al.*, 2008)(Synek et al., 2006;Hála et al., 2008). The local accumulation of PIP_2 has also been shown to

correlate with vesical secretion (Ischebeck *et al.*, 2008). Apart from exocytosis, endocytosis is also important for cell growth and regulation and PIP_2 has been implicated in clathrin-mediated endocytosis (CME) as clathrin-adaptor proteins bind and are recruited by PIP_2 (Zhao *et al.*, 2010; Baisa *et al.*, 2013; Ischebeck *et al.*, 2013). To deliver and return vesicles to and from the cell expanding areas, the actin cytoskeleton also plays crucial roles and PIP_2 has been suggested to participate too (Gungabissoon *et al.*, 1998; Wasteneys and Yang, 2004; Logan and Mandato, 2006). An important PIP_2 target in regulating this is Rho GTPases (Ras-related monomeric small G proteins), which in plant are called Rop (Rho of plants). They are crucial regulators of tip growth (Kost, 2008) proposed to control the actin cytoskeleton and membrane trafficking (Lee *et al.*, 2008) and there is evidence that PIP_2 regulates Rop in organizing this (Kost *et al.*, 1999; Klahre *et al.*, 2006; Kost, 2008; Ischebeck *et al.*, 2011). In summary, it is clear that PIP_2 can regulate root hair growth via various cellular processes and it is not unlikely that PLC(s), plays a role it its attenuation and/or additional second messenger formation. Clearly, further studies are required to decipher the molecular downstream components of PPIs, IPPs and PA, and what the role of PLC is herein. Characterization of KO, KD and OE mutants provides a start at least.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana (Columbia-0) T-DNA insertion mutant *plc5-1* (SALK_144469), was obtained from SALK collection (signal.salk.edu). Homozygous plants were identified by PCR in F2- and F3 generations using gene-specific primers (forward primer 5'-TGGAAACTCGCAGGATATGTC-3'; reverse primer 5'-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 F2- and F3 generations. The *pPLC5::GUS* line was kindly provided by Dr. Julie E. Gray (Hunt *et al.*, 2004).

Root growth

Seeds were surface sterilized in a desiccator by using 20 ml thin bleach and 1 ml 37% HCl for 3 hours, and then sowed on square petri dishes containing 30 ml ½-strength Murashi-Skoog (½MS) medium (pH 5.8), supplemented with 0.5 % sucrose, and 1.2 % Daishin agar (Duchefa Biochemie). Plates were stratified at 4 °C in the dark for two nights and then transferred to long-day conditions (22 °C, 16 h of light, 8h of dark), placed vertically under an angle of 70°. Four-day-old seedlings with comparable size were then transferred to ½MS-ager plates without sucrose and scanned 10 - 12 days after germination (Epson Perfection V700 scanner). Primary root length, lateral root number, and average lateral root length was quantified for each genotype through imageJ-analysis software (National Institutes of

Health). For root hair visualization, seedlings were grown on ½MS with 0.5% sucrose for seven days and viewed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera). More than 200 root hairs per seedling were quantified and ~10 seedlings for each genotype were used for the measurement. For inducible overexpression, seedlings were grown on ½MS with 0.5% sucrose plate for four days and then transferred to agar medium supplemented with 10 μM β-estradiol for another three days.

Cloning and plant transformation

MultiSite Gateway Three-Fragment Vector Construction Kit (www.lifetechnologies.com) was used to generate *pPLC5::PLC5*. Oligonucleotide primers (5'-GGGGACAACTTTGTATAGAAAAGTTGCTTTATAATAGATTAAGAAGCTTCATATC-3' and 5'-GGGGACTGCTTTTTTGTACAAACTTGCTCTTCAAAAAGTTCCTGCAATTTAG-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'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGAGAGATATGGGGAGTTAC -3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGAAAGTGAAACCGCATGAGAA -3'), including attB1 and attB2 sites, were used to PCR amplify *PLC5* CDS. The amplified fragment was cloned into the donor vector by using BP Clonase II enzyme mix to create entry clone BOX2. BOX3 was pGEM-TNOS entry clone containing attR2 and attL2 sites, which was obtained from university of Amsterdam Plant Physiology published construct resources. The three entry clones and a destination vector (pGreen0125) were used in MultiSite Gateway LR recombination reaction to create expression clone (Multi gateway protocol).

To generate *PLC5* overexpression line, *pUBQ10::PLC5* was constructed. The PLC5 CDS was amplified from cDNA using the following primers: *AtPLC5-BsrG*I-fw (5'-GAGCTGTACAATGAAGAGAGATATGGGG-3') and *AtPLC5-T-BamHI*(5'-CGGGATCCTTAAAGAAAGTGAAACCGCATGAG-3'). The PCR product was transformed into pJET1.2, sequenced and digested with *BsrGI* and *BamHI*. After gel extraction the *BsrG*I-AtPLC5-BamHI fragment was cloned into the *BsrGI*/*BamHI* 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 T3 and used for further experiments.

RNA extraction and Q-PCR

The primer pairs to measure the *PLC5* (At5g58690) expression level were: 5'-CTTTCAACATGCAGGGCTATGGAAG-3' and 5'- GAGATTATTGTTCATCATAAAGTCCGG-3'. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) as described (Pieterse, 1998).

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

Histochemical analyses for GUS activity

Transgenic plants carrying p*PLC5::GUS* were grown for the times indicated in the legend. Specific tissues were taken and incubated in X-Gluc reaction solution containing 1mg/ml 5-bromo-4-chloro-3 indolyl-β-D-glucuronic acid (X-gluc), 50 mM phosphate buffer pH 7.0 and 0.1% TX-100 and incubated overnight at 37°C as described before (Chapter 2; Zhang et al., 2017). The next day, solutions were replaced by 70% ethanol to de-stain the tissue after which the material was analysed under a stereo microscope (Leica MZFLIII) and photographed (ThorLabs CCD camera).

Stomatal aperture

Stomatal-aperture measurements were performed according to 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 8h of dark, and immediately floated in opening buffer (5 mM MES-KOH, pH 6.1, and 50 mM KCl) for 3 h. Strips were then treated with (0-10 μM) Stomata were digitized using a Nikon DS-Fi 1 camera, coupled to a Nikon Eclipse Ti microscope. The stomatal-aperture width was subsequently measured using ImageJ software (National Institute of Health).

$^{32}P_i$-phospholipid labelling, extraction and analysis

Different types of tissues were used, whole seedlings and epidermal leaf peels. For Seedlings: Five-day-old seedlings were transferred to 200 μl labeling buffer (2.5 mM MES-KOH, pH 5.8, 1 mM KCl) containing $^{32}P_i$ (5-10 μCi) in 2 ml Eppendorf tubes and labeled overnight (~16 h). Samples were treated the next day by adding 200 μl labeling buffer with or without sorbitol for the times and concentrations indicated. For epidermal leaf peels: Leaves of 3-week-old plants grown at 22°C under 16 h of light and 8 h of dark were stripped and immediately floated on 100 μl opening buffer (10 mM MES, pH 6.1 and 50 mM KCl) containing $^{32}P_i$ (5-10 μCi) in a 48-wells cell culture plate (Greiner bio-one) for 3 h. All treatments were stopped by adding perchloric acid at a final concentration of 5% (v/v) for 5-10 min, after which the material was transferred to 400 μl of $CHCl_3$/MeOH/HCl [50:100:1 v/v)] to extract the

lipids. After 15 min, 400 µl of $CHCl_3$ was added, followed by 200 µl of 0.9 % (w/v) NaCl to induce two phases. The organic lipid fractions were washed and concentrated as described earlier (Munnik & Zarza, 2013). Lipids were separated by thin-layer chromatography (TLC) using an alkaline solvent system, containing: chloroform/methanol/28% ammonia/water [90:70:4:16 (v/v)] (Munnik et al., 1994). Radioactive phospholipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA). Individual phospholipid levels are expressed as the percentage of the total ^{32}P-lipid fraction.

Drought tolerance

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

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

Soluble carbohydrates measurement in seedlings

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

Phloem sap soluble carbohydrates measurement

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

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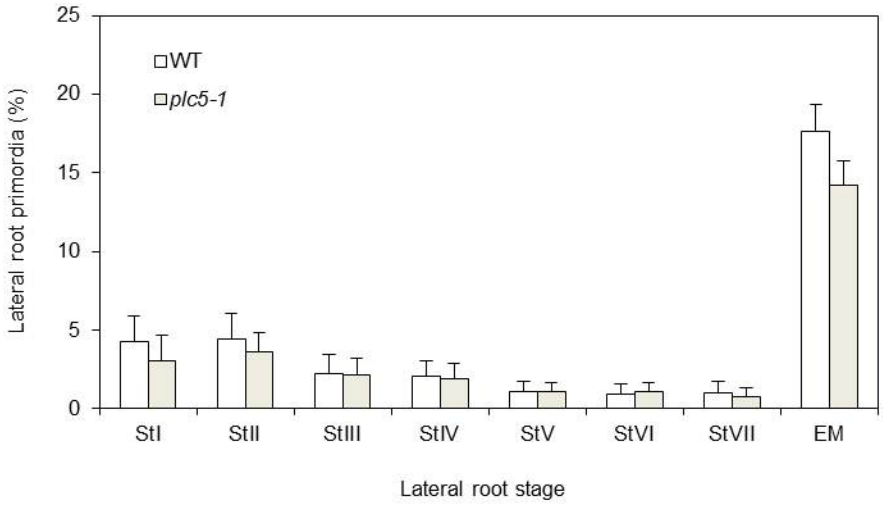
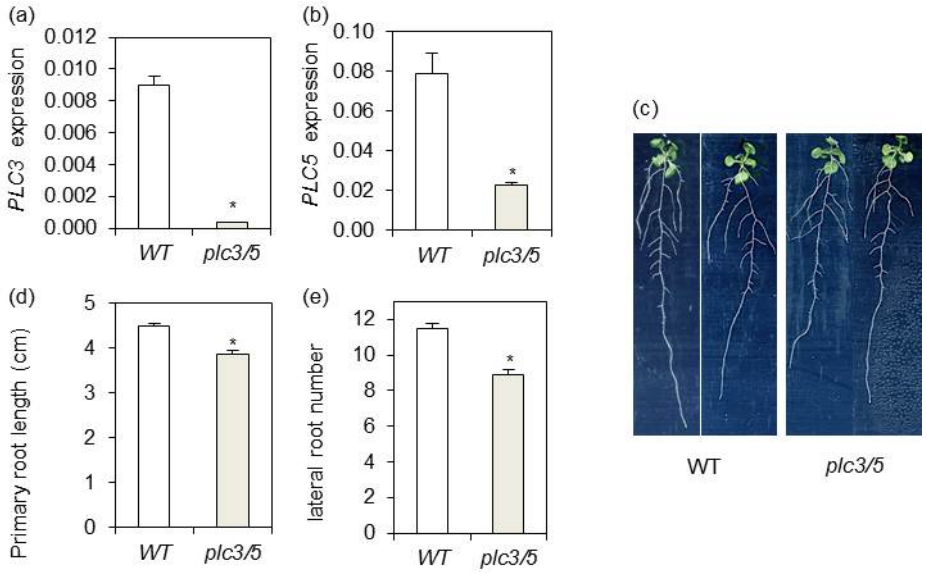
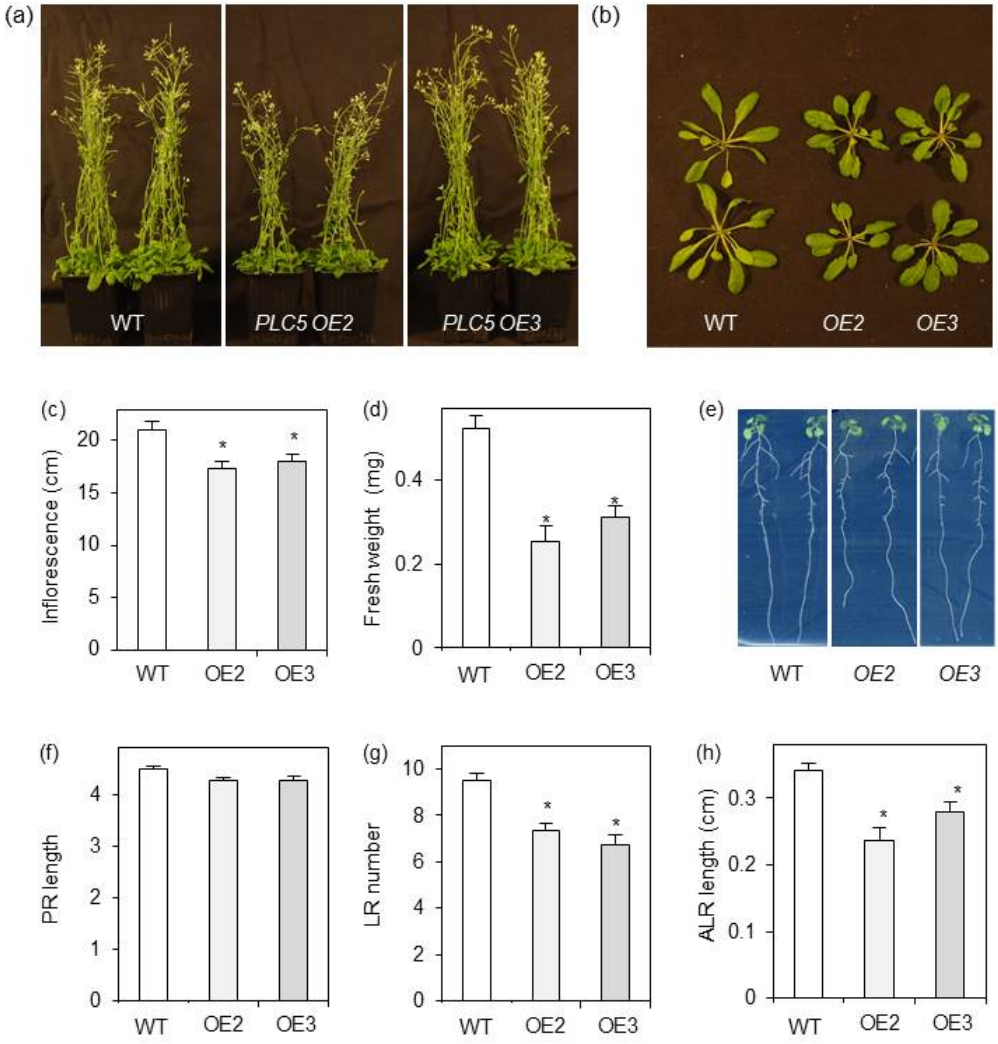
SUPPLEMENTAL DATA



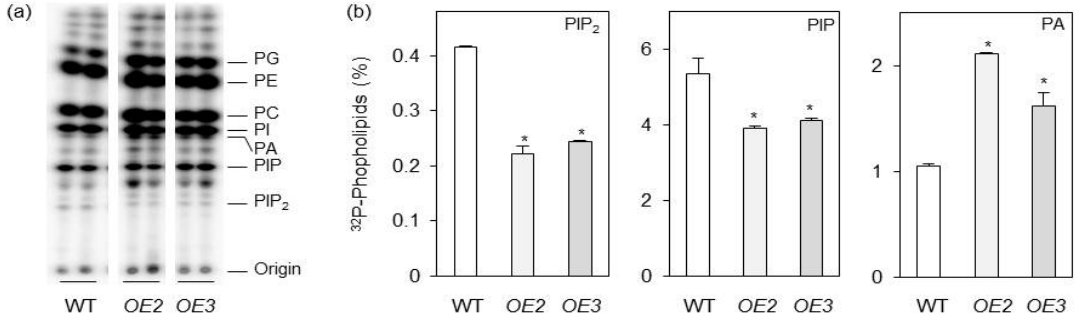
Supplemental Figure S1. Developmental stages of lateral root formation in *plc5-1* and wild-type seedlings.
Developmental stages of lateral root primordia from wild type and *plc5-1* seedlings. Values are means ± SE of three independent experiments (n>20)



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

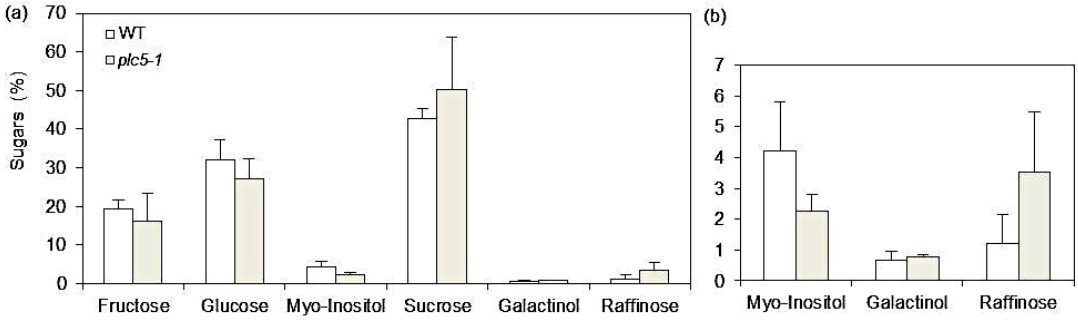


Supplemental Figure S3. Phenotypic analysis of wild type- and *PLC5 OE* lines grown on soil or agar plates.
Eight-weeks old wild type- and *PLC5 OE* plants were grown on soil under long day condition and the whole plants (a) and rosettes without inflorescences (b) were photographed. The inflorescence length was measured (c), and the fresh weight of rosette was determined (d). (e) Seedling morphology of wild type and *PLC5-OE* lines grown on agar plates. Seeds were germinated on ½ MS with 0.5% sucrose for 4 days, then transferred to ½ MS plates without sucrose. Photographs were taken 10 days after germination (DAG). (f) Primary root (PR) length, lateral root (LR) number and average lateral root (ALR) length at 10 DAG. All experiments were repeated at least three times. Values are means ± SD for one representative experiment (n=36). Asterisk (*) marks that PLC5 OEs value is significantly different from wild-type based on Student's t-test ($P < 0.05$)



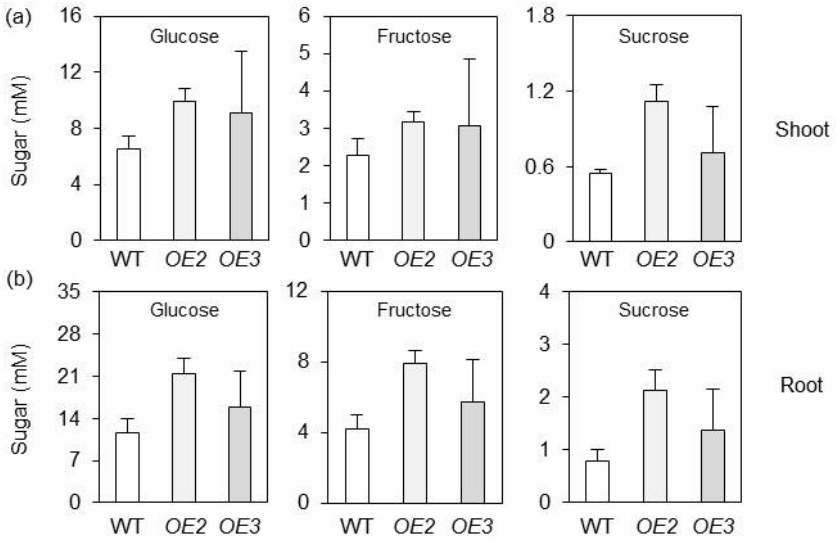
Supplemental Figure S4. PPI- and PA levels in wild type and *PLC5-OE* lines.

Five-days old seedlings were $^{32}P_i$-labelled overnight, and the next day their lipids extracted, separated by TLC and quantified by phosphoimaging. (a) Autoradiograph of a typical TLC, each lane representing 1/5th of the extract of 3 seedlings. (b) Quantification of ^{32}P-labeled PIP_2-, PIP- and PA levels in wild type or *PLC5 OE* lines. Values are calculated as the percentage of total ^{32}P-labeled phospholipids, and are represented as means ± SD (n=3). This experiment was repeated twice with similar results.



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

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



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

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