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 PLCs, 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 PLCs in both vegetative (shoot and root) and reproductive organs (flower). Cell-specific gene profiling analyses in roots revealed that these PLCs 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 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.

<table>
<thead>
<tr>
<th>expression</th>
<th>vascular tissue</th>
<th>segment expression in root</th>
<th>developing seed chalazal</th>
<th>developing seed coat</th>
<th>mature seed embryo</th>
<th>trichome</th>
<th>guard cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC3</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>(base ony) yes</td>
</tr>
<tr>
<td>PLC5</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>PLC7</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
PLC is expressed throughout 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 PLCs 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 PLCs 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-SYPF 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-SYPF 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 ³H-Inositol pre-labeled seedlings and HPLC analyses, *plc3* mutants did not any show change in IP₃ or IP₆ levels, but a small decrease in either IP₇ or IP₈, was found depending on the seedling age. Plants contain plenty of IP₆, which is predominantly formed from MIPS-generated Ins3P (Munnik and Vermeer, 2010). The possible changes
in PLC-derived \( \text{IP}_6 \) 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

<table>
<thead>
<tr>
<th>( plc ) mutant phenotype</th>
<th>delayed seed germination</th>
<th>insensitive to ABA</th>
<th>shorter primary root</th>
<th>fewer lateral roots</th>
<th>loose seed coat mucilage</th>
<th>stronger leaf serration</th>
</tr>
</thead>
<tbody>
<tr>
<td>plc3</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc5</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc7</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>plc3/5</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>plc5/7</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

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 \( \text{IP}_6 \) 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 \( \text{IP}_6 \), 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 IP6 presence will be validated by its conversion into 32P-IP7 by incubating shortly-boiled fractions with an IP6 kinase (yeast KCS1, fused to GST and expressed in E.coli) and 32P-γATP. Radioactive labeling is very sensitive, and this might allow us to detect the decreased IP6 levels in the plc mutant backgrounds that may not be detected by other methods. In this way, we can link PLC activity directly with IP6 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, PIP2) 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 Ca2+ 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.

<table>
<thead>
<tr>
<th>PLC-OE phenotype</th>
<th>drought tolerance</th>
<th>stunted root hairs</th>
<th>smaller stomatal aperture</th>
<th>ABA sensitivity stomata</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC3</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>less</td>
</tr>
<tr>
<td>PLC5</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>less</td>
</tr>
<tr>
<td>PLC7</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no change</td>
</tr>
</tbody>
</table>

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 PIP₂ and PIP signaling attenuator (Munnik and Nielsen, 2011). PIP₂ 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 IP₃ is either phosphorylated to IP₆ to release Ca²⁺, 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 (IP₄, IP₅, IP₆, IP₇ and IP₈) function as signaling molecules independently of Ca²⁺ 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 PIP₂ 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 PIP₂. The formed IP₂ can still be further phosphorylated to IP₃ 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 root hair growth phenotypes.
General discussion

REFERENCES


