Novel roles for phospholipase C in plant stress signalling and development

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Chapter 5

General discussion

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Comparison of Arabidopsis *PLC3*, *PLC5* and *PLC7* -- similarities and differences

In this thesis, we explored the roles of three *PLC* genes - *PLC3*, *PLC5* and *PLC7* - in Arabidopsis development and stress responses at the molecular, physiological and biochemical level. Arabidopsis contains 9 *PLC*s, which share several conserved protein domains, including an EF-hand domain, the catalytic X- and Y-domains, and a C2 domain (Tasma *et al.*, 2008; Munnik, 2014). In addition, the genes for *PLC3*, *PLC5* and *PLC7* localize to chromosome 3, 4 and 5 respectively and may have evolved differently (Hunt *et al.*, 2004; Tasma *et al.*, 2008). The difference in protein structure and evolutionary history is likely to result into functional differences between the nine PLC homologs of Arabidopsis

The discussion below will focus on the similarities and differences between *PLC3*, *PLC5* and *PLC7* in terms of their function in plant development and stress. This will help to put the results presented in thesis, in a broader perspective.

Expression

The *PLC*-expression analyses in this thesis were based on promoter-GUS analysis. The expression patterns of *PLC3*, *PLC5* and *PLC7* have similarities, but also some differences (see Table 1). The most striking communality in expression is that they are all vascular-expressed *PLC*s in both vegetative (shoot and root) and reproductive organs (flower). Cell-specific gene profiling analyses in roots revealed that these *PLC*s are expressed in phloem-related cells, *e.g.* companion cells (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Phloem is responsible for transferring soluble organic compounds, especially sucrose made by photosynthesis in the leaves, to tissues where it is required, such as roots (Turgeon and Wolf, 2009; De Schepper *et al.*, 2013). PLC expression detected in phloem suggests a potential role in plant development. A closer look at the expression in root vasculature revealed a typical "segmented-expression" pattern for *PLC3* and *PLC5* in roots, but not for *PLC7*. This could to be linked to the lateral root formation phenotype in plants that have lost the function of PLC3 or PLC5, but not PLC7 (see discussion below).

Table 1. *Promoter-GUS* analysis of *PLC* expression.

expression	vascular tissue	segment expression in root	developing seed chalazal	developing seed coat	mature seed embryo	trichome	guard cell
PLC3	yes	yes	yes	no	yes	yes (base only)	yes
PLC5	yes	yes	yes	yes	yes	yes	yes
PLC7	yes	no	yes	yes	yes	yes	no

PLC is expressed though out the whole life cycle, based on current knowledge (Hunt *et al.*, 2004; Tasma *et al.*, 2008; Munnik, 2014; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We found all three *PLC*s were expressed in the chalazal during seed development. This is the tissue that serves as a channel for passing nutrients from the mother plant to the embryo (Xu *et al.*, 2016). Therefore, PLCs might exert the same function here as they do in the vasculature. *PLC5* and *PLC7* were expressed in the developing seed coat but *PLC3* was not expressed in this tissue. The *plc5plc7* double mutant exhibited a non-adherent seed coat mucilage phenotype, which might have to do with the expression of both *PLC5* and *PLC7* in the seed coat. The expression of all three genes was also detected in germinating seeds. *pPLC3::GUS-YFP* embryo was intensively stained by X-Gluc (Chapter 2, Figure 4b), this was much less obvious for *PLC5::GUS* (Chapter 3, Figure 2a) and *PLC7::GUS-SYPF* (Chapter 4, Figure 1a), which could be a link to the slower germination rate of the *plc3* mutants only.

Interestingly, all three *PLC*s were expressed in trichomes. The expression of *PLC5* and *PLC7* was detected in the whole trichome body, but *PLC3* only in the basal cells of the trichome, which connect to the leaf-vascular tissue and seems to be part of the vasculature. In Arabidopsis, trichomes are non-secreting epidermal cells, which protect plants from adverse conditions and also serve as a source of phytochemicals (Schwab *et al.*, 2000; Pattanaik *et al.*, 2014). A numbers of genes have been identified to be involved in trichome development. The consistent expression of *PLC5* and *PLC7* in trichomes hints at a possible role in morphogenesis and/or function, even though we did not observe any phenotype in the single- and double *plc* mutants that were viable; obviously the *PLCs* may be redundant. An inducible-amiPLC3 line in a *plc5/7* background may reveal more on this.

In guard cells, both *PLC3* and *PLC5* were expressed, which confirms data provided by the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). However, *PLC7::GUS-SYFP* staining was not detected in guard cells. According to online database from eFP browser, *PLC7* could be induced in the guard cells after exogenous ABA application. We performed GUS staining on the leaf peel from *pPLC7::GUS-SYFP* plants after ABA treatment, but found no GUS signal (data not shown). The online data is based on microarray technique and guard cell protoplasts were used as plant material, which is different with our GUS staining analysis using seedlings, or freshly peeled leaf epidermis.

The promoter-GUS analysis and light microscopy studies cannot provide information about the PLC localization at the cellular level. The precise location in the cell will help to understand how plant PLCs work in various biological events. Thus, making transgenic plants that express *PLC-GFP* fusions driven by their own promoters is essential and will be our next step. We attempted by complementing the *plc5-1* mutant with an N-terminal YFP-PLC5, driven by the *PLC5* promoter, but no fluorescence could be observed anymore in T2- and T3 generations while antibiotic resistance, genotyping and phenotyping (root architecture) suggested that the wild-type gene was there. We suspect that the N-terminal FP-fusion is lethal/interferes too much and that we select for plants that have been able to loose the FP, as similar results were obtained for PLC2 (R. van Wijk and T. Munnik, unpublished).

Currently, the lab is testing this for new N- and C-terminal FP-PLC fusions (M. van Hooren, T. Munnik).

Role for PLCs in seed germination

We discovered a number of phenotypes in *plc3*, *plc5* and *plc7* single mutants, but also double *plc3/5* and *plc5/7* mutants (table 2). Among the mutants studied, only *plc3* exhibited a delayed seed germination phenotype (Chapter 2, Figure 4a). Seed germination is a very complex process, involving many factors, such as light, hormones and sugars etc. (Bentsink and Koornneef, 2008). Among these, abscisic acid (ABA) and gibberellin (GA) are best known for their crucial roles. ABA inhibits seed germination (Nambara et al., 2010; Nakashima & Yamaguchi-Shinozaki, 2013) while, GA acts stimulates germination (Yamaguchi and Kamiya, 2001). We compared the responses of *plc3* mutants to both ABA (Chapter 2; Fig.5) and GA (Chapter 2; Supplemental Fig. S7) with that of wild-type seeds. *plc3* mutant seeds germinated faster than wild-type when treated with exogenous ABA, indicating an increased ABA insensitivity. Therefore, the slower germination of *plc3* compared to wild type under normal condition cannot be caused by hypersensitivity to ABA. In response to GA, *plc3* mutants seemed to be hypersensitive, which again does not explain their slower germination phenotype. One possibility is that the endogenous GA level in *plc3* mutants is lower. Similarly, *plc3* mutants' insensitivity to ABA might correspond to a higher level of ABA in seeds. Hence, checking hormone level in *plc3* mutant seeds will be essential. Apart from hormones, sugar is another important factor during seed germination. The link between sugar metabolism and PLC signaling could be the Raffinose Family Oligosaccharides (RFOs), whose synthesis might require PLC-dependent inositol (Chapter 2 discussion). However, the analysis of soluble carbohydrate composition in seeds showed no significant differences between wild type and *plc3* mutants. Of course, changes could be very local, so it is also possible that these differences remain unobserved (Chapter 2; Fig. 4c).

A role for PLC in ABA signaling

Both *plc* mutants and PLC-overexpression lines of *PLC3*, *5* and *7* appear to have changes in ABA related physiological responses. In addition, *PLC3* and *PLC5* are strongly expressed in guard cells. Both *plc3* (Chapter 2; Fig. 5c) and *plc7* (Chapter 4; Supplemental Fig. 3) KO mutants did not close their stomata as much as wild type did during ABA treatment of epidermal leaf strips, which indicates an insensitivity for ABA. Furthermore, in both *PLC3* and *PLC5* overexpressor lines stomata were more closed in control conditions and while the guard cells in epidermal peels appeared to be less responsive to ABA. PLC7-OE lines did not show these changes. Together, these results hint to an involvement of PLC in ABA-induced stomatal closure. The T-DNA insertion *plc* mutants provide a tool to investigate the link between PLC and IPPs. According to our observation, using ^{3}H-Inositol pre-labeled seedlings and HPLC analyses, *plc3* mutants did not any show change in IP_3 or IP_6 levels, but a small decrease in either IP_7 or IP_8, was found depending on the seedling age. Plants contain plenty of IP_6, which is predominantly formed from MIPS-generated Ins3P (Munnik and Vermeer, 2010). The possible changes

in PLC-derived IP_6 in guard cells will be difficult to observe in a huge background other cells. Nevertheless, our results suggest that higher IPP and PP-IPP levels could be involved in PLC signaling. The measurement of IPPs and PP-IPPs will be continued in other *plc* mutants with ABA and other stresses application, in this way we will get closer to the real *PLC* signaling pathway in plant! Alternatively, fluorescent biosensors expressed in guard cells could help studying changes in PIP_2 or PIP levels during ABA induced stomatal closure.

Table 2. Phenotypes that are exhibited in *plc* knock-out or knock-down mutants

plc mutant phenotype	delayed seed germination	insensitive to ABA		shorter primary root	fewer lateral roots	loose seed coat mucilage	stronger leaf serration
		seed germination	stomatal closure				
plc3	yes	yes	yes	yes	yes	no	no
plc5	no	no	no	yes	yes	no	no
plc7	no	no	yes	no	no	no	no
plc3/5	no	no	no	yes	yes	no	no
plc5/7	no	no	no	no	no	yes	yes

The role for PLC in lateral root formation

PLC3::GUS-YFP and *PLC5::GUS* displayed a segmented-expression pattern in roots, which could be associated with the decrease in lateral root formation in *plc3*- and *plc5* mutants. *PLC7::GUS-SYFP* did not have this expression pattern, and *plc7* mutants did not display the lateral root phenotype either. The double mutant *plc3/5* had a similar root phenotype as the single mutants, with only a minor increase in severity (Chapter 3; Fig. S2), whereas no changes were found in the root architecture of the *plc5/7* mutant (Chapter 4; Fig. 3), suggesting redundancy of other PLC family members. The combination of *plc3*- and *plc7*-KO mutations was not viable, as we could not obtain homozygous lines from this cross. Generation of induced silencing lines for *PLC3* and *PLC7* could help to overcome this problem.

The possible role of *PLC* in lateral root formation has been discussed in Chapter 2 and 3. Two options were considered: PLC might affect the root architecture through the generation of IP_6 to influence auxin-TIR1 signaling, or via the metabolism, transport or storage of raffinose oligosaccharides (RFOs). One function for RFOs is loading sucrose to lateral roots, which could either facilitate their development or functions to store sugars. In *plc3* mutants, phloem sap sucrose had increased and inositol was slightly reduced in *plc3* mutants compared to wild type. However, *plc5* only displayed a slightly lower inositol level (had no change in phloem sucrose). Further investigation of the phloem sap sugar composition in *plc3/5* and multiple *plc* mutants is required to confirm the PLC/inositol/RFOs/LR hypothesis. Alternatively, PLC might regulate lateral root formation by PLC-dependent IP_6, which could affect TIR1 activity, resulting in less efficient auxin perception and fewer lateral root initiation. To confirm this hypothesis, an FP-tagged version of TIR1 in a *tir1* mutant

background (*pTIR1::gTIR-mVENUS*) will be crossed with *plc3*- and *plc5* mutants. TIR-mVENUS will then be immunoprecipitated and IP_6 presence will be validated by its conversion into ^{32}P-IP_7 by incubating shortly-boiled fractions with an IP_6 kinase (yeast KCS1, fused to GST and expressed in *E.coli*) and ^{32}P-γATP. Radioactive labeling is very sensitive, and this might allow us to detect the decreased IP_6 levels in the *plc* mutant backgrounds that may not be detected by other methods. In this way, we can link PLC activity directly with IP_6 production and auxin signaling through TIR1.

Overexpression of PLC enhances drought tolerance

Drought stress is a major threat that limits plant productivity in agriculture. It does not only cause hyperosmotic stress, but also oxidative stress on cells (Zhu, 2016). Plant responses to drought stress are complex, including the sensing and transduction of the stress signal, but also the adaptation to the adverse environment, which involves numerous physiological-, structural-, morphological-, and biochemical changes. PLC signaling is activated under drought stress (Munnik and Meijer, 2001; Munnik and Vermeer, 2010; Hou *et al.*, 2016). Both PLC's substrates (PIP, PIP_2) and products (IPP, PA converted by DAG) have been reported to be implicated in important cellular events, such as the reorganization of the cytoskeleton, endo- and exocytosis, vesicular trafficking, and ion-channel regulation, including intracellular Ca^{2+} release (Stevenson et al., 2000; Martin, 2001; van Leeuwen et al., 2007; Heilmann, 2016). Recently, overexpression of a *PLC* in maize, tobacco and canola have been shown to improve drought tolerance (Wang *et al.*, 2008; Georges *et al.*, 2009; Tripathy *et al.*, 2011).

Overexpression of *PLC3*, *PLC5* or *PLC7* were all found to enhance the drought tolerance of Arabidopsis, with *PLC5-OE* showing the strongest phenotype (Table 3). Similarly, overexpression of *PLC2* and *PLC4* showed an increased drought-tolerant phenotype (Munnik lab, unpublished), indicating that overexpression of any *PLC* might improve the plant's tolerance to drought. When plants experience drought, several physiological changes happen: e.g stomata close, root systems become denser and grow deeper, compatible solutes accumulate, photosynthesis decreases, etc. (Comas *et al.*, 2013; Singh *et al.*, 2015; Basu *et al.*, 2016; Joshi *et al.*, 2016). *PLC3-OE* and *PLC5-OE* exhibited a smaller stomatal aperture than wild type, which could be a reason for their enhanced drought tolerance. However, two *PLC7-OE* lines #9 and #12 showed no difference in stomatal aperture, but they did exhibit the drought tolerance phenotype. It is possible though, that the difference in stomatal opening in *PLC7-OE* lines #9 and #12 is too small to be observed. Therefore, more independent *PLC7-OE* lines should be used for further confirmation, and preferably, we would like to measure stomatal conductance *in vivo*, on a growing plant that experiences drought stress(Andrés *et al.*, 2014). The closure of stomata is induced by the phytohormone, ABA and this signaling pathway is central to the drought-stress response. How *PLC* could be involved in ABA signaling has been well summarized in Chapter 2 (Figure 10).

Table 3. Phenotypes that are exhibited in *PLC-OE lines*.

PLC-OE phenotype	drought tolerance	stunted root hairs	smaller stomatal aperture	ABA sensitivity stomata
PLC3	yes	no	yes	less
PLC5	yes	yes	yes	less
PLC7	yes	no	no	no change

The root system of *PLC-OE* lines appears to be neither denser nor larger that would account for absorbing more water, and thus more drought tolerant. *PLC5-OE* lines even grow a smaller root system with very short root hairs, which was not found in other *PLC-OE* lines. Thus, the increased tolerance of the *PLC-OE* lines to drought is probably not through alteration of their root systems. In *PLC5-OE* lines, an increase in the accumulation of sugars was detected, which could be another possibility for drought resistance. Sugar content of the phloem should be carried out with other *PLC-OE* lines. The drought response in plants involves many genes with potential cross-talk (Basu *et al.*, 2016; Zhu, 2016) and *PLC* could be one of them. Knowing the transcripts of drought-responsive genes in *PLC-OE* plant will help to discover the link between *PLC* and other genes in plant's response to drought stress.

An overview of *PLC3*, *PLC5* and *PLC7* phenotypes

All phenotypes found in manipulated *PLC* lines (KO mutants and OE lines) are summarized in **Figure1**. We discovered several novel roles for *PLC* in plant stress and development, which provides a step forward in understanding how *PLC* functions in plants. Here, we emphasize some new scenarios for plant PLC signaling.

Firstly, apart from second messengers (IPPs and DAG/PA) producer, PLC could also function as PIP_2 and PIP signaling attenuator (Munnik and Nielsen, 2011). PIP_2 and PIP are emerging as second messenger themselves, involved in various stress- and developmental responses (Stevenson *et al.*, 2000; Meijer and Munnik, 2003; Vermeer *et al.*, 2009; Ischebeck *et al.*, 2010; Munnik and Nielsen, 2011; Gillaspy, 2013; Rodriguez-Villalon *et al.*, 2015; Heilmann, 2016; Simon *et al.*, 2016).

Secondly, PLC follows the traditional signaling pathway to generate second messengers, except that IP_3 is either phosphorylated to IP_6 to release Ca^{2+}, or dephosphorylated to inositol, which is the precursor for RFO sugar metabolism. The DAG that originates from PLC activity is converted into PA to activate downstream targets (Munnik and Nielsen, 2011; Testerink and Munnik, 2011; Munnik, 2014).

Thirdly, higher IPPs and PP-IPPs (IP_4, IP_5, IP_6, IP_7 and IP_8) function as signaling molecules independently of Ca^{2+} signaling, such as ion-channel regulation (Zonia *et al.*, 2002), hormone

perception (Tan *et al.*, 2007; Sheard *et al.*, 2010; Laha *et al.*, 2015, 2016), mRNA transport (Lee *et al.*, 2015) and phosphate homeostasis (Kuo *et al.*, 2014; Puga *et al.*, 2014; Wild *et al.*, 2016).

Last but not the least, PI4P exerts great potential to be PLC's substrate in non-stressed cells. The authentic PIP_2 is missing from most plant plasma membranes, whereas PI4P is much more abundant (30-100 times), and most (>90%) of the PLC activity is associated with the plasma membrane fraction. *In vitro,* PI4P is hydrolyzed equally well as PIP_2. The formed IP_2 can still be further phosphorylated to IP_6 by the same two IPKs, and the DAG be converted into PA (Munnik et al., 1998a, 1998b; Meijer and Munnik, 2003; van Leeuwen et al., 2007; Vermeer et al., 2009; Vermeer and Munnik, 2013; Munnik, 2014; Simon et al., 2014; Tejos et al., 2014; Simon et al., 2016). Hence, we should keep our eyes open for everything!

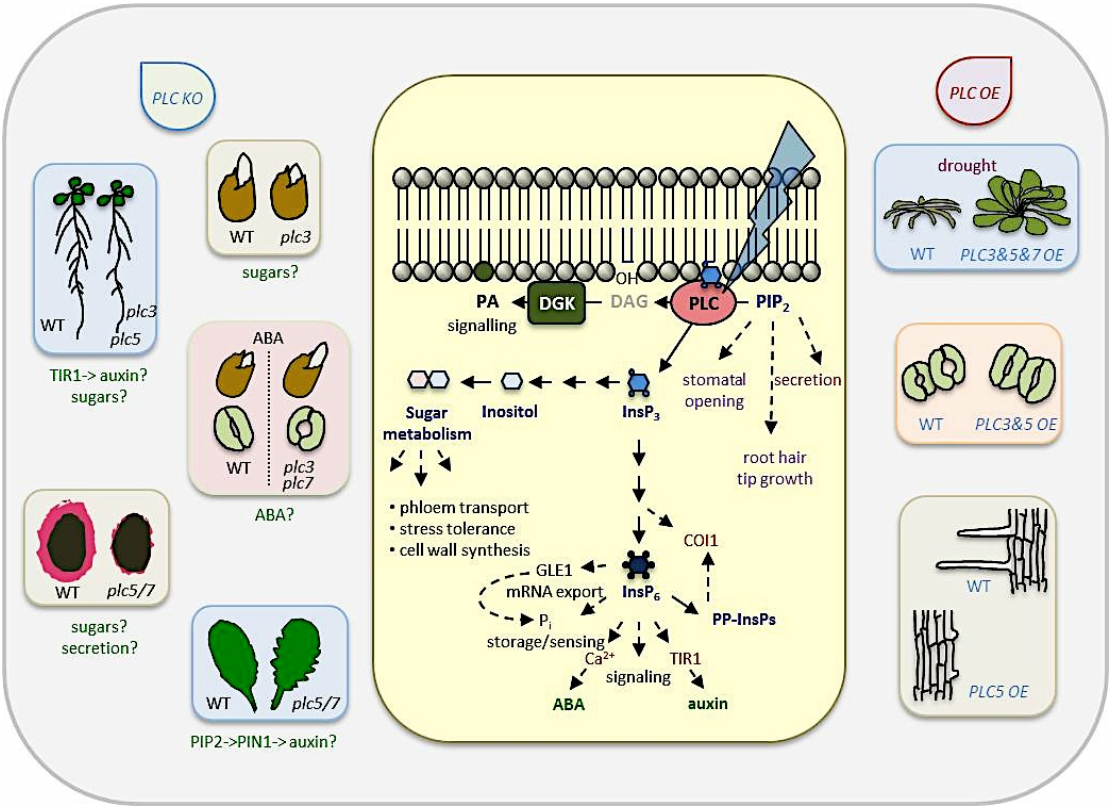

Figure 1. Summary of phenotypes in plc knockout and PLC overexpression lines.
Left: plc knockout mutants exhibit delayed germination; fewer lateral roots; insensitivity to ABA during seed germination and ABA-induced stomatal closure; non-adherent seed coat mucilage and enhanced leaf serration phenotypes.
Middle: proposed PLC signaling in plant development and stress responses
Right: PLC-OE display increased drought tolerance; less opened stomata and stunted roothair growth phenotypes.

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